Volume 305, number 2, 160–162 FEBS 11234 June 1992

Stimulation of human nitric oxide synthase by tetrahydrobiopterin and selective binding of the cofactor

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Received 21 April 1992

To check the stimulatory potency of the tetrahydro forms of the two major pteridines occurring in human tissues, neopterin and biopterin, NO synthase was purified 6000-fold from human cerebellum. Tetrahydrobiopterin stimulated the activity up to 4.5-fold in a concentration dependent manner with a maximum above 1 μM, whereas tetrahydroneopterin was completely inactive in concentrations up to 100 μM. Tetrahydrobiopterin, but not neopterin derivatives, were copurified with the NO synthase activity. Our results demonstrate that human cerebellum contains a tetrahydrobiopterin dependent NO synthase activity.

Nitric oxide synthase; Human cerebellum; Neopterin; Tetrahydrobiopterin

1. INTRODUCTION

Nitric oxide (NO) is a labile, diffusible molecule, which is thought to be involved in a variety of biologically important phenomena such as neurotransmission, blood pressure regulation and cytotoxicity (for review see e.g. [1]). Nitric oxide is formed from L-arginine by NO synthase, an enzyme which has been found to occur in at least two different forms, a cytokine-inducible, calcium independent and a constitutive, calcium dependent type. With the cytokine inducible type from murine macrophages, tetrahydrobiopterin has been identified as the cytosolic component required for full activity of the enzyme [2,3]. Subsequently, also the calcium dependent, constitutive form of NO synthase isolated from porcine cerebellum [4], rat cerebellum [5] or bovine endothelial cells [6] was found to be stimulated by tetrahydrobiopterin. Moreover, studies with intact cells revealed that intracellular concentrations of tetrahydrobiopterin limit the amount of nitric oxide formed from L-arginine in cytokine treated murine fibroblasts [7] and brain endothelial cells [8] as well as in porcine endothelial cells [9]. The studies investigating stimulatory action of pteridines to NO synthase preparations from non-human sources revealed a remarkable specificity for 6-*R*-tetrahydrobiopterin. In addition, tetrahydrobiopterin was found to be bound to the enzyme purified from porcine cerebellum [10].

Like other mammals, humans are able to synthesize tetrahydrobiopterin from guanosine 5′-triphosphate (GTP). Pteridine metabolism of humans is, however, peculiar in the accumulation of neopterin derivatives in addition to biopterin in cells [11], tissues and fluids [12]. The finding of 7-neopterin derivatives in human urine suggests that a tetrahydroneopterin species might be involved in hydroxylation-type reactions in humans [13]. We were interested, therefore, whether human NO synthase can bind and utilize tetrahydroneopterin or tetrahydrobiopterin for stimulation of its activity. For this purpose, we purified NO synthase from human cerebellum, compared the stimulatory potency of the tetrahydro forms of biopterin and neopterin and checked the pteridine content of the cerebellum homogenate versus that of the purified preparation.

Abbreviations: NO, nitric oxide; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis.

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1. MATERIALS AND METHODS

Human cerebella were obtained from the Department of Forensic Medicine of the University of Innsbruck, which had been dedicated to scientific research observing the respective Austrian laws. Cerebella were collected 10–40 h post-mortem and stored for up to 30 days at −80°C. Preparation of the enzyme was carried out using a protocol optimized for porcine cerebellum [4]. Briefly, the tissue (900 g) was homogenized with an Ultra Turrax in 3 volumes of a 50 mM triethanolamine- HCl buffer, pH 7.5, containing 0.5 mM EDTA (buffer A), and the homogenate was centrifuged at 10,000 × *g* for 30 min. Solid ammonium sulfate (176 g/l; 30% saturation) was added to the supernatant, followed by centrifugation at 10,000 × *g* for 15 min. The pellet was washed once with buffer A containing ammonium sulfate (30%

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saturation) and was subsequently resuspended in a 20 mM triethanolamine-HCl buffer, pH 7.5, containing 0.5 mM EDTA and 10 mM mercaptoethanol (buffer B) followed by a further centrifugation for 40 min at 10000 × g. The supernatant was incubated for 30 min with 10 ml of 2',5'-ADP-Sepharose (Pharmacia, Freiburg, Germany) and then poured into a column. The Sepharose was washed with 100 ml buffer B containing 1 mM NADPH. NO synthase was eluted with 30 ml of buffer B containing 10 mM NADPH and concentrated by ultrafiltration (Centricon 30 from Amicon, Witten, Germany). Protein was determined according to Bradford [14], with pure bovine serum albumine (Serva, Heidelberg, Germany) as standard.

Sodium dodecylsulfate/polyacrylamide gel electrophoresis (SDS/PAGE) and immunoblotting were performed as previously described [15]. Purified NO synthase (5–20 ng) was subjected to SDS/PAGE using 8% slab gels according to Laemmli [16] and electrophoretically transferred to nitrocellulose membranes in 25 mM Tris-HCl, pH 8.3, containing 192 mM glycine, 0.02% (*m/v*) SDS and 20% (by vol.) methanol at 250 mA for 100 min. The membranes were incubated with a polyclonal antiserum raised against the porcine cerebellum enzyme [4], followed by incubation with horseradish peroxidase labelled anti-rabbit IgG antibodies. The bands were visualized with the ECL Western blotting detection system (Amersham Buchler, Braunschweig, Germany) according to the recommendations of the company.

Determination of pteridines was performed subsequent to oxidation by iodine in acidic or alkaline media according to Fukushima and Nixon [17]. Briefly, 100 μl were mixed with 5 μl 1 N HCl (for determination of the total amount of pteridines) or 5 μl 1 N NaOH (for the determination of alkali stable 7,8-dihydrobiopterin and biopterin) and 5 μl of 0.1 M iodine (solubilized in 0.25 M potassium iodide) added. Subsequent to incubation at room temperature in the dark for 1 h, 10 μl 1M HCl were added to the alkaline incubation mixture and 10 μl 0.1 M ascorbic acid were added to both mixtures. These acidic solutions were then applied to solid phase cartridges (SCX, Varian, Palo Alto, CA, USA), processed and directly introduced into an HPLC system by an AASP module (Varian) as described [18]. Pteridines were separated with a reversed phase C-18 column (Lichrospher, 250 mm × 4 mm i.d., 5 μm particle size, Merck, Darmstadt, Germany) eluted with 0.015 M potassium phosphate buffer, pH 6.0 at a flow rate of 0.8 ml/min and detected by fluorescence (excitation 350 nm, emission 440 nm).

NO synthase activity was determined as formation of [^{3}H]citrulline from [^{3}H]L-arginine as described [19]. NO synthase incubations were carried out at 37°C for 15 min in total volumes of 0.1 ml of a 50 mM triethanolamine-HCl buffer, pH 7.0, containing 0.1 mM L-[2,3-^{3}H]-arginine (50,000 to 100,000 cpm), 0.1 mM NADPH, 5 μM FAD, 10 μg/ml calmodulin, 3 μM free Ca^{2+} and 10 μM 6-*R*-tetrahydrobiopterin. In some cases (Fig. 1), varying concentrations of 6-*R*-tetrahydrobiopterin or 6-*R,S*-tetrahydroneopterin (both from Dr. Schircks Laboratories, Jona, Switzerland) were used instead of 10 μM 6-*R*-tetrahydrobiopterin. Free calcium concentrations were adjusted using mixtures of $MgCl_2$, $CaCl_2$, EDTA and EGTA as described [20]. For the determination of the stoichiometry of nitrite plus nitrate to citrulline, conditions were modified to 40 mM Tris-HCl, pH 8.0, containing 25 μM FAD, 25 μM FMN, 2 mM NADPH, and an NADPH regenerating system (0.25 U/ml glucose 6-phosphate dehydrogenase (Serva) and 20 mM glucose 6-phosphate) was added and the incubation carried out for 3 h at 37°C. Nitrate was reduced by nitrate reductase (Serva, 1.25 U/ml plus 2.5 mM freshly dissolved NADPH for 1 h at room temperature) and nitrite was subsequently determined by the Griess reaction with the method of Green et al. [21], using the stable Griess reagent form Merck.

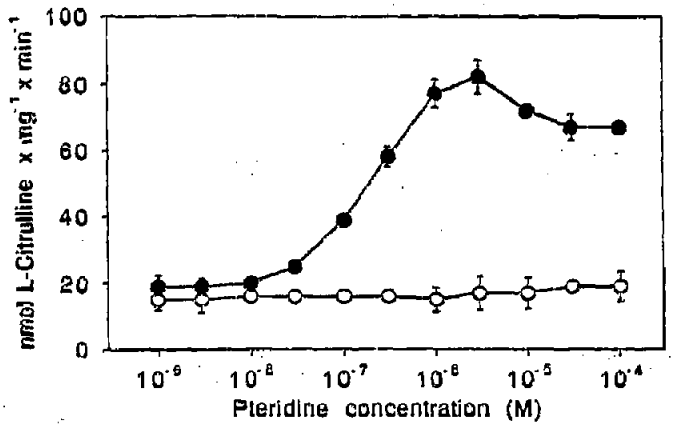

Fig. 1. Stimulation of human NO synthase by the tetrahydroforms of neopterin (○) and biopterin (●). Data represent mean values±S.E.M. (n=3).

3. RESULTS AND DISCUSSION

As shown in Table I, NO synthase was purified 6,000-fold from a 10,000 × *g* supernatant of human cerebella with a recovery of 3%. From 900 g of tissue, 80 μg of protein were obtained with a specific activity of 180 nmol citrulline × mg^{-1} × min^{-1}. The purity of the enzyme preparation was about 20% as judged from Coomassie blue stained SDS gels. In Western blots, a rabbit antiserum raised against purified porcine cerebellar NO synthase recognized one single band with an apparent molecular mass of 160 kDa. The human enzyme converted L-arginine into equal amounts of citrulline and NO_2^-/ NO_3^- in a Ca^{2+}-dependent manner. Tetrahydrobiopterin stimulated NO synthase up to 4-fold with an EC_{50} of about 0.2 μM and a maximally effective concentration of 1 μM, but tetrahydroneopterin was completely inactive in concentrations up to 100 μM (Fig. 1). The cerebellum homogenate contained both neopterin and biopterin derivatives (6.2±0.7 and 5.6±0.4 pmol/mg of protein, respectively, mean±SD, n=3). In contrast, only biopterin (227 pmol/mg of protein) remains bound to the enzyme upon purification (Fig. 2). Loss of the

Table 1

Purification of NO synthase from human cerebellum

Fraction	Protein (mg)	Specific activity (nmol·mg^{-1}·min^{-1})	Recovery (%)	Purification factor
10,000 × *g* supernatant	15,600	0.03	100	1
Ammonium sulfate precipitate	1170	0.14	35	4.7
2',5'-ADP-Sepharose eluate	0.26	81.1	4.5	2703
Ultrafiltration concentrate	0.08	181.3	3.1	6043

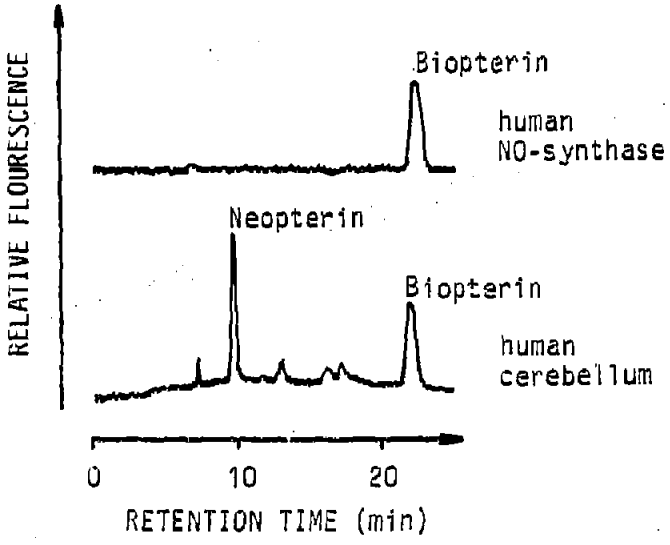

Fig. 2. HPLC profiles of pteridines in the human NO synthase preparation and the crude cerebellum homogenate.

biopterin peak upon alkaline oxidation indicates that more than 90% of the biopterin bound to human NO synthase is present in the tetrahydro form.

These results demonstrate that human cerebellum contains a Ca^{2+}-dependent NO synthase activity, which is selectively stimulated by tetrahydrobiopterin. Using calmodulin antagonists, Schmidt and Murad have shown that the calcium dependence is mediated by calmodulin [22].

The clear failure of neopterin derivatives to bind to NO synthase or to stimulate its activity render unlikely the possibility that 7-neopterin derivatives found in human urine [13] originate from a turnover of the NO synthase reaction. Tetrahydrobiopterin deficiency in human brain can occur in inherited disorders of the biosynthesis of tetrahydrobiopterin from GTP as well as by deficiencies of the dihydropteridine reductase enzyme, which keeps the cofactor in its active tetrahydro form. It is well known that these inherited disorders of tetrahydrobiopterin synthesis and/or recycling lead to severe mental retardation (reviewed in [23]). Milder forms of tetrahydrobiopterin deficiency have been observed in several neurological disorders [23]. Our results suggest that, in addition to the known roles of tetrahydrobiopterin in the hydroxylation of aromatic amino acids in neurotransmitter synthesis [24–26], tetrahydrobiopterin deficiency might also lead to impaired NO formation in human brain and this might contribute to the neurological symptoms observed.

Acknowledgements: Supported by the Deutsche Forschungsgemeinschaft (Bo-888/2-1), Österreichische Nationalbank (3474), Österreichische Forschungsgemeinschaft (support of P.K.) as well as Österreichischer Fonds zur Förderung der Wissenschaftlichen Forschung (8231), is gratefully acknowledged.

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