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Identification of imidazole as L-arginine-competitive inhibitor of porcine brain nitric oxide synthase

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Abstract

Imidazole acts as a heme-site inhibitor of nitric oxide synthase (NOS). We used this compound to investigate whether the substrate L-arginine binds directly to the heme or to a separate domain of brain NOS. Enzyme kinetic experiments showed that imidazole enhanced the apparent K_m for L-arginine without affecting maximal enzyme activity, and binding studies revealed that the inhibitor displaced the radioligand N^G-nitro-L-[^{3}H]arginine in a concentration-dependent fashion. These results demonstrate that imidazole exerts its effects on NOS in an L-arginine-competitive manner and that the substrate site of the enzyme may be identical with the prosthetic heme group.

Key words: Nitric oxide synthase; Cytochrome P_{450}; Hydrogen peroxide; Imidazole; Radioligand binding; Enzyme kinetics

1. Introduction

Nitric oxide synthase (NOS) isozymes catalyze oxidation of the guanidino group of L-arginine to NO and L-citrulline [1–3]. The reaction involves activation of molecular oxygen by a cytochrome P_{450}-like heme group located in the catalytic center of the enzyme [4–7] and requires the pteridine H_4biopterin as a cofactor [8–10]. Reduction of oxygen requires NADPH-derived electrons shuttled to the heme by a flavin-containing reductase domain, and this electron transfer appears to represent the calmodulin-dependent step of NO synthesis [11,12]. The reductase activity of NOS can be assayed independently of NO synthesis, because the flavins also reduce exogenously added acceptor molecules such as cytochrome *c* or nitroblue tetrazolium [11]. When brain NOS is activated by Ca^{2+}/calmodulin in the presence of subsaturating concentrations of L-arginine or H_4biopterin, electron transfer from NADPH to the heme still proceeds, but uncoupling of oxygen activation from substrate metabolism results in the generation of superoxide anions and hydrogen peroxide under these conditions [13–16].

As a cytochrome P_{450}, NOS is inhibited by imidazole and various imidazole derivatives [7,17]. It has been shown that recombinant rat brain NOS exhibits type II difference spectra upon binding of imidazole, indicative of the occurrence of a low spin heme species [18]. This effect of imidazole has been attributed to displacement of a bound, as yet uncharacterized ligand of the heme. We recently found that 7-nitro-indazole, another putative heme-site inhibitor of NOS, antagonized both substrate and H_4biopterin binding to the purified brain enzyme, suggesting that the pteridine site of the enzyme may be located proximal to the heme [19]. The present study was performed to investigate: (i) whether imidazole binds to the substrate site of NOS; and (ii) whether the unknown ligand displaced from the enzyme by imidazole is identical with H_4biopterin.

2. Materials and methods

2.1. Materials

NOS was purified from porcine brain as previously described [10]. L-[2,3,4,5-^{3}H]Arginine hydrochloride (57 Ci/mmol) and N^G-nitro-L-[2,3,4,5-^{3}H]arginine hydrochloride (59 Ci/mmol) were purchased from MedPro (Amersham), Vienna, Austria. Labelled L-arginine was purified monthly by cation exchange HPLC with 50 mM sodium acetate (pH 6.5) as eluent to reduce blank levels in the citrulline assay. 3′-[^{3}H](6*R*)-5,6,7,8-Tetrahydro-L-biopterin was synthesized enzymatically from [8,5′-^{3}H]GTP [20], unlabelled H_4biopterin was obtained from Dr. B. Schircks Laboratories, Jona, Switzerland. Stock solutions of imidazole (1 M) were adjusted to pH 7.0 with hydrochloric acid and diluted with H_2O. All other chemicals were from Sigma, Deisenhofen, Germany.

2.2. Determination of NOS activities

NOS activity was determined as formation of L-[^{3}H]citrulline from L-[^{3}H]arginine. Unless otherwise indicated, purified brain NOS (0.1–0.5 μg) was incubated in total volumes of 0.1 ml for 10 min at 37°C in the presence of 50 mM triethanolamine/HCl buffer (pH 7.0), 0.1 mM L-[^{3}H]arginine (50,000–70,000 cpm), 0.5 mM $CaCl_2$, 10 μg/ml calmodulin, 0.2 mM NADPH, 10 μM H_4biopterin, 5 μM FMN, and 5 μM FAD. [^{3}H]Citrulline was separated from [^{3}H]arginine by chromatography over a Dowex 50W cation exchange resin, and radioactivity was determined by liquid scintillation counting. Blanks were determined in the absence of enzyme. For calculation of enzyme activities, total amounts of L- [^{3}H]citrulline formation were corrected for blank levels and recovery (83 ± 3% as determined with authentic L-[^{14}C]citrulline).

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Abbreviations: NOS, nitric oxide synthase; L-NNA, N^G-nitro-L-arginine; H_4biopterin, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; IC_{50}, concentration producing half-maximal inhibition.

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NOS-catalyzed formation of H_2O_2 and reduction of cytochrome *c* were assayed spectrophotometrically against calmodulin-deficient blanks as previously described [11,14].

2.3. Binding studies

Protein binding studies were performed as described [19,21]. Briefly, purified brain NOS (2–4 μg) was incubated for 10 min at 37°C with a fixed concentration of the radioligand (20 nM [^{3}H]H_4biopterin, 18 nCi or 10 nM N^G-nitro-L-[2,3,4,5-^{3}H]arginine, 60 nCi) in 0.1 ml of 50 mM triethanolamine/HCl buffer (pH 7.0). Saturation experiments were performed in the presence of increasing concentrations of the respective unlabelled ligands. Separation of bound from free radioligand was performed by polyethylene glycol precipitation and rapid vacuum filtration over Whatman glass fiber filters (GF/B). Radioactivity on the filters was determined by liquid scintillation counting.

2.4. Data evaluation

Enzyme kinetic parameters were calculated from individual double-reciprocal plots. Data obtained from binding experiments were analyzed by the GIPMAX non-linear least-squares regression curve fitting program [22] to obtain equilibrium constants. All data are mean values ± S.E.M. of three experiments.

3. Results

To investigate the mechanisms accounting for inhibition of brain NOS by imidazole, we have compared the effects of the drug on three different catalytic activities of the purified enzyme: (i) formation of L-citrulline from L-arginine; (ii) formation of H_2O_2 in the absence of L-arginine; and (iii) reduction of cytochrome *c*. As shown in Fig. 1, imidazole seemed to be a less potent inhibitor in the citrulline assay as compared to its effects on uncoupled oxygen activation. Cytochrome *c* reduction was not affected by up to 10 mM imidazole, confirming that this compound does not interfere with redox-cycling of the flavins [17]. Complete concentration–response curves recorded in the presence of 0.1 mM L-arginine (not shown) revealed an IC_{50} value for inhibition of L-citrulline formation of 6.2 ± 0.58 mM, whereas approximately 20- fold lower concentrations of the drug were sufficient to block generation of H_2O_2 (IC_{50} = 83 ± 38 μM). Since formation of H_2O_2 was measured in the absence of both L-arginine and H_4biopterin, the data indicated to us that imidazole may compete for either substrate or pteridine binding to NOS. Thus, we have performed enzyme kinetic experiments to settle the type of NOS inhibition by imidazole as well as binding studies using labelled L- NNA and H_4biopterin as radioligands [19,21] to see whether the drug interferes with substrate or pteridine binding. For enzyme kinetics, formation of L-citrulline was measured in the presence of 1–100 μM L-arginine with and without 1 mM imidazole. The representative double-reciprocal plot shown in Fig. 2 identified imidazole as a purely competitive inhibitor of brain NOS. Evaluation of the data revealed an apparent K_m for L-arginine of 6.4 ± 0.65 μM, which was increased about 4-fold to 23.6 ± 3.12 μM by 1 mM imidazole, whereas V_{max} was not significantly affected (0.62 ± 0.041 and 0.56 ± 0.076 μmol L-citrulline·mg^{-1}·min^{-1} in the absence and presence of 1 mM imidazole). From five individual plots we have calculated a kinetic K_i of 390 ± 45 μM.

These data pointed to a purely L-arginine-competitive type of NOS inhibition by imidazole, but this was in apparent conflict with previous work reporting the opposite, i.e. purely non-competitive actions of the drug [17,18]. Since recent work from our laboratory demonstrated that L-[^{3}H]NNA is a useful tool for reversible labelling of the substrate site of NOS in protein binding studies [21], we applied this method for competition experiments with imidazole to substantiate and extend the observations made in functional studies. As shown in Fig. 3 (filled symbols), imidazole antagonized binding of L-[^{3}H]NNA in a concentration-dependent manner with an IC_{50} of approximately 0.1 mM. Analysis of the data with the GIPMAX non-linear least-squares regression curve fitting program [22] yielded a K_i of 198 ± 63 μM imidazole. Saturation experiments revealed that imidazole reduced the affinity of NOS for the radioligand but had no effect on maximal binding (not shown). Finally, we used ^{3}H-labelled H_4biopterin as a radioligand to test for possible effects of imidazole on pteridine binding. However, as shown in Fig. 3 (open symbols), the drug was an only weak antagonist of [^{3}H]H_4biopterin binding and produced about 50% inhibition at 100 mM with an estimated K_i of ~50 mM.

4. Discussion

Inhibition by imidazole of reactions involving the P_{450}-like heme moiety of brain NOS and lack of effect on

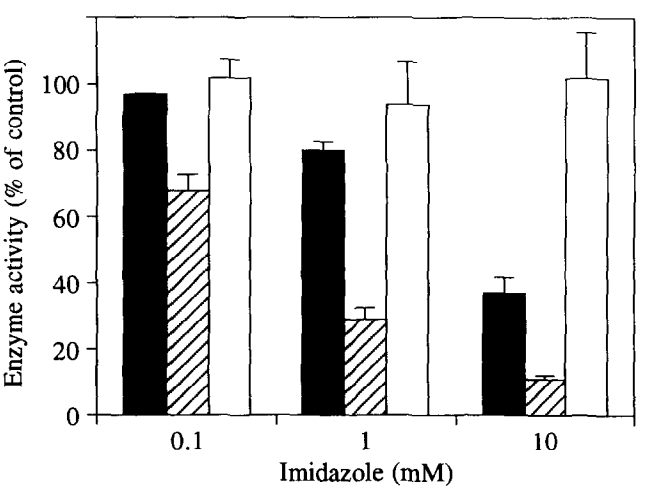

Fig. 1. Effects of imidazole on different catalytic activities of brain NOS. Formation of L-citrulline (filled) and H_2O_2 (hatched) as well as reduction of cytochrome *c* (open) were determined in the presence of the indicated concentrations of imidazole as described in section 2.2. Results are expressed as percent of enzyme activities determined in the absence of imidazole and represent means ± S.E.M. of three experiments.

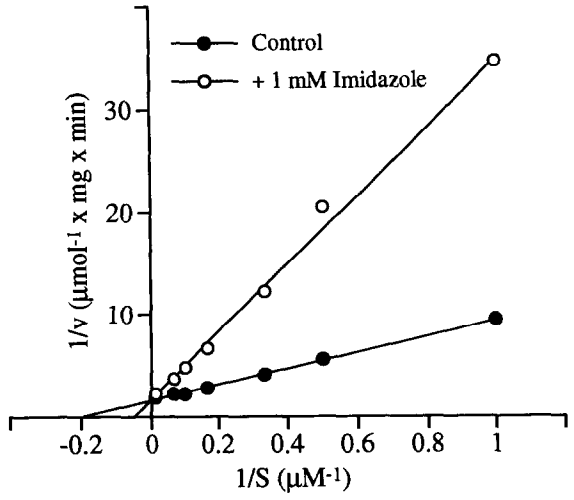

Fig. 2. Double-reciprocal plot of L-arginine concentration (1–100 μM) *versus* L-citrulline formation by brain NOS. Data are representative for five similar experiments.

reduction of cytochrome *c* is in agreement with previous reports describing the effects of imidazole or various imidazole derivatives on NOS and other cytochromes P_{450} [7,17,23–27], The present study suggests that binding of imidazole to the prosthetic heme group of brain NOS is competitive with the substrate L-arginine. This is evident from enzyme kinetic experiments and from binding studies showing that imidazole enhanced the apparent K_m for L-arginine and antagonized L-[^{3}H]NNA binding, respectively, whereas V_{max} and binding of H_4biopterin were not markedly affected by the drug. The calculated kinetic K_i (390 μM) for inhibition of citrulline formation is in accordance with the affinity constant obtained in binding studies (200 μM) and with the IC_{50} for inhibition of H_2O_2 formation (280 μM). The latter enzyme activity was assayed in the absence of L-arginine and may therefore reflect directly the affinity of imidazole for brain NOS. The equilibrium constants we have determined are similar to the K_i value reported for inhibition of purified bovine brain NOS [17] and to the spectral binding constant of the drug determined with the purified recombinant rat brain enzyme [18].

Imidazole also antagonized [^{3}H]H_4biopterin binding, but this effect became significant at high concentrations only and may therefore not contribute to inhibition of NOS activity. These data also render it unlikely that H_4biopterin represents the unidentified ligand of the heme McMillan et al. have proposed to be displaced by imidazole [18]. Since recent results point to an allosteric interaction of the substrate and pteridine domains of NOS [19], it may be concluded that the effect of imidazole on [^{3}H]H_4biopterin binding results from binding of the drug to the heme site rather than from a direct interaction with the pteridine binding domain.

Miconazole and other imidazole derivatives were identified as purely competitive inhibitors of steroid aromatase [24], and several related compounds were found to be competitive or mixed-type inhibitors of various cytochrome P_{450} mediated hydroxylation reactions [25–27]. Thus, substrate-competitive inhibition of a cytochrome P_{450} by imidazole or its derivatives is not surprising per se. However, our results are in conflict with two previous studies which have described imidazole as purely non-competitive inhibitor of brain NOS [17,18]. We have considered several possible explanations for this intriguing discrepancy but obtained no experimental evidence to support them. Importantly, different NOS preparations were used in each of the pertinent studies, and in a recent abstract Dawson et al. reported on the identification of two novel isoforms of neuronal NOS [28]. It may be speculated, therefore, that the enzyme we have used represents a distinct NOS isoform. However, experiments performed with purified rat brain NOS and crude cytosols of RAW 264.7 macrophages confirmed the present data obtained with the porcine enzyme (unpublished results). Moreover, virtually identical biochemical properties have been reported so far for neuronal NOS prepared from various sources (for reviews see [1–3]). Alternatively, different protocols applied for enzyme purification could account for the observed differences, but this is unlikely because similar results were obtained when the initial ammonium sulfate precipitation step was omitted and the ADP-Sepharose eluate was chromatographed over a gel filtration column as final purification step (not shown). Thus, at present we are unable to provide a reliable explanation for the controversial results, but future studies using other NOS preparations and additional biochemical methods may shed light on this issue.

In summary, our results demonstrate that imidazole

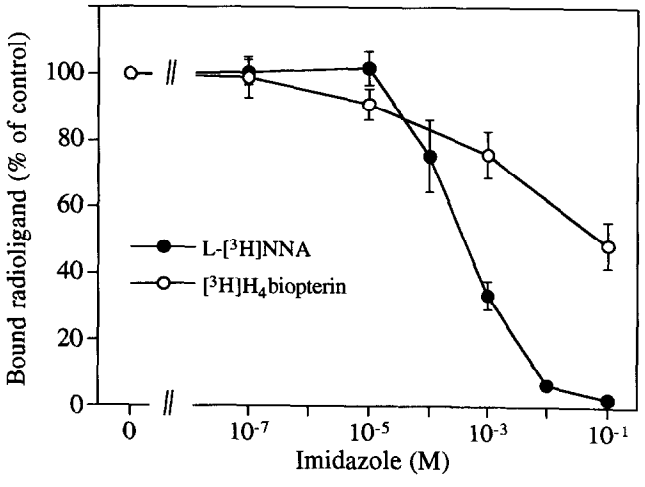

Fig. 3. Effects of imidazole on binding of L-[^{3}H]NNA (●) and [^{3}H]H_4biopterin (○) to brain NOS. Protein binding studies were carried out in the presence of increasing concentrations of imidazole as described in section 2.2. Data are expressed as percent of radioligand binding determined in the absence of imidazole and represent mean values ± S.E.M. of three experiments.

inhibits heme-catalyzed activities of NOS and binds to the enzyme isolated from porcine brain in an L-arginine-competitive manner, indicating that the substrate site of NOS may be identical with the prosthetic heme group.

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References

[1] Marletta, M.A. (1993) J. Biol. Chem. 268, 12231–12234.
[2] Mayer, B. (1993) Semin. Neurosci. 5, 197–205.
[3] Knowles, R.G. and Moncada, S. (1994) Biochem. J. 298, 249–258.
[4] White, K.A. and Marletta, M.A. (1992) Biochemistry 31, 6627–6631.
[5] Stuehr, D.J. and Ikeda-Saito, M. (1992) J. Biol. Chem. 267, 20547–20550.
[6] McMillan, K., Bredt, D.S., Hirsch, D.J., Snyder, S.H., Clark, J.E. and Masters, B.S.S. (1992) Proc. Natl. Acad. Sci. USA 89, 11141–11145.
[7] Klatt, P., Schmidt, K. and Mayer, B. (1992) Biochem. J. 288, 15–17.
[8] Tayeh, M.A. and Marletta, M.A. (1989) J. Biol. Chem. 264, 19654–19658.
[9] Kwon, N.S., Nathan, C.F. and Stuehr, D.J. (1989) J. Biol. Chem. 264, 20496–20501.
[10] Mayer, B., John, M. and Böhme, E. (1990) FEBS Lett. 277, 215–219.
[11] Klatt, P., Heinzel, B., John, M., Kastner, M., Böhme, E. and Mayer, B. (1992) J. Biol. Chem. 267, 11374–11378.
[12] Abu-Soud, H.M. and Stuehr, D.J. (1993) Proc. Natl. Acad. Sci. USA 90, 10769–10772.
[13] Mayer, B., John, M., Heinzel, B., Werner, E.R., Wachter, H., Schultz, G. and Böhme, E. (1991) FEBS Lett. 288, 187–191.
[14] Heinzel, B., John, M., Klatt, P., Böhme, E. and Mayer, B. (1992) Biochem. J. 281, 627–630.
[15] Pou, S., Pou, W.S., Bredt, D.S., Snyder, S.H. and Rosen, G.M. (1992) J. Biol. Chem. 267, 24173–24176.
[16] Culcasi, M., Lafoncazal, M., Pietri, S. and Bockaert, J. (1994) J. Biol. Chem. 269, 12589–12593.
[17] Wolff, D.J., Datto, G.A., Samatovicz, R.A. and Tempsick, R.A. (1993) J. Biol. Chem. 268, 9425–9429.
[18] McMillan, K. and Masters, B.S.S. (1993) Biochemistry 32, 9875–9880.
[19] Klatt, P., Schmid, M., Leopold, E., Schmidt, K., Werner, E.R. and Mayer, B. (1994) J. Biol. Chem. 269, 13861–13866.
[20] Werner, E.R., Schmid, M., Werner-Felmayer, G., Mayer, B. and Wachter, H. (1994) Biochem. J. (in press).
[21] Klatt, P., Schmidt, K., Brunner, F. and Mayer, B. (1994) J. Biol. Chem. 269, 1674–1680.
[22] Brunner, F. and Kukovetz, W.R. (1991) Br. J. Pharmacol. 102, 373–380.
[23] Klatt, P., Schmidt, K., Uray, G. and Mayer, B. (1993) J. Biol. Chem. 268, 14781–14787.
[24] Mason, J.I., Murry, B.A., Olcott, M. and Sheets, J.J. (1985) Biochem. Pharmacol. 34, 1087–1092.
[25] Lee, F.Y.F., Workman, P. and Cheeseman, K.H. (1987) Biochem. Pharmacol. 36, 1349–1355.
[26] Back, D.J., Tjia, J.F., Karbwang, J. and Colbert, J. (1988) Br. J. Clin. Pharmacol. 26, 23–29.
[27] Gemzik, B. and Parkinson, A. (1992) Arch. Biochem. Biophys. 296, 366–373.
[28] Dawson, T.M., Steiner, J.P., Mong, J.A. and Snyder, S.H. (1993) Soc. Neurosci. Abstr. 19, 905A.