Identification of the 4-amino analogue of tetrahydrobiopterin as a dihydropteridine reductase inhibitor and a potent pteridine antagonist of rat neuronal nitric oxide synthase

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The binding of tetrahydropteridines with 6-di- and trihydroxypropyl side chains to recombinant rat neuronal nitric oxide (NO) synthase (EC 1.14.13.39) was determined by competition with 6*R*-[3′-³H]-5,6,7,8-tetrahydro-*L*-erythro-biopterin (6*R*-[3′-³H]₄biopterin). Although all but one of the derivatives exhibited only poor affinities (*K*_i 50 μM), the 4-amino analogue of 6*R*-H₄biopterin was a potent antagonist of 6*R*-H₄biopterin binding (*K*_i 13.2 nM). The 4-amino analogue of 6*R*-H₄ biopterin inhibited

NO synthase stimulation by the natural cofactor 6*R*-H₄biopterin with an IC₅₀ of 1 μM without affecting the basal activity observed in the absence of added 6*R*-H₄biopterin. Because the 4-amino analogue of 6*R*-H₄biopterin also inhibited dihydropteridine reductase (EC 1.6.99.7; IC₅₀ 20 μM), our results support the hypothesis that redox cycling of H₄biopterin might be required for the NO synthase reaction.

INTRODUCTION

Nitric oxide (NO) is synthesized in mammals by at least three different isoforms of NO synthase (EC 1.14.13.39) [1] and serves a surprisingly heterogeneous spectrum of biological activities, ranging from neurotransmission to blood pressure regulation and cytotoxicity [2]. All of these NO synthases are known to contain enzyme-bound 6*R*-5,6,7,8-tetrahydro-*L*-erythro-biopterin (6*R*-H₄biopterin), which in addition to haem, FAD, FMN and NADPH is required for fully active conversion of the substrate *L*-arginine to NO [3]. In addition to *L*-arginine and haem, 6*R*-H₄biopterin is also required for dimerization of the enzyme [4,5]. The mechanism of stimulation of NO synthases by 6*R*-H₄biopterin is still a matter of debate [6]. Because recycling of the free cofactor had not been detected, it was suggested that 6*R*-H₄biopterin served exclusively as an allosteric cofactor of the enzyme [7]. More recently, reduction of 6*R*-6,7-[8H]dihydrobiopterin by NO synthase has been demonstrated but this reaction required significantly higher concentrations 6*R*-6,7-[8H]dihydrobiopterin to occur at the rate of the overall reaction than are required for full stimulation of NO synthase by 6*R*-H₄biopterin [8]. Previously we characterized the pteridine-binding site of neuronal NO synthase [9] by using enzymically prepared 6*R*-[3′-³H]₄biopterin [10]. In this study we used 2-amino-4-hydroxy-5,6,7,8-tetrahydropteridines (H₄pterins) with di- and trihydroxypropyl side chains to define further the stereochemical requirements for binding and catalysis. In addition, we chose 6*R*-2,4-diamino-5,6,7,8-tetrahydro-6-(*L*-erythro-1,2,-dihydroxypropyl)pteridine (6*R*-H₄aminobiopterin) to test for the effect of replacing the C4-oxo group with an C4-amino group (Figure 1). The rationale for this choice was based on previous findings demonstrating that the side chain at C6 of 6*R*-H₄biopterin is required for binding, and that redox catalysis in addition to

binding might be required for stimulation of the enzyme activity [9]. Owing to the structural analogy of 6*R*-H₄aminobiopterin to folic acid derivatives having an amino instead of the oxo group on position 4 of the pteridine ring (e.g. aminopterin or amethopterin), which in high concentrations are capable of inhibiting dihydropteridine reductase (EC 1.6.99.7), we speculated that this substitution might interfere with recycling of the cofactor and thereby inhibit the enzyme.

EXPERIMENTAL

Materials

Recombinant rat brain NO synthase was purified from baculovirus-infected Sf9 cells as described [11,12], sheep liver dihydropteridine reductase was from Sigma (Vienna, Austria), and *L*-[2,3,4,5-³H]arginine (57 Ci/mmol) was from Amersham, purchased through MedPro (Vienna, Austria), and further purified by cation exchange HPLC before use [13]. 6*R*-[3′-³H]₄biopterin (14 Ci/mmol) was prepared enzymically from [8,5′-³H]GTP as described [10]. All pteridines were obtained from B. Schircks Laboratories, Jona, Switzerland. 6*R*-H₄biopterin (which was pure enough for use), 6-(*R,S*)-5,6,7,8-tetrahydro-*D*-erythro-neopterin [6-(*R,S*)-H₄neopterin] (which was not separated into diastereomers because it had no effect) and 6*R*-H₄aminobiopterin (77% 6*R*, 23% 6*S*; we could not obtain enough for further purification) were used as supplied by the manufacturer. 6*S*-H₄biopterin was purified from residual 6*R*-H₄biopterin, and 6-(*R,S*)-5,6,7,8-tetrahydrodictyopterin [6-(*R,S*)-H₄dictyopterin] was separated into the *R*- and *S*- diastereomers by HPLC. For H₄dictyopterin, the *R*- and *S*- diastereomers were assigned by the aid of chromatographic behaviour and CD spectra [kindly recorded by H. P. Bächinger (Portland, OR, U.S.A.) on a Jasco J-500 A (Jasco, Tokyo, Japan)]. Stereochemical

Abbreviations used: H₄aminobiopterin, 2,4-diamino-5,6,7,8-tetrahydro-6-(*L*-erythro-1,2,-dihydroxypropyl)pteridine; H₄biopterin, 5,6,7,8-tetrahydro-*L*-erythro-biopterin; H₄dictyopterin, 5,6,7,8-tetrahydrodictyopterin; H₄neopterin, 5,6,7,8-tetrahydro-*D*-erythro-neopterin.

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Figure 1 Structural formulae of pteridines used in this study

[I], H_4 biopterin = 5,6,7,8-tetrahydro-6-(*L*-erythro-1,2-dihydroxypropyl)pterin; [II], H_4 amino-biopterin; [III], H_4 dictyopterin = 5,6,7,8-tetrahydro-6-(*D*-threo-1,2-dihydroxypropyl)pterin; [IV], H_4 neopterin = 5,6,7,8-tetrahydro-6-(*D*-erythro-1,2,3-trihydroxypropyl)pterin.

purity after this processing was more than 95% 6*S* for 6*S*- H_4 biopterin, more than 95% 6*R* for 6*R*- H_4 dictyopterin and 80% 6*S* and 20% 6*R* for 6*S*- H_4 dictyopterin. All other chemicals were from Merck (Darmstadt, Germany) or Sigma (Vienna, Austria) and of the highest purity grade available.

HPLC of tetrahydropteridines

On a 1050 HPLC system (Hewlett Packard, Waldbronn, Germany), a Nucleosil 10 SA column (250 mm long, 4 mm internal diam.; Marcherey Nagl, Düren, Germany) was eluted isocratically at 0.8 ml/min with 10 mM KH_2PO_4/H_3PO_4 buffer, pH 3.0, containing 100 mM NaCl, employing an injection volume of 100 μ l. Tetrahydropteridines were monitored by UV absorption at 254 nm.

Binding of tetrahydropteridines to NO synthase

Purified rat brain NO synthase (0.5 μ g) was incubated for 10 min at 37 °C with 10 nM 6*R*-[3'- 3H] H_4 biopterin (14 nCi) with increasing concentrations of unlabelled pteridines in 0.1 ml of a 50 mM triethanolamine/HCl buffer, pH 7.0, followed by rapid vacuum filtration with the MultiScreen Assay System from Millipore (Bedford, MA, U.S.A.) and determination of the radioactivity retained on the filters by liquid-scintillation counting. Data were corrected for non-specific binding determined in the presence of 1 mM unlabelled H_4 biopterin. K_i and maximal binding (B_{max}) values were calculated from individual experiments with the GIPMAX curve-fitting program [14], by taking the K_d for 6*R*- H_4 biopterin as 250 nM [9,15].

Assay of NO synthase activity

NO synthase activity was determined as the formation of L-[2,3,4,5- 3H]citrulline from L-[2,3,4,5- 3H]arginine [13]. Incubations were performed for 10 min at 37 °C in 0.1 ml of 50 mM triethanolamine/HCl buffer, pH 7.0, containing 0.1 μ g of purified neuronal rat NO synthase, 0.1 mM L-[2,3,4,5- 3H]arginine (approx. 60000 counts per min), 0.5 mM $CaCl_2$, 10 μ g/ml calmodulin, 0.2 mM NADPH, 10 μ M 6*R*- H_4 biopterin, 5 μ M FAD, 5 μ M FMN and 0.2 mM CHAPS.

Assay of dihydropteridine reductase activity

The amount of dichlorophenol-indophenol reduced by tetrahydrobiopterin, which is generated by the dihydropteridine reductase reaction, was monitored by measuring the absorption at 620 nm for 2 min at room temperature with a microplate reader (Anthos Labtec, Salzburg, Austria). Typical assay mixtures contained 0.1 μ g of dihydropteridine reductase (specific activity 50 μ mol/min per mg), 3 μ M 6*R*- H_4 biopterin, 50 μ M dichlorophenol-indophenol, 100 μ M NADH and 0–1 mM of inhibitor in 50 mM Tris/HCl buffer, pH 7.2 (modified from [16]). For competition experiments with 6*R*- H_4 biopterin (up to 100 μ M), the amount of dichlorophenol-indophenol was increased to 150 μ M. The reaction was started by the addition of dichlorophenol-indophenol.

All values shown are means $\pm$ S.E.M. for three experiments, unless indicated otherwise.

RESULTS

Figure 2 shows the competition of tetrahydropteridines for 6*R*-[3'- 3H] H_4 biopterin binding to recombinant rat neuronal NO synthase. 6*R*- H_4 aminobiopterin (K_i 13.2 $\pm$ 1.1 nM) exhibited a 20-fold higher affinity than the natural cofactor 6*R*- H_4 biopterin itself (K_d 250 nM [9]). The other pterins were up to three orders of magnitude less potent with K_i values of 89 $\pm$ 10 μ M (6*R*- H_4 dictyopterin), 52 $\pm$ 7 μ M (6*S*- H_4 dictyopterin), 125 $\pm$ 24 μ M (6*S*- H_4 biopterin) and 338 $\pm$ 13 μ M [6(*R,S*)- H_4 neopterin]. Amethopterin (methotrexate) was inactive in concentrations up to 0.3 mM (results not shown).

Figure 2 Effects of tetrahydropteridines on 6*R*-[3'- 3H] H_4 biopterin binding to NO synthase

Purified NO synthase (0.5 μ g) was incubated for 10 min at 37 °C with 6*R*-[3'- 3H] H_4 biopterin in the presence of increasing concentrations of 6*R*- H_4 aminobiopterin (■); 6*R*- H_4 biopterin (●); 6*R*- H_4 dictyopterin (▲); 6*S*- H_4 biopterin (□, broken line); 6*S*- H_4 dictyopterin (○); 6(*R,S*)- H_4 neopterin (△). Enzyme-bound 6*R*-[3'- 3H] H_4 biopterin was determined as described in the Experimental section. Results are means $\pm$ S.E.M. for three experiments.

Figure 3 Effect of 6*R*-H₄aminobiopterin on NO synthase activity

Activity of recombinant rat neuronal NO synthase was determined with (●) or without (○) the addition of 10 μ M 6*R*-H₄biopterin in the presence of the indicated concentrations of 6*R*-H₄aminobiopterin. NO synthase activity was determined by measuring the radioactivity of [³H]citrulline formed from [³H]arginine as described in the Experimental section. Results are means $\pm$ S.E.M. for three experiments.

Figure 4 Effect of 6*R*-H₄aminobiopterin and amethopterin on dihydropteridine reductase activity

Sheep liver dihydropteridine reductase was incubated with the indicated concentrations of 6*R*-H₄aminobiopterin (●) and amethopterin (○) under standard assay conditions as described in the Experimental section. IC₅₀ values estimated from these curves were 20 μ M for 6*R*-H₄aminobiopterin and 100 μ M for amethopterin. Results are means $\pm$ S.E.M. for eight incubations.

The effects on the activity of NO synthase observed were in accordance with binding, i.e. the tetrahydropterins, which bound to NO synthase only weakly, stimulated enzyme activity only weakly. Compared with 10 μ M 6*R*-H₄biopterin (2.31 $\pm$ 0.12-fold stimulation), at 10 μ M concentration only minor stimulatory activities were observed [6*R*-H₄dictyopterin 1.18 $\pm$ 0.4-fold, 6*S*-H₄dictyopterin 1.11 $\pm$ 0.2-fold, 6*S*-H₄biopterin 1.13 $\pm$ 0.03-fold, 6(*R,S*)-H₄neopterin 1.01 $\pm$ 0.02-fold].

6*R*-H₄aminobiopterin strongly inhibited the stimulation of NO synthase by 6*R*-H₄biopterin (IC₅₀ 1 μ M), whereas the basal activity observed in the absence of added 6*R*-H₄biopterin was only slightly inhibited at concentrations as high as 1 mM (Figure 3).

As shown in Figure 4, 6*R*-H₄aminobiopterin inhibited dihydropteridine reductase activity in a concentration-dependent manner (IC₅₀ 20 μ M; complete inhibition at 300 μ M). In our hands 6*R*-

H₄aminobiopterin was more potent than amethopterin (IC₅₀ 100 μ M). For both compounds, this inhibition could not be reversed by increasing the 6*R*-H₄biopterin concentration from 3 μ M to up to 100 μ M. This is in agreement with results on other pteridine inhibitors of dihydropteridine reductase, which also did not show classical Michaelis–Menten kinetics (reviewed in [17]).

DISCUSSION

With the use of novel tetrahydropteridine derivatives, the present study underlines the high selectivity of rat neuronal NO synthase for the stereochemical configuration of the side chain at C6 of the natural cofactor, 6*R*-H₄biopterin. Even epimerization of the 2'-hydroxy group as in 6*R*-H₄dictyopterin leads to a decrease in affinity of three orders of magnitude, and an almost complete loss of ability to stimulate the reaction. A replacement of the oxo group at C4 of the pteridine ring of 6*R*-H₄biopterin with an amino group had two consequences: the affinity for NO synthase was enhanced 20-fold and the compound was converted to a fairly potent dihydropteridine reductase and very potent NO synthase inhibitor. It antagonized stimulation of the enzyme by 10 μ M 6*R*-H₄biopterin with an IC₅₀ of 1 μ M, which is in good accordance with the *K*_i of 13 nM determined in radioligand-binding studies. As found previously with low-affinity pteridine antagonists [9], 6*R*-H₄aminobiopterin did not affect basal NO synthase activity, indicating that it does not displace tightly bound endogenous 6*R*-H₄biopterin. The slight decrease in basal activity caused by high concentrations of the amino analogue (1 mM) might have been due to effects unrelated to pteridine binding and was not further investigated. The findings that NO synthases do not utilize stoichiometric quantities [7,18] of 6*R*-H₄biopterin and are not inhibited by amethopterin (methotrexate) seemed to argue against redox cycling of 6*R*-H₄biopterin. However, the results presented here shed a new light on this issue: amethopterin does not bind with appreciable affinity to the pteridine site of NO synthase because the side chain at C6, which seems to be crucial for binding, differs considerably from the side chain of 6*R*-H₄biopterin. With the amino substitution at C4, 6*R*-H₄aminobiopterin resembles the pteridine part of amethopterin and is accordingly rendered an inhibitor of dihydropteridine reductase. In contrast with amethopterin, 6*R*-H₄aminobiopterin contains the same side chain as the natural cofactor 6*R*-H₄biopterin, which explains its high affinity for the pteridine site of NO synthase. Thus 6*R*-H₄aminobiopterin exhibits the structural features essential for both inhibition of dihydropteridine reductase and binding to NO synthase. Identification of this compound as a potent pteridine antagonist of the enzyme supports the hypothesis that redox cycling of 6*R*-H₄biopterin is required for enzyme catalysis, but further studies are needed to settle this issue unequivocally. 6*R*-H₄aminobiopterin might represent the prototype of a novel class of NO synthase inhibitors that act as potent pteridine antagonists of the enzyme.

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