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Stimulation of dopamine biosynthesis in cultured PC 12 phaeochromocytoma cells by the coenzyme nicotinamide adeninedinucleotide (NADH)

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Summary. The activity of the tyrosine hydroxylase, the enzyme which is diminished in the brains of Parkinson patients, has been measured in cultured PC 12 rat phaeochromocytoma cells. In the same way dopamine content in the medium after incubating these cells with or without NADH was assayed. The experiment shows that NADH is able to increase the activity of the tyrosine hydroxylase and dopamine – production in PC 12 cells up to 6 times.

The results provide evidence that NADH is able to stimulate dopamine – biosynthesis directly.

Keywords: Parkinson's disease, tyrosine hydroxylase, nicotinamide adenine dinucleotide (NADH), dopamine, PC 12 cells.

Introduction

The biochemical cause of Parkinson's disease (PD) is a degeneration of dopaminergic nerve cells of substantia nigra. Previous studies have shown that the first and rate limiting step in the biosynthesis of dopamine (D), namely the conversion of the amino acid tyrosine into L-DOPA, catalyzed by the enzyme tyrosine hydroxylase is concerned (Mc Geer et al., 1971). The activity of this enzyme is considerably reduced in the brain of parkinsonian patients (PP) (Loyd et al., 1975). Tyrosine hydroxylase is an iron containing protein with tetrahydrobiopterin (BH4) as cofactor (Nagatsu et al., 1964). As shown by Nagatsu and his group BH4 itself is also diminished in the brain of PP. This BH4 deficiency could be due either to a decreased biosynthesis or to a lack in the biologically active form of BH4. We know that BH4 is synthesized from dihydropterin (BH2) by the quinonoid-dihydropteridine reductase. This enzyme is coupled on the NAD/NADH redox system (Nichol et al., 1985). If BH4 in active reduced form is not

sufficiently available, NADH should lead to an increased synthesis of BH4 and due to this to an increased L-DOPA synthesis via activation of tyrosine hydroxylase.

With this concept in mind PP were treated with NADH (reduced form), in order to enhance the endogenous L-DOPA and D-production in the brain (Birkmayer et al., 1989). The classical therapy is treatment with L-DOPA, the immediate precursor of D (Cotzias et al., 1967). This is a substitution therapy where the endproduct of the reaction of the tyrosine hydroxylase, L-DOPA was given exogenously (Birkmayer and Riederer, 1983). Biochemically an overload with L-DOPA will lead to the well known phenomenon of endproduct inhibition resulting in a further decrease of endogenous L-DOPA biosynthesis and in a reduction of the enzymatic activity of tyrosine hydroxylase itself (Ames et al., 1978). Therefore a new strategy in the treatment of PD was envisaged, namely the stimulation of the endogenous L-DOPA biosynthesis via activation of the key enzyme tyrosine hydroxylase.

The PP treated with NADH showed a very good clinical improvement of their disabilities (Birkmayer et al., 1989). The mechanism of NADH action however was unclear, as NADH is the coenzyme also of many other enzyme systems in addition to tyrosine hydroxylase. Therefore the beneficial effects of NADH in Parkinson's disease might include other enzymatic reactions too.

In order to elucidate the mechanism of NADH action, the stimulation of L-DOPA biosynthesis by NADH was studied in cultured phaeochromocytoma (PC 12) cells, which are known to produce L-DOPA in rather large quantities.

Material and methods

Rat phaeochromocytoma cells, clone PC 12, were cultured in RPMI medium (Roswell Park Memorial Institute, RPMI 1640 from Boehringer Mannheim) with 10% horse serum and 5% fetal calf serum in 75 cm^2 tissue culture flasks (Szabo Vienna) at 37°C in 5% CO_2 –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. In the first experiment a known number of cells were incubated for three hours with 15 ml culture medium. After incubation the medium was collected and stored for a few days at –70°C. New medium together with 250 mcg NADH/ml was added for another three hours to the same cells. In all other experiments cells were parallely incubated with standard medium or medium with different amounts of NADH.

The D content in the medium was determined by HPLC using electrochemical detection. The Coulochem electrochemical detector Modell 5100 A from ESA was used in connection with a high sensivity analytical cell ESA-Modell 5011. Separation was achieved by a catecholamine HR – 80 column (4,6 × 80 mm, packed with the micron C 18 stationary phase) purchased from ESA (45 Wiggins Avenue, Bedford, MA). In the same mode the effectiveness of the DOPA decarboxylase inhibitor M-hydroxy benzylhydrazine (final concentration in the incubation medium 5×10^{-4} M) on the D-synthesis in the PC 12 cells was tested. For each figure in the result section five experiments were carried out. The data are presented as means ± SEM. Statistically the data were subjected to the Student's t test.

The enzyme tyrosine hydroxylase was estimated according to the method of

Mc Geer et al. (1967) with some modifications. The PC 12 cells were homogenated with an Ultrathurax (Labsonic 1510, B. Braun) in 0.25 M sucrose and incubated for 20 minutes in a potassium phosphate buffer pH 6,4, containing 3×10^{-3} M DMPH4 (6,7 Dimethyl 5,6,7,8 – tetrahydropterin hydrochloride and 6×10^{-2} M 2-mercaptoethanol. 10 mM final concentration of iron was added as iron sulfate ($FeSO_4 \cdot 7H_2O$). The reaction was started by addition of 0,1mcCi L-U-^{14}C tyrosine (specific activity 463 mCi/mmol), purchased from Amersham. The reaction was stopped by adding perchloric and acetic acid containing as carrier each 0,2mcg of L-DOPA, D and noradrenaline. Sodium hydroxyde increased the pH to 9 and precipitated the protein. The supernatant was extracted by acidified aluminium oxide. The bound L-DOPA was reextracted by acetic acid and counted with 10 ml of scintillation liquid (Hi Safe II, LKB) in a β-counter. NADH (β-nicotinamide adenine dinucleotide, reduced form, disodiumsalt), M-hydroxybenzylhydrazine, DMPH4 and D were purchased from Sigma (St. Louis, MO, USA). All other reagents used were obtained form MERCK and were of reagent grade.

Results

In the first experiment a known number of cells were incubated for three hours with culture medium, after this time the medium was collected and new medium together with 250 mcg NADH/ml was added for another three hours. The medium was collected and the D content was determined (Fig. 1). The D content in control medium was 6.32 ± 1.3 ng/ml, in NADH containing medium 48.0 ± 9.6 ng/ml ($p < 0.01$).

D content was found increased by about 500% in the medium with NADH. This was a first indication that D-biosynthesis could be stimulated by NADH.

In the next experiment three vials with each 40×10^6 PC 12 cells were incubated for two hours with 15 ml control medium. Two ml of the medium were collected and another two ml of medium with NADH were added in such a mode that the end concentration of NADH in the first vial was 50 mcg NADH/ml medium (D in control medium was 80.0 ± 13.6 ng/ml, in medium with 50 mcg NADH/ml 112.0 ± 22.4 ng/ml, $p. < 0.05$), in the second 100 mcg/ml (D in control medium was 98.0 ± 17.4 ng/ml, in medium with 100 mcg NADH/ml 232.0 ± 47.3 ng/ml, $p < 0.001$) and in the third 200 mcg/ml (D in control medium was 110.0 ± 19.9 ng/ml and in medium with 200 mcg/ml 449.0 ± 85.4 ng/ml, $p < 0.01$) for further two

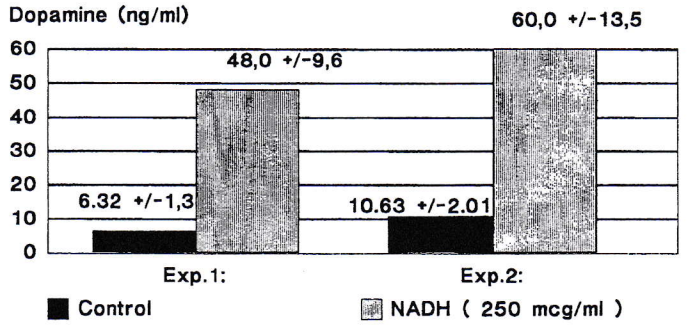

Fig. 1. Increase of dopamine production by NADH

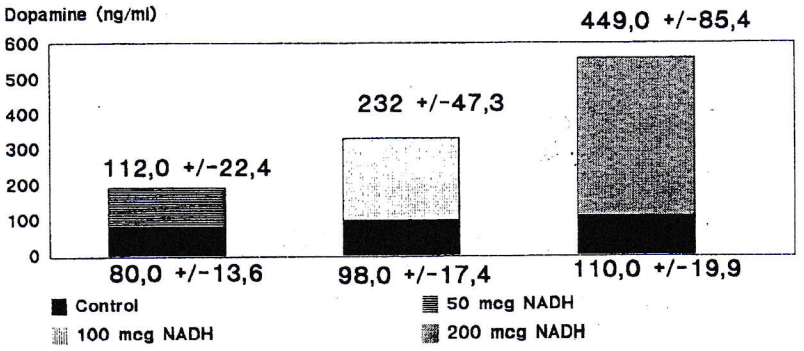

Fig. 2. Increase of dopamine production by NADH – concentration dependance

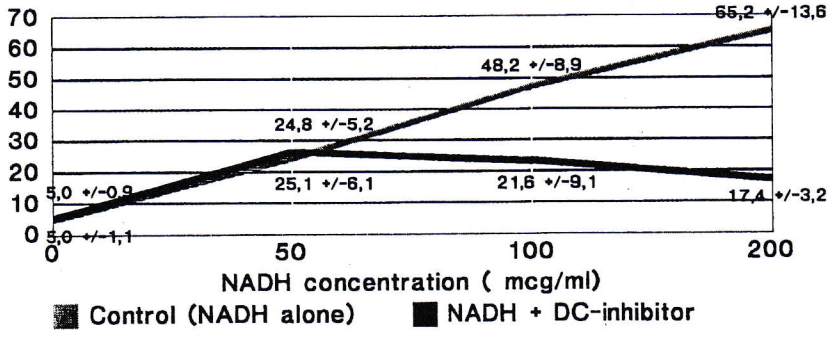

Fig. 3. Increase of dopamine production by NADH – effect of decarboxylase inhibitor

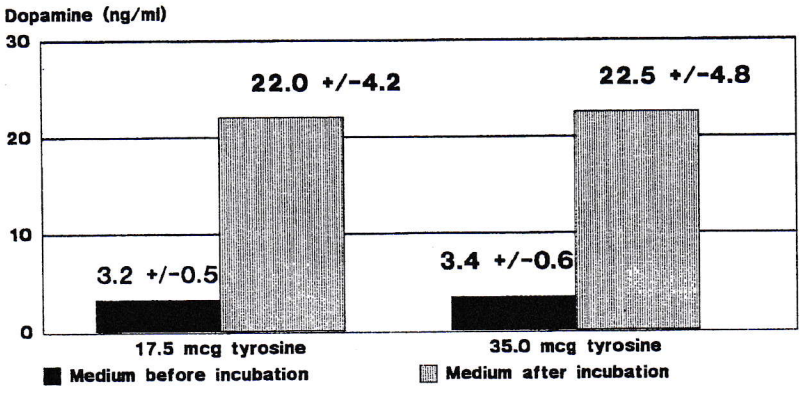

Fig. 4. Effect of tyrosine on dopamine production of PC 12 cells

hours. Under this condition a linear rise of D-biosynthesis was observed (Fig. 2).

M-hydroxybenzylhydrazine, an inhibitor of DOPA decarboxylase decreases D-biosynthesis (Fig. 3). Three vials with each 40×10^6 PC 12 cells were parallely incubated with 50, 100 and 200 mcg NADH/ml medium for two hours. Another three vials were incubated under the same conditions

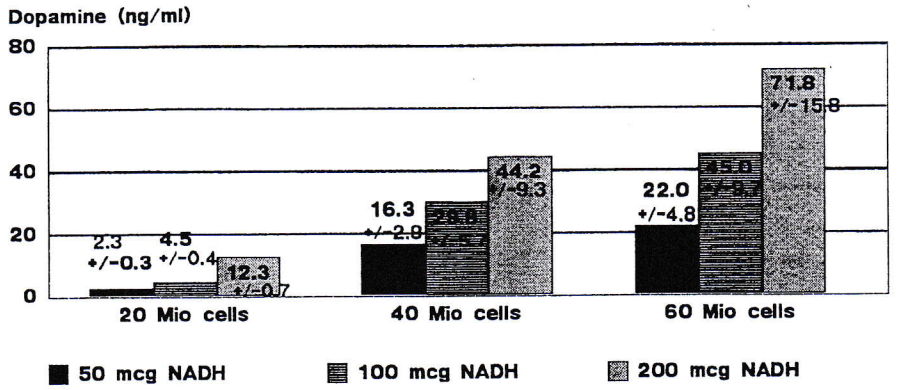

Fig. 5. Increase in dopamine production by NADH – effect of number of cells

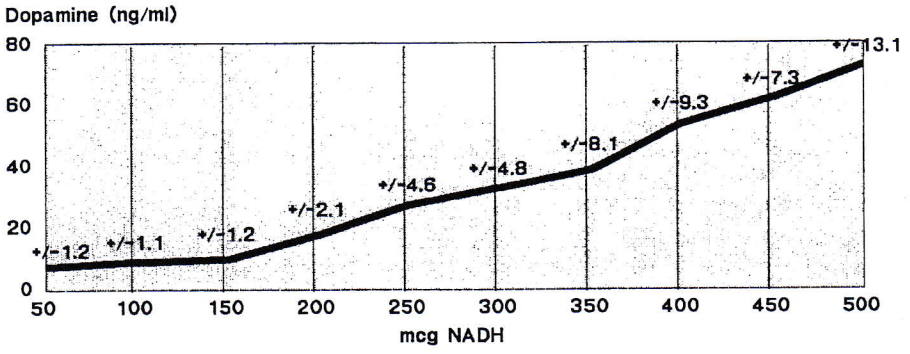

Fig. 6. Increase in dopamine production by NADH – effect of NADH concentration

only M-hydroxybenzylhydrazine was added to a final concentration of 5×10^{-4} M. D in medium with 100 mcg NADH/ml was 48.2 ± 8.9 ng/ml and in medium with 100 mcg NADH and 5×10^{-4} M M-hydroxybenzylhydrazine 21.6 ± 9.1 ng/ml, $p < 0.01$. D content in medium with 200 mcg NADH/ml was 65.2 ± 13.0 ng/ml and in medium with 200 mcg NADH and 5×10^{-4} M M-hydroxybenzylhydrazine 17.4 ± 3.2 ng/ml, $p < 0.001$. As we can see depression was the greater the higher the NADH concentration in the medium was.

In order to find out whether the level of the D-precursor tyrosine has an influence on the D-biosynthesis the tyrosine concentration in the medium was doubled. No increase in D-biosynthesis was found which confirms the assumption that sufficient substrate is available (Fig. 4).

Further experiments showed that also a certain number of cells is necessary to obtain a linear enhancement of D synthesis under the influence of NADH. The best results are achieved with 40×10^6 and 60×10^6 cells per 15 ml medium (Fig. 5). D was 2.3 ± 0.3 ng/ml medium with 50 mcg NADH and 20×10^6 cells, 16.3 ± 2.9 ng/ml medium with 50 mcg NADH and 40×10^6 cells $p < 0.001$, and 22.0 ± 4.8 ng/ml medium with 50 mcg NADH and 60×10^6, $p < 0.001$ compared with the D content in medium

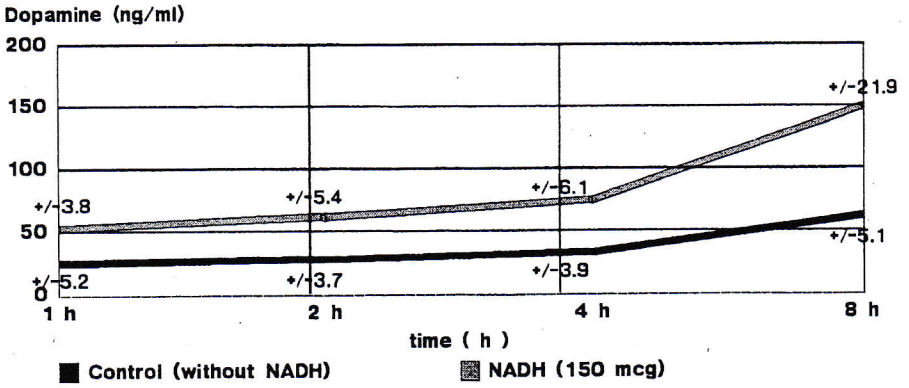

Fig. 7. Increase of dopamine production by NADH – effect of incubation time

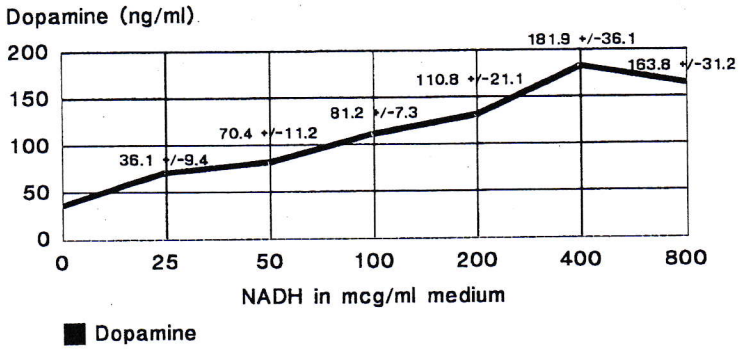

Fig. 8. Increase of dopamine production – effect of NADH, 8 h incubation time, 40 million cells

with 20×10^6 cells, $p < 0.05$ compared with D in medium with 40×10^6 cells. D was 4.5 ± 0.4 ng/ml medium with 100 mcg NADH and 20×10^6 cells, 29.8 ± 5.7 ng/ml in medium with 100 mcg NADH and 40×10^6 cells, $p < 0.001$, and 45.0 ± 9.7 ng/ml medium with 100 mcg NADH and 60×10^6 cells, $p < 0.001$ compared with D contant in medium with 20×10^6 cells, $p < 0.05$ compared with D content in medium with 40×10^6 cells. D was 12.3 ± 0.7 ng/ml medium with 200 mcg NADH and 20×10^6 cells, 44.2 ± 9.3 ng/ml medium with 200 mcg NADH and 40×10^6 cells, $p < 0.001$, 71.8 ± 15.8 ng/ml medium with 200 mcg NADH and 60×10^6 cells, $p < 0.001$ compared with D content in medium with 20×10^6 cells and $p < 0.01$ compared with D content in medium with 40×10^6 cells.

All the experiments reported above were carried out with an incubation time of two or three hours. If 40×10^6 cells are incubated for 30 minutes only a linear rise of D synthesis could be seen up to a concentration of 500 mcg NADH/ml (Fig. 6).

The next experiments were performed in order to see whether a time dependence of NADH action exists. 40×10^6 cells were incubated with

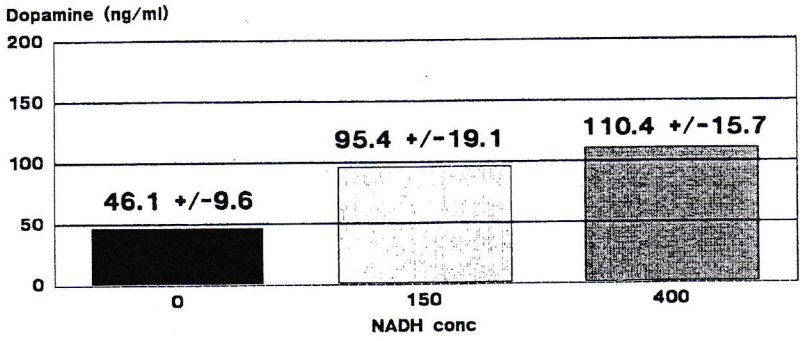

Fig. 9. Increase of dopamine production in PC 12 cells (40 mio) – 8 h incubation time

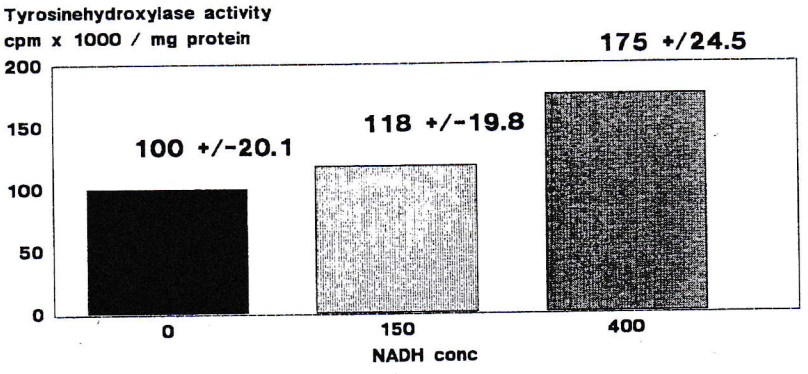

Fig. 10. Tyrosine hydroxylase activity – stimulation by NADH

150 mcg NADH/ml medium for 1,2,4 and 8 hours. The D production increased with time (Fig. 7). All values in medium with NADH compared with control medium were significant different at all time points ($p < 0.001$). No decrease in D-production could be observed up to 8 hours.

Now with a cell number of 40×10^6/15 ml medium parallely in each vial and 8 hours incubation time, the test with 25 mcg NADH/ml, 50, 100, 200, 400 and 800 mcg was carried out (Fig. 8). Under this condition the D production was linearly until a concentration of 400 mcg NADH/ml.

All these experiments show that NADH stimulates D biosynthesis. In order to elucidate the mechanism of NADH further we looked whether NADH is able to activate tyrosine hydroxylase directly. This was shown by incubating three samples of 40×10^6 cells parallely for 8 hours. One sample contained only normal medium, the other 150 mcg NADH/ml and the third 400 mcg/ml medium. The D content in the medium was measured (Fig. 9) as well as the activity of tyrosine hydroxylase in the cell homogenate after incubating the cells in NADH enriched medium (Fig. 10). The D content increased in the medium about 107% ($p < 0.001$) and 140% ($p < 0.001$) respectively. The activity of tyrosine hydroxylase was stimulated to 18% by 150 mcg NADH (n.s.) and to 75% by 400 mcg NADH ($p < 0.001$). This

last experiment clearly shows that NADH acts on the enzyme tyrosine hydroxylase directly.

Discussion

Our findings support the concept that the clinical improvement of PP after administration of NADH is caused by activation of the endogenous D-biosynthesis. As mentioned above, NADH is not only coenzyme of dihydropteridine reductase, but also of various other enzyme systems. Furthermore its beneficial effect may be a peripheral rather than a central one.

On the other site the direct increase of tyrosine hydroxylase activity in PC 12 cells shows that NADH is effective at the site of transforming L-tyrosine to L-DOPA and further to D and may trigger a new therapeutic concept for PD. Our observation is the very first evidence that NADH stimulates D-biosynthesis directly. In this context a number of questions arise; for example: 1) Does NADH cross the blood brain barrier? 2) Why is the NADH – cytochrome reductase deficient in mitochondria of substantia nigra of PP? Is this a cause or a consequence of PD? 3) Does NADH act directly or indirectly?

It should be pointed out here, that the endogenous NADH concentration in the organism in particular in cells and tissues is rather high. For example erythrocytes contain 3,5 mcg/g, brain tissue has 50 mcg/g and liver 71,5 mcg/g weight. Furthermore NADH is quite stable in the blood and not degraded in between seconds as stated occassionally in lectures of Parkinson researchers (Klingenberg, 1960).

Many suppositions were made with regard to the diminished activity of the key enzyme of D-biosynthesis, tyrosine hydroxylase (TH). One must know that a change in the concentration of D in the incubation medium is not only a function of the activity of tyrosine hydroxylase. However D concentrations in the medium may reflect synthesis, transport, release and metabolism changes in other pathways (e.g. norepinephrine synthesis). Nagatsu and coworkers found a decrease of total biopterin, the coenzyme of TH, in postmortem material of substantia nigra of PP (Nagatsu et al., 1981a,b). Due to this we are tempted to assume that the action of NADH is connected with the increasing formation of BH4 via activation of dihydropteridine reductase. If sufficient active BH4 is available the TH can work with optimal activity. BH4 itself, when administered orally had no clinical effect in PP most likely, because BH4 is not able to cross the blood brain barrier (Kapatos and Kaufmann, 1981; Leeming et al., 1983).

TH needs also iron as cofactor for its optimal activity (Rausch et al., 1988). 1986 Birkmayer W. showed that iron as a special ferric-ferrous complex called oxyferriscorbone shows a good clinical response in treatment of PP (Birkmayer and Birkmayer, 1986, 1987).

Maryama W. and coworkers (Maruyama et al., 1991) found that a carcinogenic, food derived heterocyclic amine, namely 3-amino-1,4-dimethyl-5H-pyride (4,3-b) indole (Trp-P-1) inhibits the TH activity by reducing its

affinity to tetrahydrobiopterin. Naoi (Naoi et al., 1988) showed that the addition of Trp-P-1 to culture medium also reduced the TH activity of rat PC 12 cells.

Ascorbate also decreased TH activity in cultured PC 12 cells (Wilgus and Roskoski, 1988).

On the other site the role of the monoamine oxidase B (MAO B) is not clear. MAO B is the enzyme which degrades D and which is elevated in many PP (Schneider et al., 1981; Riederer and Jellinger, 1983). It has been reported that L-DOPA decreases the MAO B activity whereas D enhances it in human platelets (Demisch et al., 1983).

It could be that the MAO B degrades D too rapidly that only too little amounts of D are available for the dopaminergic neurotransmission. If this is actually the case than monoamine oxidase should be higher in PC 12 cells after incubation with NADH due to the increased D-production. Studies in this direction are in progress.

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