BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **232,** 88–90 (1997)
ARTICLE NO. RC976228

Competitive Inhibition of Nitric Oxide Synthase by *p*-Aminobenzamidine, a Serine Proteinase Inhibitor

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Received January 22, 1997

***p*-Aminobenzamidine competitively inhibits bovine trypsin, human and bovine thrombin, and human plasmin, all of which act on substrates containing preferentially the L-arginyl side chain at their P_1 position. Considering the structural and functional similarity between *p*-aminobenzamidine and the L-arginyl side chain in trypsin-like serine proteinases, we investigated the interaction of *p*-aminobenzamidine with mouse brain nitric oxide synthase (NOS), which uses L-arginine as the substrate for generating NO and L-citrulline. *p*-Aminobenzamidine is a competitive NOS inhibitor ($K_i = 1.2\times10^{-4}$ M, at pH 7.5 and 37.0°C), but not an NO precursor. Therefore, *p*-aminobenzamidine affects the NO production and the trypsin-like serine proteinase action.** © 1997 Academic Press

Serine proteinases play a central role in several physiological processes and are also recognized as pathogenic factors in many diseases. Therefore, the possibility of selectively influencing serine proteinase activities by specific inhibitors appears to be of considerable interest in view of their potential therapeutic value as drugs [1-4]. In this respect, *p*-aminobenzamidine and related compounds are competitive inhibitors of trypsin-like serine proteinases. These chemicals bind to the enzyme primary specificity subsite, which recognizes preferentially the L-arginyl side chain present at the P_1 position of cationic substrates and inhibitors [1,4-10]. Considering the close structural and functional similarity between *p*-aminobenzamidine and the L-arginyl side chain in serine proteinases, we investigated the interaction of *p*-aminobenzamidine with mouse brain nitric oxide synthase (NOS), which uses L-arginine as the substrate to generate NO, regulating blood flow, neurotransmission and cytotoxicity [11]. Present results indicate that *p*-aminobenzamidine is a competitive NOS inhibitor, but not an NO precursor.

MATERIALS AND METHODS

NOS activity was analyzed in homogenates of whole brain, freshly obtained from male Swiss mouse killed by decapitation. The tissue was homogenized in 5 vol. of ice cold 5.0×10^{-2} M Hepes buffer pH 7.5, containing 5.0×10^{-4} M EDTA, 1.0×10^{-3} M dithiothreitol, and 6.0×10^{-4} M phenylmethanesulfonyl fluoride. The homogenate was centrifuged at 30,000 *g* for 60 min and the supernatant was passed through a 1.0 ml column of Dowex AG50WX-8 (Na^+ form) to remove endogenous L-arginine [12].

NOS activity was measured by production of [^{3}H]L-citrulline from [^{3}H]L-arginine. Samples (340 μl) of homogenate were incubated for 30 min at 37.0°C in a reaction mixture (final volume 400 μl) containing 1.0×10^{-3} M NADPH, 1.0×10^{-4} M [^{3}H]L-arginine (1.0 μCi/ml) and 4.5×10^{-4} M $CaCl_2$, in the absence and presence of NOS inhibitors Nω-nitro-L-arginine methyl ester, agmatine and *p*-aminobenzamidine. After incubation, the reaction was stopped by mixing 150 μl of the mixture with 2.0 ml of ice cold 2.0×10^{-2} M Hepes buffer pH 5.5, containing 2.0×10^{-3} M EDTA. The mixture was passed through a 1.0 ml column of Dowex AG50WX-8 (Na^+ form) to separate [^{3}H]L-citrulline from [^{3}H]L-arginine. The radioactivity present in the eluate was determined by liquid scintillation counting [12]. Protein content of homogenate was determined according to Bradford [13].

NO production was also assessed by measuring the conversion of oxygenated mouse hemoglobin (Hb), present in the whole brain homogenate, to its ferric derivative by reaction with nitrites. Aliquots (340 μl) of tissue homogenate were incubated with the reaction mixture containing ions and cofactors as described above, in a final volume of 400 μl, and saturating amounts (i.e., 1.0×10^{-3} M) of either unlabeled L-arginine or *p*-aminobenzamidine. After 60 min at 37.0°C, samples were centrifuged at 30,000 *g* for 15 min and the absorbance spectrum of mouse Hb recorded between 450 nm and 650 nm [14].

Dowex AG50WX-8 (Na^+ form) was purchased from Fluka Chemie AG (Buchs, CH). [^{3}H]L-arginine was obtained from Amersham International plc (Amersham, UK; 63 Ci/mmol). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

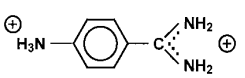

FIG. 1. Chemical structure of *p*-aminobenzamidine.

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Abbreviations used: NOS, nitric oxide synthase (from mouse brain); Hb, hemoglobin.

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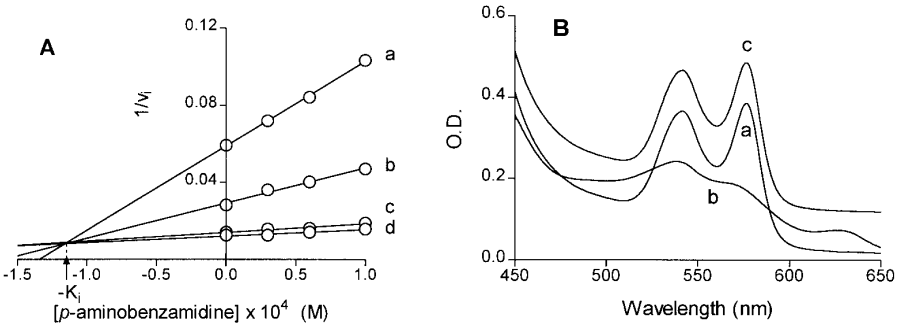

FIG. 2. (A) Dixon plot [22] for mouse brain NOS inhibition by *p*-aminobenzamidine (v_i, nmol × mg^{-1} × min^{-1}). The analysis of data allowed to evaluate a K_i value of 1.2×10^{-4} M, at pH 7.5 and 37.0°C. L-arginine concentration was 3.0×10^{-6} M (a), 1.0×10^{-5} M (b), 3.0×10^{-5} M (c), and 1.0×10^{-4} M (d). (B) Absorbance spectra of oxygenated Hb present in the mouse brain homogenate (spectrum a), of ferric Hb obtained by reaction of the oxygenated derivative with nitrites accompanying the NOS catalyzed conversion of L-arginine to NO and L-citrulline (spectrum b), and of oxygenated Hb obtained in the presence of *p*-aminobenzamidine, instead of L-arginine (spectrum c). For the sake of clarity, spectrum c was up shifted by 0.1 O.D. L-arginine and *p*-aminobenzamidine concentration was 1.0×10^{-3} M. All spectra were obtained at pH 7.5 and 37.0°C. For further details, see text.

RESULTS AND DISCUSSION

NOS activity, measured as production of L-citrulline from L-arginine and as conversion of oxygenated Hb to its ferric derivative by reaction with nitrites, is constitutively present in the whole mouse brain. As expected, the enzyme activity is inhibited by NOS inhibitors Nω-nitro-L-arginine methyl ester and agmatine [11,15,16].

p-Aminobenzamidine (see Fig. 1) competitively inhibits mouse brain NOS activity with a K_i value of 1.2×10^{-4} M, at pH 7.5 and 37.0°C (see Fig. 2, panel A, and Table 1). Multiple competitive NOS inhibitors, developed in the last few years to unravel the physiology of NO as well as to specifically block the pathological production of this diatomic molecule, are L-arginine analogues displaying K_i values ranging between 1×10^{-6} M and 1×10^{-2} M [15,16]. K_i values of most NOS inhibitors are similar to K_m values for L-arginine, ranging between 1×10^{-6} M and 1×10^{-4} M [15].

The affinity of *p*-aminobenzamidine for the mouse brain NOS is similar to that observed for inhibitor association to bovine and human thrombin as well as human plasmin, being lower than that reported for bovine trypsin [1,5,6] (see Fig. 2, panel A, and Table 1). Competitive synthetic inhibitors of trypsin-like serine proteinases display K_i values ranging between 1×10^{-8} M and 1×10^{-2} M [1,4-7,10]. K_i values of most inhibitors for bovine trypsin, bovine and human thrombin as well as human plasmin are similar to K_m values for synthetic substrates, ranging between 1×10^{-7} M and 1×10^{-4} M [1,4,7,10,17].

As already reported for most NOS inhibitors [15,16], NO does not originate from *p*-aminobenzamidine. In fact, oxygenated Hb present in mouse brain homogenates (see Fig. 2, panel B, spectrum a) was converted to its ferric derivative by reaction with nitrites accompanying the NO production from L-arginine catalyzed by NOS (see Fig. 2, panel B, spectrum b), the ferrous to ferric Hb conversion being not observed in the presence of *p*-aminobenzamidine, instead of L-arginine (see Fig. 2, panel B, spectrum c).

As a whole, present results clearly indicate that *p*-aminobenzamidine affects the NO production and the trypsin-like serine proteinase action. In this respect, it may be observed that agmatine inactivates competitively NOS [16], and agmatine derivatives inhibit bovine trypsin and thrombin [1,18,19]. Moreover, *p*-aminobenzamidine and related compounds might also affect arginase, L-arginine-glycine transaminase, kyotorphine synthase and L-arginine decarboxylase, all using L-arginine as the substrate [20]. Therefore, the use of NOS inhibitors, based on the L-arginine structure, may affect (un)related function(s). Enzyme inhibition may also occur *in vivo*, indeed *p*-aminobenzamidine derivatives permeate cell membranes [21].

TABLE 1

Values of K_i for *p*-Aminobenzamidine Binding to Mouse Brain NOS and Trypsin-like Serine Proteinases

Enzyme	K_i (M)
Mouse brain NOS[a]	1.2×10^{-4}
Bovine trypsin[b]	6.1×10^{-6}
Bovine thrombin[c]	8.0×10^{-5}
Human thrombin[b]	6.5×10^{-5}
Human plasmin[c]	1.3×10^{-4}

[a] pH 7.5 and 3.7.0°C; present study. A S.E. of ±8% was evaluated as the standard deviation for the K_i value.
[b] pH 7.4 and 25.0°C [6].
[c] pH 8.0 and 25.0°C [5].

ACKNOWLEDGMENTS

The authors thank Professor A. Bosia, Professor L. Calabrese, and Professor R. Federico for helpful discussions.

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