

# NADH stimulates endogenous dopamine biosynthesis by enhancing the recycling of tetrahydrobiopterin in rat phaeochromocytoma cells

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## Abstract

Treatment of Parkinson patients with L-DOPA (3,4-dihydroxy-L-phenylalanine) leads to endproduct inhibition of tyrosine hydroxylase, the key enzyme in dopamine biosynthesis and the enzyme needing tetrahydrobiopterin and iron as cofactors. To overcome this problem an alternative treatment was investigated which attempted to stimulate endogenous dopamine biosynthesis. Incubation of rat PC 12 cells with NADH ( $\beta$ -nicotinamide adeninedinucleotide) leads to increased dopamine production. We investigated the possibility that this increase of dopamine biosynthesis was due to stimulation of quinonoid dihydropteridine reductase, the enzyme which recycles the inactive dihydrobiopterin to the active tetrahydrobiopterin. The experiments showed that whereas NADH is able to increase dopamine production in PC 12 cells (rat phaeochromocytoma cells, clone PC 12) up to three-fold, no influence is exerted by NADH on pteridine metabolism; neither are tetrahydrobiopterin concentrations nor the de novo-biosynthesis of pteridines from guanosine triphosphate altered by NADH. Further no influence of NADH on protein de novo synthesis of quinonoid dihydropteridine reductase was observed. However, NADH was able to directly increase the catalytic activity of this enzyme. Our results suggest that the stimulation of dopamine biosynthesis by NADH is due to more rapid regeneration of quinonoid dihydrobiopterin to tetrahydrobiopterin.

*Keywords:* Parkinson's disease; Dopamine biosynthesis; NADH; Quinonoid dihydropteridine reductase; Tetrahydrobiopterin; PC 12 cell; (Rat)

## 1. Introduction

Parkinson's disease is characterized by a functional deficit of the neurotransmitter dopamine in the striatum and a loss of dopaminergic cells in the zona compacta of the substantia nigra. The result is a diminished presence of the tyrosine hydroxylase, the key enzyme in dopamine biosynthesis [1,2], an enzyme which needs tetrahydrobiopterin as cofactor.

Investigations regarding the localisation of tetrahydrobiopterin have suggested that it is concentrated in nigrostriatal dopaminergic neurons [3,4]. The concentration of the cofactor tetrahydrobiopterin [5–8] as well as the activity of the DHPR (quinonoid dihydropteridine reductase), the enzyme which recycles the inactive quinonoid dihydrobiopterin to tetrahydrobiopterin, are diminished, too, in the basal ganglia of Parkinson patients [9].

Clinical experiments using tetrahydro(bio)pterin have been contradicting: Nagatsu et al. [10] and

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Nichol et al. [11] demonstrated that oral administration of tetrahydrobiopterin to Parkinson patients does not lead to improvement of clinical symptoms, because tetrahydrobiopterin is not able to cross the blood brain barrier. However, Kaufmann et al. [12] reported an increased content of tetrahydropterin in CSF after administering tetrahydropterin peripherally and suggested that the administration of the tetrahydropterin may prove to be a treatment not only for the impaired peripheral phenylalanine metabolism, but also for neurologic disorders of the variant forms of hyperphenylalaninaemia. Tetrahydrobiopterin replacement therapy has also been tried with only limited success in several neuropsychiatric diseases like endogenous depression [13] and familial dystonia [14–16]. Further attempts to increase intraneuronal tetrahydrobiopterin content through intracerebral injections of tetrahydrobiopterin in rats have been successful [17]. Systemic administration of tetrahydrobiopterin to rats increases brain tetrahydrobiopterin [18] but not to levels sufficiently high to enhance brain biogenic amine synthesis [19]. Anastasiadis et al. [20] reported a linear and concentration dependent uptake of tetrahydrobiopterin in cultured PC12 cells. Although there was only one clinical case of Parkinson's disease, Kondo et al. [21] reported that oral administration of tetrahydropterin improved the clinical symptoms of Parkinson's disease.

On the other hand, dihydropteridine reductase is coupled to the  $\text{NAD}^+/\text{NADH}$  redox system. With this knowledge in mind we studied dopamine and tetrahydrobiopterin biosynthesis under the influence of NADH *in vitro* in rat phaeochromocytoma cells (clone PC 12), which have a metabolism comparable to the substantia nigra nerve cells (they develop from the same germ layer).

These experiments were performed firstly in order to find out whether NADH is able to stimulate endogenous dopamine biosynthesis. If this would be the case, the well known endproduct inhibition of L-DOPA which is used as classical medication in Parkinson patients [22], could be eliminated. If the endogenous L-DOPA biosynthesis could be stimulated in addition to the already increased activity of the residual tyrosine hydroxylase in the remaining neuronal cells of basal ganglia, L-DOPA would not be produced in such a surplus that the endogenous activity of tyrosine hydroxylase could be influenced.

Secondly, the influence of NADH on tetrahydrobiopterin *de novo* synthesis was tested by measuring the activity of the key enzyme of pteridine biosynthesis, e.g., GTP-cyclohydrolase I (E.C. 3.5.4.16) and the concentrations of biopterin itself. Further the influence of NADH on protein *de novo* synthesis of the DHPR (E.C. 1.6.99.7) was investigated. Finally, a possible effect of NADH on catalytic activity of DHPR was studied.

## 2. Materials and methods

PC 12 cells were cultured in RPMI medium (Roswell Park Memorial Institute, RPMI 1640 from Boehringer, Mannheim, Germany) with 10% horse serum and 5% fetal calf serum in 75 cm<sup>2</sup> tissue culture flasks (Szabo, Vienna, Austria) at 37°C in 5% CO<sub>2</sub>–95% air and 95% humidity. The cells were cultured as monolayers. When the experiments were started, the PC 12 cells were in a stationary phase referring to growth. This was achieved by changing the medium during cultivation every second day. The time interval between first and last recultivation before the experiment was always the same. 48 h before starting the experiment the cells were centrifuged at low speed, the medium discarded and  $1 \cdot 10^7$  cells were recultivated in new culture flasks with 20 ml fresh medium. On the day of carrying out the experiments, the cells were washed with RPMI medium (without horse and calf serum) and subsequently each of  $4 \cdot 10^7$  cells were incubated for 0, 4, 8 or 24 h parallelly with 15 ml RPMI medium alone or together with 400 µg NADH/ml medium. After incubation the medium was collected and stored until analysis at –70°C. The dopamine content in the medium was determined, after deproteinisation with 0.4 M perchloric acid, by HPLC (high performance liquid chromatography) using electrochemical detection [20,23]. The Coulochem electrochemical detector Model 5100 A (ESA, Wiggins Avenue, Bedford, MA) was used in connection with a high sensitivity analytical cell (ESA-Model 5011). Separation was achieved by a catecholamine HR-80 column (4.6 · 80 mm, packed with the micron C 18 stationary phase and purchased from ESA) with ESA mobile phase for catecholamines.

The cells were washed with saline, the cell pellet

resuspended in 1 mM dithioerythrol and 0.1 mg/ml Pefabloc and subsequently frozen in liquid nitrogen to break up the cells. For analysis the cells were thawed and homogenized (UltraThurax, Model 1510, B. Braun, Melsungen, Germany) and subsequently an aliquot of these cell extracts was freed from low molecular weight compounds by chromatography on small Sephadex G-25 desalting columns NAP-5 (Pharmacia, Uppsala, Sweden) using the assay buffer as eluent, which contained 0.1 M 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride (Tris-HCl), pH 7.8; 5 mM EDTA, 0.3 M KCl and 10% (v/v) glycerol.

In these purified cell extracts GTP-cyclohydrolase I was estimated according to the method of Viveros et al. [24]. An aliquot of the obtained protein fraction was incubated with 2 mM guanosine triphosphate (GTP) for 90 minutes at 37°C in the dark. The reaction was terminated by addition of 0.1 M (final concentration) HCl and the 7,8-dihydroneopterin triphosphate oxidized to neopterin phosphates by 0.01 M KI · I<sub>2</sub> by incubation for 1 h in the dark at room temperature. Insoluble material was removed by centrifugation (10000 × *g*, 2 min), the iodine excess destroyed by 0.01 M ascorbic acid. After neutralisation, using NaOH, the phosphates were cleaved by alkaline phosphatase for one hour at 37°C in the dark. Afterwards neopterin was determined by HPLC (Waters 474 Scanning Fluorescence Detector, Waters, Vienna, Austria) using fluorescence detection (353 nm excitation, 438 nm emission wavelengths). As analytical column a ready-to-use cartridge was employed (Hibar LiChroCart, 125 × 4 mm, E. Merck, Darmstadt, Germany), packed with 7 μm reversed phase C-18 material (LiChroSorb, RP 18, E. Merck). For protection of the analytical column, a guard cartridge was used (Hibar LiChroCart, 4 × 4 mm, E. Merck) packed with the same material.

Aliquots of sonicated cell homogenates were oxidized in acidic and in alkaline solution. During alkaline oxidation only dihydrobiopterin is converted into biopterin, tetrahydrobiopterin undergoes side chain loss and conversion to compounds different from biopterin. Alkaline oxidation must therefore be carried out very quickly by addition of NaOH · I<sub>2</sub> (1 N NaOH with 0.1 M I<sub>2</sub> solved in 0.25 M KI in a mixture of 1:1) and incubation for 1 h in the dark at room temperature. If one carries out the alkaline

oxidation step not quickly enough, dihydrobiopterin may be formed from tetrahydrobiopterin by air oxidation; the analysis in that case would suggest too low tetrahydrobiopterin values. After incubation 1 N HCl is added, insoluble material centrifuged and the iodine excess destroyed by 0.1 M ascorbic acid. During acid oxidation dihydro- and tetrahydrobiopterin are oxidized by 0.01 M iodine solution (1 N HCl with 0.1 M I<sub>2</sub> in 0.25 M KI<sub>2</sub> in a mixture of 1:1) to the fluorescent biopterin [25]. After acidic or alkaline oxidation of aliquots of sonicated cell homogenate, biopterin was measured by HPLC, using fluorescence detection and the same chromatographic conditions as for determination of neopterin (which of course has a different retention time). Thus, the amount of tetrahydrobiopterin is estimated by subtracting the biopterin concentration found after alkaline oxidation from the value obtained after acidic oxidation.

In another aliquot of sonicated cell homogenate DHPR activity was measured. The principle of the assay is the reaction of tetrahydrobiopterin formed in the DHPR reaction with dichlorophenol-indophenol to yield quinonoid dihydrobiopterin, which is again substrate for DHPR. By this redox reaction, the blue colour of dichlorophenol-indophenol disappears in proportion to the DHPR activity. The assay endconcentration was as following: 0.05 M Tris, pH = 7.3; 10 μM tetrahydrobiopterin; 0.1 mM NADH, 0.002–0.05 U DHPR and 0.1 mM dichlorophenol indophenol. Absorbance decrease was measured at 620 nm and 25°C during the first ten minutes of the reaction after addition of dichlorophenol indophenol to the incubation assay. To test whether the enzymatic activity of DHPR can be increased by higher concentrations of NADH than used in the classical assay, the DHPR determination was performed in further experiments by adding 1000, 2000, and 3000 μg NADH to the assay in the cuvette for three replicas.

NADH (β-nicotinamide adenine dinucleotide, reduced form, disodium salt) was purchased from Boehringer, dopamine, 2,6-dichlorophenol-indophenol, sodium salt (2,6-dichloro-*N*-(4-hydroxyphenyl)-1,4-benzochinonimin, sodium salt) and dithioerythritol from Sigma (St. Louis, MO, USA), (6R)-5,6,7,8-tetrahydro-L-biopterin dihydrochloride from Schircks (Jona, Switzerland). Pefabloc and all other reagents were obtained from E. Merck and were of reagent grade.

Statistical evaluation of results was performed by two-way analysis of variance (ANOVA). Here, concentrations of dopamine and tetrahydrobiopterin, and activity of GTP-cyclohydrolase I were used as dependent variables. The effects of the factors NADH (absence versus presence) and incubation time (4, 8, 24 h) were determined. For analysis of the effect of NADH on DHPR activity ANOVA for repeated measurements was used. We employed program BMDP2V, BMDP Statistical Software (Cork, Ireland).

### 3. Results

As shown in Fig. 1 the dopamine content in the culture medium increased up to threefold after incubating  $4 \cdot 10^7$  cells with  $400 \mu\text{g}$  NADH/ml medium, compared with controls. By two-way ANOVA, both incubation time and NADH were shown to be highly significant factors determining dopamine concentration ( $P < 0.0001$  for both variables).

As determined by alkaline and acidic oxidation of cell extracts, all the biopterin we found in the cell homogenate occurred in the tetrahydro form (details not shown). Whereas incubation time significantly influenced tetrahydrobiopterin levels ( $P < 0.0001$ ; ANOVA), no significant difference ( $P = 0.72$ ) was seen between controls and cells incubated with  $400 \mu\text{g}$  NADH/ml medium (Fig. 2).

Similarly lack of influence of NADH ( $P = 0.88$ ) but significant effect of incubation time ( $P < 0.0001$ )

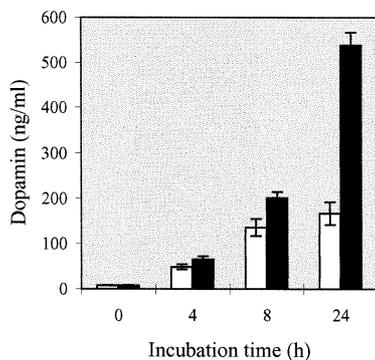


Fig. 1. Dopamine concentrations in ng/ml medium (mean and error bars SD) after incubation of  $4 \cdot 10^7$  PC 12 cells in 15 ml RPMI medium without NADH (white bars) and with  $400 \mu\text{g}$  NADH/ml (black bars) for 0, 4, 8 and 24 h.

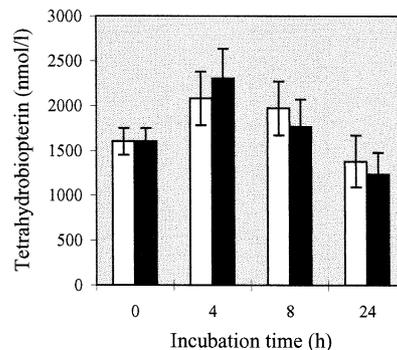


Fig. 2. Tetrahydrobiopterin concentration in nmol/l cell homogenate (means and error bars SD) after incubation of  $4 \cdot 10^7$  PC 12 cells in 15 ml RPMI medium without NADH (white bars) and with  $400 \mu\text{g}$  NADH/ml (black bars) for 0, 4, 8, and 24 h.

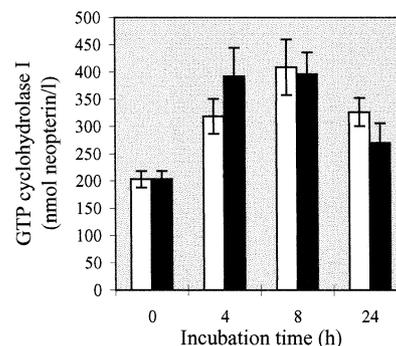


Fig. 3. Activity of GTP-cyclohydrolase I in nmol neopterin/l (means and error bars SD) in purified cell extracts after incubation of  $4 \cdot 10^7$  PC 12 cells in 15 ml RPMI medium without NADH (white bars) and with  $400 \mu\text{g}$  NADH/ml (black bars) for 0, 4, 8 and 24 h.

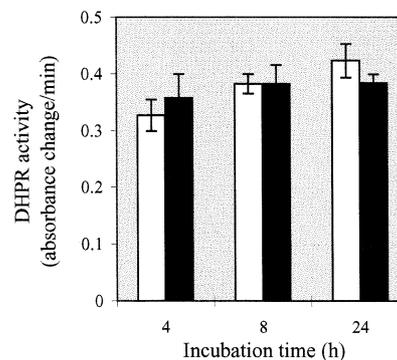


Fig. 4. DHPR activity in sonicated cell homogenates after incubation of  $4 \cdot 10^7$  PC 12 cells in 15 ml RPMI medium without NADH (white bars with SD) and with  $400 \mu\text{g}$  NADH/ml (black bars with SD) for 0, 4, 8 and 24 h.

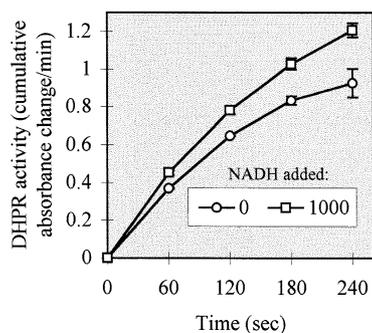


Fig. 5. DHPR activity after further addition of 0 or 1000  $\mu\text{g}$  NADH directly to 1 ml of PC 12 cell homogenate to the classical assay and subsequent measurement of enzymatic activity (decrease of absorbance at 620 nm during the first 4 min of the assay).

were observed for the activity of GTP-cyclohydrolase I (Fig. 3).

Nearly the same result was obtained by measuring the activity of DHPR after having incubated the cells with or without NADH. Thus we found no evidence for an influence of NADH on de novo synthesis of DHPR (Fig. 4). To investigate whether the enzymatic activity of DHPR can be stimulated directly by NADH, different amounts of NADH were added to the cell homogenate to the classical assay in the cuvette. Addition of 1000  $\mu\text{g}$  NADH to 1 ml homogenate increased absorbance change significantly ( $P = 0.015$ ; ANOVA for repeated measurements) (Fig. 5). Addition of higher concentrations of NADH (2000 or 3000  $\mu\text{g}$ , data not shown in detail) led to a saturation of this substance in the assay; only a small additional absorbance change was observed in comparison to the 1000  $\mu\text{g}$  value.

#### 4. Discussion

This study confirms that NADH is able to stimulate endogenous dopamine biosynthesis. The significant increase of dopamine concentration in the culture supernatants after incubation of the cells with 400  $\mu\text{g}$  NADH/ml shows that NADH influences the activity of the key enzyme tyrosine hydroxylase. In previous experiments we found that the activity of tyrosine hydroxylase is stimulated up to 75% by 400  $\mu\text{g}$  NADH/ml incubation medium [26]. However we are aware of the possibility that the change of

dopamine concentration in the incubation medium is not only a function of the activity of tyrosine hydroxylase, it may also reflect synthesis, transport, release and metabolism changes in other pathways. Further the increase of dopamine concentration could be due to the possibility of the inhibitory effect on dopamine autooxidation by tetrahydrobiopterin/DHPR-mediated antioxidation system. Shen R.S. reported that tetrahydrobiopterin, in combination with NADH and DHPR, acted as an antioxidation system during dopamine autooxidation and this antioxidation system required the simultaneous presence of tetrahydrobiopterin, NADH and DHPR [27]. PC12 cells are susceptible to toxicity caused by xanthine/xanthine oxidase, hydrogen peroxide and dopamine. Shen et al. also reported that the toxicities of these agents were reduced by the simultaneous presence of tetrahydrobiopterin, NADH and DHPR — tetrahydrobiopterin antioxidation system [28].

We indicate that the stimulating effect of NADH on dopamine biosynthesis is mainly based on supplying the tyrosine hydroxylase with sufficiently tetrahydrobiopterin cofactor by speeding up the recycling pathway from dihydrobiopterin to tetrahydrobiopterin via the DHPR system. A further argument that the increased dopamine concentration is due to increased activity of tyrosine hydroxylase is, that only NADH is offered to the incubation medium of PC12 cells in surplus. If the endogenous concentrations of tetrahydrobiopterin, NADH and DHPR of PC12 cells would be sufficiently to reduce the susceptibility of these cells to toxicity caused by xanthine/xanthine oxidase and hydrogen peroxide, the PC12 cells incubated as controls without NADH should be able to produce dopamine to the same extent as PC12 cells incubated with NADH. Besides we found that whether the quantity of DHPR enzyme protein is influenced by NADH nor the absolute concentration of tetrahydrobiopterin cofactor, which is in all probability consumed by the tyrosine hydroxylase immediately.

Previous studies have shown that the GTP cyclohydrolase I activity in mononuclear blood cells from patients with juvenile Parkinsonism is normal as compared to healthy controls [29]. This indicates that the decreased dopamine level in the basal ganglia of juvenile Parkinsonism is not due to decreased activity of GTP cyclohydrolase I, the enzyme responsible for the de novo synthesis of the tetrahydrobiopterin co-

factor of tyrosine hydroxylase. The question remains why the concentration of the tetrahydrobiopterin cofactor is diminished in the striatum and cerebrospinal fluid of Parkinson patients as previous studies have demonstrated [5–8].

Under normal physiological conditions, this reduced form constitutes approximately 80–90% of total biopterin in tissues [30,31].

Anastasiadis et al. [20] also measured catecholamine levels in PC 12 cells following exposure to tetrahydrobiopterin. They found that the intracellular levels of dopamine and L-DOPA were significantly increased following incubations with tetrahydrobiopterin. Concentrations of norepinephrine, dihydroxyphenyl acetic acid and homovanillic acid were not changed by tetrahydrobiopterin in either the intracellular or extracellular compartments. These results demonstrate that tetrahydrobiopterin is actually entering the PC 12 cells, where it is interacting with tyrosine hydroxylase to enhance L-DOPA and dopamine production. Further they suggest that the uptake of tetrahydrobiopterin is a saturable process, because neither glucose nor sodium could enhance it. Moreover, the uptake of tetrahydrobiopterin into PC 12 cells becomes asymptotic over time. After twenty minutes incubation time of PC 12 cells together with tetrahydrobiopterin it appeared that the intracellular concentration had reached equilibrium with the extracellular concentration.

In our experiments the concentration of tetrahydrobiopterin as well as the activity of GTP-cyclohydroxylase I in the PC 12 cells were not influenced by NADH. These findings suggest that NADH is not able to stimulate the de novo synthesis of tetrahydrobiopterin from guanosine triphosphate.

Further, lack of influence of incubating the cells with NADH on DHPR de novo synthesis was found. We thought that NADH perhaps is able to force mitochondrial oxidative phosphorylation and further its enzyme systems could be able to influence gene expression for de novo synthesis of DHPR enzyme protein, because during mitochondrial oxidative phosphorylation four enzymes (complexes I–IV) transport electrons from NADH (or succinate) to oxygen and pump protons out of the mitochondria to form an electrochemical gradient. The fifth enzyme (complex V) uses the electrochemical gradient to synthesize adenosine triphosphate (ATP) from adenosine

diphosphate (ADP). These five enzymes are assembled from 13 polypeptides coded by the mitochondrial DNA and approximately 50 polypeptides coded by the nuclear DNA. In addition the mitochondrial DNA codes two ribosomal ribonucleic acids (RNA) and 22 transfer RNAs necessary for mitochondrial DNA gene expression [32,33]. As Parker et al. [34] and Shapira et al. [35] described complex I defects in platelets and substantia nigra from patients with Parkinson's disease, NADH could be effective at this site. But the increase of enzymatic activity after addition of higher amounts of NADH directly to the enzyme assay suggests that NADH increases dopamine biosynthesis simply by its reductive capacity via enhancing the recycling of quinonoid dihydrobiopterin to tetrahydrobiopterin.

The comparison of these studies in PC12 cells to what may occur in Parkinson neurons is unclear. But previous studies have shown that NADH is able to stimulate dopamine biosynthesis in Parkinsonian patients too [36,37]. Measurement of L-DOPA and dopamine concentrations in the brain, in particular in substantia nigra in Parkinsonian patients before and after NADH treatment is impossible for the time being. Therefore homovanillic acid, the metabolic product of dopamine was measured in the urine in these studies, and the level of this substance increased after NADH treatment parallelly to the improvement in disability.

Summarizing our results, we found no explanation for the well established stimulation of dopamine biosynthesis by NADH other than the cosubstrate function of this compound in the DHPR reaction.

Since this direct action of NADH was found in PC12 cells in vitro, further experiments are scheduled to inquire into the details of NADH effects upon intact cells.

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