Inhibition of Endothelial Nitric Oxide Synthase by Cytochrome P-450 Reductase Inhibitors (43878)

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Abstract. **Nitric oxide synthase (NOS) shows similarities to cytochrome P-450 reductase. The two enzymes catalyze the oxidation of N-ω-hydroxy-L-arginine by NADPH and oxygen to nitric oxide (NO) and citrulline. Nitric oxide synthase activity is inhibited by L-arginine analogs like N-ω-nitro-L-arginine, which does not affect cytochrome P-450 reductase. Dihydroergotamine, miconazole, and troleandomycin are classical inhibitors of cytochrome. The present study shows the concentration-dependent inhibitory effect of these compounds and of L- but not D-N-ω-nitro-arginine on the activity of constitutive nitric oxide synthase from bovine aortic endothelial cells. Activity of nitric oxide synthase was estimated by measurement of conversion of [^{3}H]arginine to [^{3}H]citrulline. The tested cytochrome P-450 inhibitors are likely to interfere with heme of nitric oxide synthase. The data confirms a similarity as well as functional differences between the enzymes.** [P.S.E.B.M. 1995, Vol 209]

Nitric oxide (NO) acts as a signal molecule in cardiovascular and central nervous systems with soluble guanylate cyclase as an effector enzyme (1, 2). NO is also formed by cytokine-activated cells of the immune system and mediates the cytotoxicity of these cells (3). Several isoforms of nitric oxide synthase (NOS), which catalyze the conversion of L-arginine to NO plus L-citrulline (4), have been identified. The intracellular Ca^{2+} and calmodulin-dependent isoforms of NOS are constitutively expressed in endothelial cells (5), brain (6), platelets (7), adrenal gland (8), and lung (9). Ca^{2+} independent, cytokine-inducible NO synthase has been purified from macrophages (10). Another inducible NOS, which is dependent on Ca^{2+} but not on calmodulin, has been obtained from neutrophiles (11).

The constitutive and inducible NO synthases have similar catalytic properties (12). Both require NADPH, tetrahydrobiopterin, and molecular oxygen as cofactors and contain FAD and FMN (1, 6, 13). Cytochrome P-450 reductase is the only known mammalian enzyme with close homology to NOS (13, 14). Similar to NOS (15), this enzyme is also known to catalyze the oxidation of N-ω-hydroxy-L-arginine to NO and L-citrulline. Cytochrome P-450 and cytochrome P-450 reductase are major components of the hepatic cytochrome P-450 monooxygenase system embedded in the lipid bilayer of the smooth endoplasmic reticulum.

The present study demonstrates that nitric oxide synthase from freshly harvested endothelial cells is inhibited by classical inhibitors of hepatic cytochrome P-450 such as miconazole, dihydroergotamine (DHE), and troleandomycin (TAO). We also confirmed that NOS was inhibited by N-ω-nitro-L-arginine, a compound which failed to affect cytochrome P-450 activity (15). Miconazole, DHE, and TAO are used in clinical pharmacology as anti-mycotic, anti-migraine, and antibacterial agents, and have chemical structures (imid-

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azole, ergopeptide, and macrocyclic lactone antibiotic, respectively) that are unrelated to each other. However, the above substances do share some properties: together with O_2 and CO they are inhibitors of cytochrome P-450, CYP3A isoforms (16). Moreover, all of the inhibitors are likely to interfere with heme binding, and since both cytochrome P-450 reductase and NOS are hemoproteins (14), this formulates the basis for the selection of the test compounds. The results confirm a close similarity between nitric oxide synthase and cytochrome P-450 reductase despite functional differences between the two proteins.

Materials and Methods

Materials. [^{3}H]Arginine (57 Ci/mmol; 1 Ci = 37 GBq) was obtained from Amersham (Arlington Heights, IL). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Endothelial Cells. The preparation of endothelial cells has been previously described (17). In brief, six bovine aortas were obtained from a local abattoir and transported to the laboratory on ice. After removing the surrounding connective tissue, the aortic branches were occluded with titanium hemostatic clips (Pilling Co., Fort Washington, PA) and with Edslab parallel jaw spring clips (Baxter Healthcare Corp., Irvine, CA). The aortic lumina was filled with 0.1% collagenase, type 1, dissolved in Krebs-Henseleit solution (K-H), prewarmed to 37°C and incubated for 45 min. The Krebs-Henseleit solution was composed of the following substances: 1.2 m*M* $H_2PO_4^{2-}$, 127.8 m*M* Cl^-, 143 m*M* Na^+, 5.9 m*M* K^+, 2.5 m*M* Ca^{2+}, 1.2 m*M* Mg^{2+}, 25 m*M* HCO_3^-, 1.2 m*M* SO_4^{2-}, 10 m*M* glucose. After incubation, the vessels were cut lengthwise and rinsed twice by gentle irrigation with K-H. The intimal surface was then delicately scraped with a rubber policeman to detach endothelial cells and further irrigated with K-H, which was collected into 50 ml capacity polypropelene centrifuge tubes and centrifuged for 10 min at 1600 rpm. Cells were finally suspended in 1 ml of K-H solution and then divided into aliquots.

Counting of Endothelial Cells. One hundred microliters of the cell suspension were incubated for 3 min with Tripan blue (final concentration 0.08%) and the viable cells were counted. Viability was approximately 90%.

Measurement of Nitric Oxide Synthase Activity. NO synthase activity was determined in both cell homogenates and intact endothelial cells by measuring the conversion of [^{3}H]arginine to [^{3}H]citrulline (6).

Homogenized Cells. Approximately 30 million freshly harvested endothelial cells were homogenized in 1 ml of Tris buffer (0.05 *M*, pH 7.4) containing EDTA (0.1 m*M*), dithiothreitol (0.5 m*M*), leupeptin (10 μg/ml), phosphoramidon (25 μg/ml), and aprotinin (100 μg/ml). The samples were sonicated for 20 sec (Biosonik III apparatus; Bronwill Scientific Inc., Rochester, NY). One hundred-microliter aliquots of the homogenates were incubated in 1.5-ml Eppendorf test tubes and shaken in a water bath at 37°C for 5 min. Each sample contained calmodulin (100 U), NADPH (1 m*M*), and $CaCl_2$ (2 m*M*). If required, N-ω-nitro-L-arginine (L-NNA, 0.1 μ*M*–1 m*M*), dihydroergotamine (DHE, 1–100 μ*M*), miconazole (1–100 μ*M*), troleandomycin (TAO, 5–50 μ*M*) or N-ω-nitro-D-arginine (D-NNA, 0.1 μ*M*–1 μ*M*) was added (all concentrations final). DHE, miconazole, and TAO were added at concentrations known to inhibit cytochrome P-450 reductase (15). DHE and miconazole were dissolved in dimethylosulfoxide, and the final concentration of DMSO was maintained below 1% (15). After 5 min, [^{3}H]arginine was added to a final concentration of 3.0 μCi/ml (50 n*M*). The reaction was stopped after 30 min by addition of 1 ml ice-cold Tris buffer, pH 5.5 containing EDTA (2 m*M*). Separation of radiolabeled citrulline from arginine was performed by cation exchange chromatography. Dowex 50WX-8, 100–200 Mesh Na^+ resin (Bio-Rad Laboratories, Hercules, CA), packed into disposable polypropelene 10-ml chromatography columns (Pierce, Rockford, IL) was used. Radioactivity of the sample was determined in a liquid scintillation counter (Beckman LS 100 SC, Irvine CA) and expressed as the percentage of net CPM count (basal activity equals 100%). The amount of [^{3}H]arginine added to each sample corresponded with approximately 100,000 CPM, and the representative basal sample had 8,000 to 10,000 CPM derived from [^{3}H]citrulline. The nonspecific background was below 600 CPM. Approximately 3.7–4.7 fmol of arginine was converted to citrulline within 10 min in the basal sample.

Intact Cells. Aliquots of 70 μl (3.0 million cells) were placed in Eppendorf tubes (1.5 ml capacity) with or without the inhibitors of D-NNA (0.1 m*M*). After 10 min of initial incubation in a shaking water bath at 37°C, 10 μl of [^{3}H]arginine was added (3.0 μCi/ml, 50 n*M* final). After 60 min, samples were removed from the water bath, placed on ice and 1 ml of ice-cold Tris buffer (0.05 *M*, pH 5.5) containing 2 m*M* of EDTA was added. The samples were sonicated for 10 sec. Thereafter, the same procedure previously described was followed.

Statistics. Data are expressed as mean ± SEM. Student's *t* test was used to analyze the data for significant differences. The differences were considered significant when $P < 0.05$ (two-tailed).

Results and Discussion

L-NNA (0.01 μ*M*–1 m*M*) significantly inhibited NOS activity in endothelial cells homogenates in a concentration-dependent manner (Fig. 1), whereas D-NNA (0.01 μ*M*–1 m*M*) showed no effect. The in-

hibitory effect of L-NNA was absent at concentrations 0.1 μ*M* and lower; maximal effect was observed at 0.1 m*M* and EC_{50} was 3.1 μ*M* (Fig. 1). All three cytochrome P-450 reductase inhibitors, DHE (1–100 μ*M*), miconazole (1–100 μ*M*), and TAO (5–50 μ*M*), significantly lowered NOS activity in cell homogenates; the inhibition was concentration dependent (Fig. 1). At the highest used concentrations, DHE, miconazole, and TAO respectively produced 43.1% ± 4.2%, 44.0% ± 5.2%, and 26.3% ± 4.9% of inhibition (Fig. 1). The compounds could not be used in higher concentration because of their weak solubility. Compared with L-NNA, all three concentration-dependent curves were shifted to the right, showing a lower potency for DHE, miconazole and TAO; their potencies were similar (Fig. 1). The efficacy of L-NNA was also higher than other inhibitors. Compared with L-NNA, DHE, miconazole, and TAO appeared as partial inhibitors of NOS.

With the exception of miconazole (0.1 m*M*), in intact endothelial cells, L-NNA (0.1 m*M*), DHE (0.05 m*M*), and TAO (0.05 m*M*) showed a significant inhibitory effect on NOS (32.9% ± 4.4%, 70.6% ± 6.4%, 66.4% ± 6.2% and 91.0% ± 18.9% of basal activity, respectively, Fig. 2). D-NNA showed no effect (107.8 ± 6.4%, Fig. 2).

The enzymes nitric oxide synthase and cytochrome P-450 reductase have some properties in common (12). The two proteins have an amino acid sequence that is 36% identical and 58% of the sequence reveals a close homology (13). Both are flavoproteins, containing 1 mol each of FAD and FMN, tetrahydrobiopterin, and iron (18). Recent evidences demonstrate a P-450-type CO-binding heme prosthetic group in purified NOS (14, 18). The conclusion that NOS was the first example of mammalian soluble isoform of cytochrome P-450 reductase (19) containing both a reductase and a heme domain on the same polypeptide was reached (14).

Several analogs of L-arginine, which is a substrate for NOS, have been shown to be competitive inhibitors of NOS (20). When N-ω-methyl- or N-ω-nitro-L-arginine are present, there is no conversion of L-arginine to N-ω-hydroxy-L-arginine (21) and subsequent to L-citrulline and NO by NOS. Cytochrome P-450 reductase also catalyzes the oxidation of N-ω-hydroxy-L-arginine by NADPH and O_2 to NO and citrulline. This reaction is strongly inhibited by classical inhibitors of cytochrome P-450 such as CO, miconazole, dihydroergotamine, and troleandomycin, but not by N-ω-methyl- and N-ω-nitro-L-arginine (15). Although miconazole, DHE, and TAO have different chemical structures, they all inhibit cytochrome P-450, CYP3A isoforms (16) by interfering with heme binding. Imidazole-related compounds have high selectivity for cytochrome heme moiety (22), and imidazole acts as a heme-site inhibitor of brain NOS (23). Dihydroergotamine interacts with cytochrome P-450 from microsomes and binds to a protein site close to the heme (24). Macrolide antibiotics, including troleandomycin, also exhibits a high affinity for cytochrome as shown by difference visible spectroscopy (25, 26).

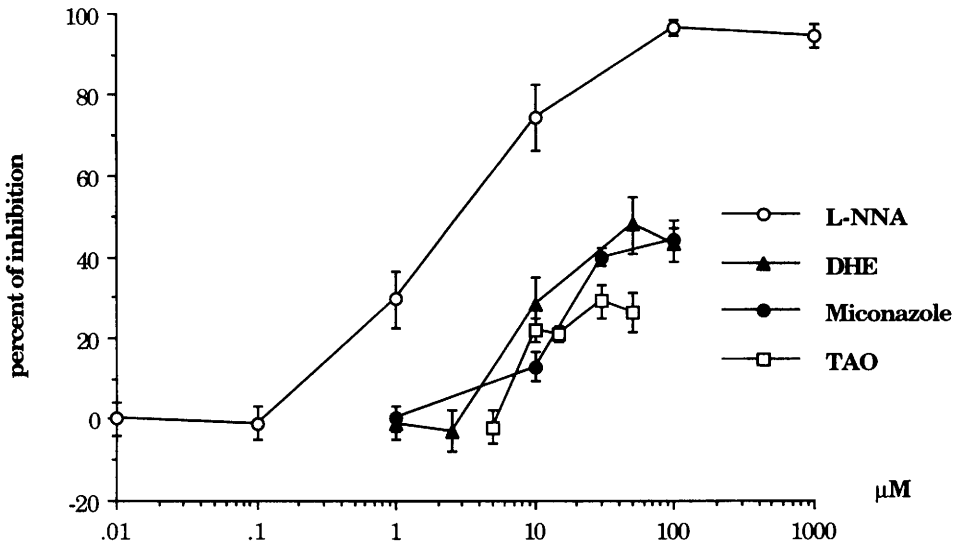

Figure 1. The effect of N-ω-nitro-L-arginine (L-NNA, 0.01 μ*M*–1 m*M*), dihydroergotamine (DHE, 1–100 μ*M*), miconazole (1–100 μ*M*), and troleandomycin (TAO, 5–50 μ*M*) on the nitric oxide synthase (NOS) activity in cell homogenates (n = 4–10 separate experiments for each point). L-NNA blocked NOS nearly completely (96.7% ± 2.1% of inhibition at 100 μ*M*, $P < 0.0001$), whereas D-NNA had no effect (7.8% ± 11.4% of inhibition at 100 μ*M*, not shown on the figure). DHE, miconazole, and TAO significantly inhibited NOS activity in concentration-dependent manner. Concentration-response curves are shifted to the right compared with L-NNA, showing lower potency of DHE, miconazole, and TAO. The efficacy of these compounds is also lower compared with L-NNA. Values are obtained by comparison to basal NOS activity. Significant inhibition was observed for concentrations of 10 μ*M* and higher of DHE, miconazole, and TAO ($P < 0.05$).

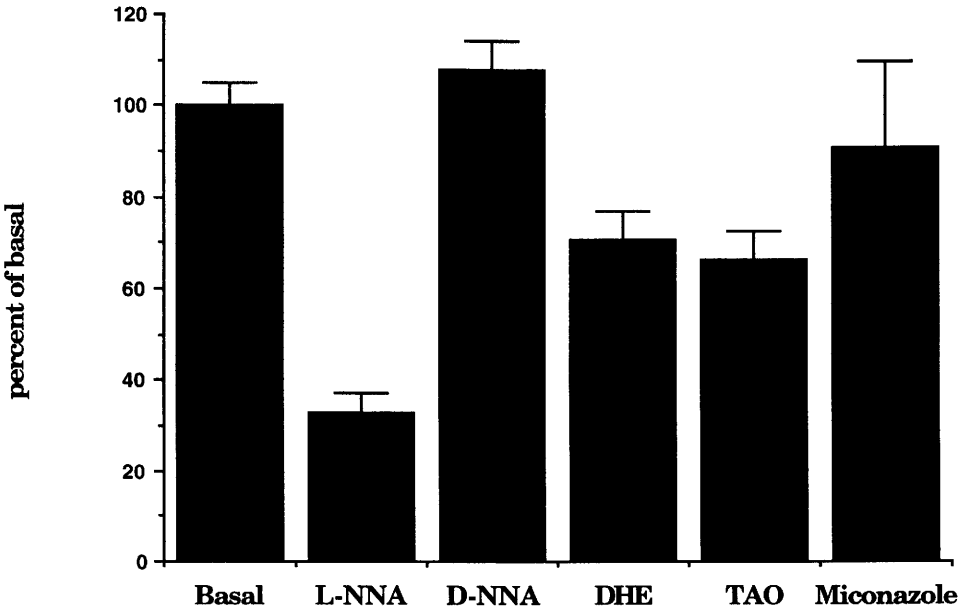

Figure 2. The effect of N-ω-nitro-L-arginine (L-NNA, 0.1 m*M*), N-ω-nitro-D-arginine (D-NNA, 0.1 m*M*), dihydroergotamine (DHE, 0.05 m*M*), miconazole (0.1 m*M*), and troleandomycin (TAO, 0.05 m*M*) on the nitric oxide synthase (NOS) activity in intact endothelial cells (n = 12 separate experiments for each column). L-NNA inhibited NOS to 32.9% ± 4.4% of basal ($P < 0.0001$), whereas D-NNA had no effect (107.8% ± 6.4%, NS). DHE and TAO significantly inhibited NOS activity to 70.6% ± 6.4% ($P < 0.005$) and 66.4% ± 6.2% ($P < 0.001$) respectively, but miconazole showed no significant effect (91.0% ± 18.9%, NS). Basal activity equals 100% of net CPM.

In the present study, it has been shown that, in case of constitutive bovine endothelial nitric oxide synthase, the enzyme is inhibited by both classical NOS and classical cytochrome P-450 inhibitors. Since miconazole, DHE, and TAO interfere with heme binding, and because NOS is a hemoprotein, similar mechanisms seem to be responsible for the inhibition of endothelial NOS by these compounds. The inhibitory effect of miconazole on the L-citrulline-forming activity of constitutive brain NOS has been previously reported, demonstrating that enzyme-bound heme is involved in NO synthesis (23). Although the inhibitory effect of miconazole, DHE, and TAO was not as potent as L-NNA, it remained significant. In intact cells, however, miconazole demonstrated no significant inhibition. The reason is not completely clear. Imidazole antimycotics, including miconazole, possess a number of pharmacological properties. For example, they interfere with Ca^{2+} influx into cells (27). Since endothelial NOS is a calcium-dependent enzyme, the effect of miconazole should be even more pronounced in intact cells. Miconazole has been shown to be effective in cell systems, and its permeability is probably no less than that of the other inhibitors. On the basis of the presented data, we are unable to explain the failure of miconazole to inhibit NOS in intact endothelial cells. D-isomer of NNA had no effect on NOS activity.

This data is in agreement with other findings on close homology and similarity between nitric oxide synthase and cytochrome P-450 reductase. However, the present results, together with the observations made by others (15), show the functional difference between cytochrome P-450 reductase and endothelial nitric oxide synthase: NOS but not cytochrome P-450 reductase is inhibited by L-arginine analogs. The pharmacological relevance of inhibition of NOS by DHE, miconazole, and TAO cannot be established on the basis of presented data.

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