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Effect of Interferon-γ and LPS on Tetrahydrobiopterin in Rat PC12 and Human KNA Cells

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Abstract

The first human phaeochromocytoma cell line KNA was tested with regard to stimulation of dopamine biosynthesis by nicotinamide adenine dinucleotide (NADH). Differently from rat phaeochromocytoma cells - clone PC12, where NADH increased dopamine biosynthesis significantly, no stimulation in KNA cells was found. NADH is effective on speeding up the formation of the active cofactor tetrahydrobiopterin by a recycling pathway via the dihydropteridinreductase system. Another way of formation of tetrahydrobiopterin is the induction of de novo synthesis by cytokines such as interferon-γ or by lipopolysaccharide via activation of GTP cyclohydrolase I, as shown in macrophages. Neither rat PC12 nor human KNA cells showed in vitro a stimulation of tetrahydrobiopterin de novo synthesis by interferon-γ and/or lipopolysaccharide.

Key words: phaeochromocytoma, dopamine, tetrahydrobiopterin, interferon-γ, lipopolysaccharide

Introduction

The reduced form of nicotinamide adenine dinucleotide (NADH) is able to stimulate endogenous dopamine biosynthesis in rat PC12 phaeochromocytoma cells (1). The stimulating effect of NADH is based on supplying the tyrosine hydroxylase (TH), the key enzyme in dopamine (D) biosynthesis, with sufficient tetrahydrobiopterin (BH_4) cofactor. NADH acts by speeding up the recycling pathway from dihydrobiopterin to the active BH4 cofactor via the dihydropteridinereductase (DHPR) system, which is coupled to the NAD^+/NADH redox system. D biosynthesis is increased significantly, although the absolute concentration of BH_4 and the concentration of DHPR in PC12 cells are not altered (2).

The following experiments were performed in order to find out whether stimulation of D biosynthesis can also be observed in human phaeochromocytoma cells (KNA). These KNA cells constitute the first continuous human phaeochromocytoma cell line, established from a sporadic phaeochromocytoma of the right adrenal gland of a 73 year-old woman (3).

Previous investigations have shown that human macrophages and monocytes produce large amounts of neopterin and biopterin upon stimulation by interferon-γ (IFN-γ), which in vivo is produced by activated T cells (4). Further, T cells are activated by lipopolysaccharide (LPS) to produce cytokines (IFN-γ tumor necrosis factor-α), which in turn leads to pteridine formation in macrophages (5). IFN-γ acts by inducing the activity of GTP cyclohydrolase I (EC 3.5.4.16). GTP cyclohydrolase I is the first and rate-limiting enzyme in pteridine synthesis (6). Therefore, we were interested whether or not BH_4 production can be stimulated in PC12 and KNA cells by IFN-γ and/or by LPS.

Materials and Methods

Cell cultures

The KNA cell line was originally grown in Ham's F12 medium (GIBCO ISRL, Grand Island, NY) with 10% fetal bovine serum (PAA Laborat. Exton, PA). The cells were adapted for the present investigation to the same medium mixture as used for PC12 cells, i.e., RPMI 1640 (Seromed, Berlin, Germany) supplemented with 10% horse serum and 5% fetal bovine serum (both sera from PAA Laboratories, Exton, PA), 100 IU

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penicillin and 100 µg streptomycin/ml medium. Experiments were performed without antibiotics. An inoculum of $5x10^5$ cells/ml medium yields a two fold increase within four days.

The rat adrenal phaeochromocytoma cell line PC12 was obtained from the American Type Culture Collection, Largo, MD (ATCC CRL 1721) and propagated in RPMI 1640, as described above. An inoculum of $5x10^5$ cells/ml medium increases by 2-fold within two days.

The cultures were maintained in vented T-12.5, T-25, T-150 and T-175 flasks (Costar, Cambridge, MA) and incubated at 37°C in a 5% CO_2 and 95% humidity incubator. Cell counting and growth analyses were performed by Casy-1 Cell Counter and Analyzer (Schärfe System, Reutlingen, Germany).

Both KNA and PC12 cell lines were stained with Hoechst dye 33258 to exclude mycoplasma contamination (DAPI-Test, Hoechst, Frankfurt, Germany) and were found negative.

Experimental conditions

Each experimental group had a total cell number of $2\text{-}3x10^7$ cells, suspended in 15 ml medium. In experiment 1, KNA cells were incubated with 400 µg NADH/ml medium for 8 hours. In the subsequent experiments KNA as well as PC12 cells were incubated for 24, 48, and 72 hours, respectively, with following substances:

2:Human IFN-γ, (purchased from Sigma, St. Louis, USA), 250 IU/ml medium
3: IFN-γ, 500 IU/ml
4: IFN-γ, 2500 IU/ml
5: LPS from E. coli (purchased from Sigma, St. Louis, USA), 1µg/ml medium
6: LPS, 2 µg/ml medium
7: IFN-γ, 500 IU + LPS, 2 µg/ml medium
8: IFN-γ, 2500 IU + LPS, 2 µg/ml medium
9: untreated cultures (control).
10: medium (control)

Determination of dopamine

After cell incubation the medium was collected and stored until analysis in liquid nitrogen. The dopamine content in the medium was determined, after deproteinisation with 0.4 M perchloric acid, employing HPLC (high performance liquid chromatography) using electrochemical detection (7, 8). The Coulochem electrochemical detector Model 5100 A (ESA, Wiggins Avenue, Bedford, MA) was used in connection with a high sensivity analytical cell (ESA-Model 5011). Separation was achieved by a catecholamine HR-80 column (4.6 x 80 mm, packed with the micron C 18 stationary phase and purchased from ESA) with ESA mobile phase for catecholamines.

Determination of tetrahydrobiopterin

After incubation the cells were washed twice with 10 ml saline, the cell pellet resuspended in 2 ml of a mixture of 1 mM dithioerythrol and 0.1 mg/ml Pefabloc and subsequently frozen in 1 ml portions in liquid nitrogen to break up the cells. For analysis the cells were thawed and homogenized (UltraThurax, Model 1510, B. Braun, Melsungen, Germany).

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 $NaOH.I_2$ (1N NaOH with 0.1 M I_2 solved in 0.25 M KI in a mixture of 1:1) and incubation for one hour 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 1N HCl is added, unsoluble material centrifuged and the iodine excess destroyed by 0.1 M ascorbic acid. During acid oxidation dihydro- and tetrahydrobiopterin are oxidized by 0.01M iodine solution (1N HCl with 0.1M I_2 in 0.25M KI_2 in a mixture of 1:1) to the fluorescent biopterin (9). After acidic or alkaline oxidation of aliquots of sonicated cell homogenate, biopterin was measured 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 x 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 x 4 mm, E. Merck) packed with the same material.

Thus, the amount of tetrahydrobiopterin is estimated by substracting the biopterin concentration found after alkaline oxidation from the value obtained after acidic oxidation.

Determination of tyrosine hydroxylase

The activity of tyrosine hydroxylase was estimated according to the method of Mc Geer et al. (10) with some modifications (1). After sonication of the PC 12 cells with an Ultrathurax (Labsonic 1510, B. Braun, Melsungen, Germany) in 0.25 M sucrose, the homogenate was incubated for 20 minutes with 0.1 µCi L-U-^{14}C tyrosine (specific activity 448 mCi/mmol,

purchased from Amersham, GB) as substrate. The reaction was stopped by adding perchloric and acetic acid containing as carrier 0.2 µg of L-DOPA, dopamine and noradrenaline each. Sodium hydroxide increased the pH to 9 and precipitated the protein. The supernatant was extracted by acidified aluminium oxide. The bound, and by the activity of tyrosine hydroxylase synthesized ^{14}C L-DOPA was reextracted by acetic acid and counted in a ß-counter (Beckman LS 6000IC, Germany).

Results

D content in medium of KNA cells (experiment 1) was 418.5 (SD=20.4) pg/ml medium and did not differ from D concentration in KNA cells incubated with NADH. Here D concentrations of 418.9 (SD=54.3) pg/ml were found.

The activity of tyrosine hydroxylase in these cells was very low; in some incubations we failed to find any activity at all, in some we found 583 (SD=79) cpm/mg protein (38; SD=5 cpm/10^6 KNA cells). NADH increased slightly the activity, namely, to a value of 790 (SD=113) cpm/mg protein (65; SD=9 cpm/10^6 KNA cells).

BH_4 concentration in human KNA cells was much lower than in PC12 cells. In untreated cultures (controls) of 20x10^6 KNA cells we found 28.5 nM BH_4 (SD=5.33) within 24 hours incubation time. In comparison, in PC12 cells we observed 912.7 nM (SD= 65.20). Furthermore, we found 22.5 nM (SD=10.84) after 48 hours and 13.0 nM (SD=2.70) in KNA cells after 72 hours incubation; in PC12 cells BH_4 concentration was 534,3 nM (SD=33.2) after 72 hours incubation (Fig. 1).

In further experiments the influence of IFN-γ and LPS on BH_4 biosynthesis in KNA and PC12 cells was tested.

Addition of IFN-γ (250; 500; 2500 U/ml) to the incubation medium did not increase BH_4 concentration significantly at different incubation times (24, 48 or 72 hours), neither in KNA nor in PC12 cells. We made the same observation by addition of IFN-γ plus LPS (1 or 2 mg/ml) or LPS alone (1 or 2 mg/ml).

Finally, the addition of IFN-γ and/or LPS had no effect on the proliferation rates of KNA and PC12 cells.

Discussion

Our earlier studies have shown that NADH is able to stimulate D biosynthesis in rat PC12 cells, although endogenous BH_4 concentration in these cells remained stable (2). A similar effect was found by Kittner B. et al (11) by treating PC12 cells with potassium chloride. Potassium chloride induced release of endogenous D. Simultaneously, DOPA synthesis was increased, but intracellular concentration of BH_4 remained stable, indicating that the catalytic recycling of the used BH_4 is much more rapid than the BH_4 consumption by tyrosine hydroxylation.

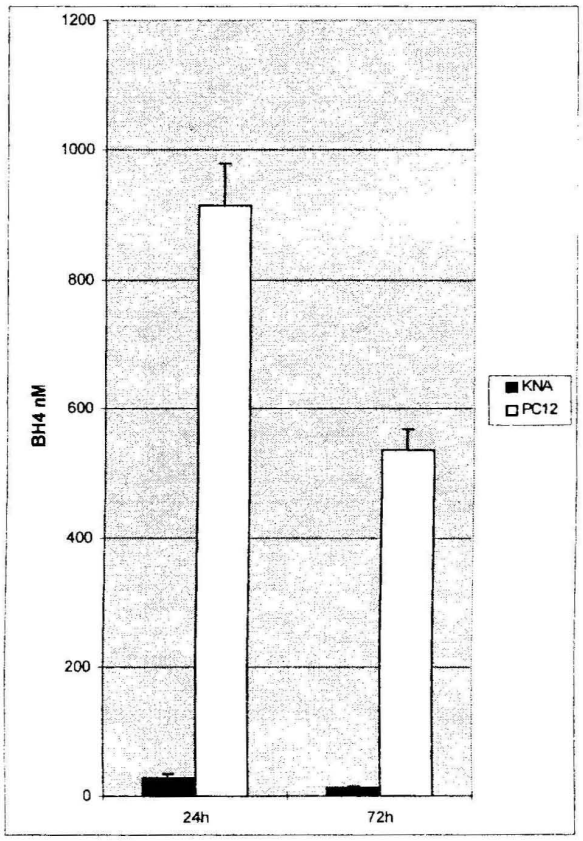

Figure 1. Tetrahydrobiopterin concentrations (nM) of human KNA and rat PC12 cells after 24 and 72 hours incubation without any addition to the incubation medium.

Incubation of the first human phaeochromocytoma cell line, KNA, with NADH failed to reproduce this effect. Apparently, KNA cells have lost their ability to synthesize D during spontaneous treatment and selection of various cleaning steps in cultivation. This assumption is supported by the very low activity of tyrosine hydroxylase and the very low concentration of BH_4 cofactor observable in these cells.

The establishment of the KNA cell line was obtained by spontaneous transformation - a rare multistage event which is superior to experimental immortalization. Spontaneous immortalization always implies that spezialized properties of the cells are lost

by dedifferentiation. The extend to which the derived cell line retains the differentiated features of the tumor of origin is unpredictable. Besides, the expression of differentiated properties in cell culture is often limited by the promotion of cell proliferation (12). Physiological inducers are necessary to control differentiation in vitro, such as the differentiation of neuroblastoma by IFN-γ and retinoic acid (13). In our earlier experiments we could demonstrate positive immunoreactivity with dopamine β-hydroxylase while catecholamine concentrations were below the detection limit. On this condition, an enhancement of dopamine production was desirable. The promotion of proliferation was imperative for producing a large amount of cells - thus promoting dedifferentiation. Further experiments are in progress in order to induce differentiation by enhanced cell-cell and cell-matrix interaction and by the presence of various differentiation factors.

Earlier studies have shown that IFN-γ being produced by activated T lymphocytes during immune response (14, 15), enhances pteridine biosynthesis in macrophages (4, 5). However, intracellular biopterin and neopterin concentrations are not only increased in macrophages by IFN-γ but also in other cell lines tested (16, 17). Werner et al (18) investigated neopterin and biopterin concentrations in various carcinoma cell lines such as A 431 (epidermoid carcinoma), A 498 (kidney carcinoma), A 549 (lung carcinoma), SK-HEP-1 (liver carcinoma), U 138 MG (glioblastoma) and T 24 (bladder carcinoma). Intracellular concentrations of neopterin and biopterin were distinctly increased by IFN-γ in all cells investigated. T 24 cells (bladder carcinoma) showed considerable pteridine synthesis also when unstimulated. The reason for this stimulation is the IFN-γ-mediated induction of GTP cyclohydrolase I (EC 3.5.4.16), the key enzyme of pteridine synthesis. D'Sa et al (19) found a time dependent increase of BH_4 biosynthesis by treating the astrocyte-derived C6 glioma cell line with LPS and/or TNFa, and proved that the stimulation of BH_4 biosynthesis is due to induction of GTP cyclohydrolase gene expression.

In our experiments with KNA and PC12 cells no stimulation of BH_4 biosynthesis by IFN-γ was found. Anastasiadis (20) investigated the regulation of catecholamine and BH_4 synthesis in cultured rat PC12 cells and found, in good agreement with our results that treatment of these cells with IFN-γ caused neither an increase of D and BH_4 biosynthesis nor an increase in activities of tyrosine hydroxylase and GTP cyclohydrolase I.

BH_4 concentration in PC12 and KNA cells was not altered by IFN-γ. As expected from this finding, treatment by IFN-γ did not produce an increase in proliferation of these cells. On the other hand, addition of BH_4 to incubation medium of PC12 cells was shown to cause a concentration-dependent increase in PC12 proliferation by others (21). These authors further showed that the BH_4 induced increase in PC12 cell proliferation was not related to elevated catecholamine synthesis.

LPS is also an effective inducer of pteridine synthesis in macrophages, even under in vitro conditions without detectable participation of IFN-γ. In vivo, LPS is acting via endogenously formed IFN-γ and other factors and mediators (5).

Like IFN-γ, in our experiments LPS did not cause any increase in BH_4 synthesis, neither in PC12 nor in KNA cells.

We conclude that a) the KNA cell line has lost some of the functions of its rat counterpart PC12 cell line, and b) that the pteridine biosynthesis and the catecholamine biosynthetic cascade in PC12 cells are truly constitutive functions and hence, cannot be modulated by cytokines like IFN-γ or LPS.

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References

1. Vrecko K, Birkmayer JGD, and Krainz J. Stimulation of dopamine biosynthesis in cultured PC12 phaeochromocytoma cells by the coenzyme nicotinamide adeninedinucleotide (NADH). J Neural Transm (P-D Sect) 1993; 5: 147-156.
2. Vrecko K, Storga D, Birkmayer JGD, Möller R, Tafeit E, Horejsi R, Reibnegger G. NADH stimulates endogenous dopamine biosynthesis by enhancing the recycling of tetrahydrobiopterin in rat phaeochromocytoma cells. Biochim Biophys Acta 1997, 1361: 59-65.
3. Pfragner R, Behmel A, Smith DP, et al. First continuous human pheochromocytoma cell line: KNA - Biological, cytogenetic and molecular characterization of KNA cells. J Neurocyt 1998, 27: 175-186.
4. Huber C, Batchelor JR, Fuchs D, et al. Immune response-associated production of neopterin. Realease from macrophages primarily under control of interferon-γ. J Exp Med 1984, 160: 310-316.
5. Werner-Felmayer G, Werner ER, Fuchs D, et al. Tumour necrosis factor-α and lipopolysaccaride enhance interferon-induced tryptophan degradation and pteridine synthesis in human cells. Biol Chem Hoppe-Seyler 1989, 370: 1063-1069.
6. Nichol CA, Smith GK, Duch DS. Biosynthesis and

metabolism of tetrahydrobiopterin and molybdopterin. Ann Rev Biochem 1985, 54: 729-764.
7. Anastasiadis PZ, Kuhn DM, Levine RA. Tetrahydrobiopterin uptake in rat brain synaptosomes, cultured PC 12 cells and rat striatum. Brain Res 1994, 665: 77-84.
8. Alburges ME, Narang N, Wamsley JK. A sensitive and rapid HPLC-ECD method for the simultaneous analysis of norepinephrine, dopamine, serotonin and their primary metabolites in brain tissue. Biomed Chromatogr 1993, 7: 306-310.
9. Fukushima T, Nixon JC. Analysis of reduced forms of biopterin in biological tissues and fluids. Anal Biochem 1980, 102: 176-188.
10. Mc Geer EG, Gibson S, Wada JA, Mc Geer PL. Distribution of tyrosine hydroxylase activity in adult and developing brain. Can J Biochem 1967, 45: 1943-1952.
11. Kittner B, Brautigam M, Herken H. PC12 cells: a model system for studying drug effects on dopamine synthesis and release. Arch Int Pharmacodyn Ther 1987, 286 (2): 181-194.
12. Freshney RI. Differentiation. In: Culture of animal cells. A manual of basic technique. 4th ed. Wiley-Liss, New York 2000, 259-267.
13. Wuarin L, Verity MA, Sidell N. Effects of interferon-γ and its interaction with retinoic acid on human neuroblastoma differentiation. Int J Cancer 1991, 48: 136-141.
14. Dinarello CA, Mier JW. Lymphokines. N Engl J Med 1987, 317 (15): 940-945.
15. Murray HW. Interferon-γ, the activated macrophage, and host defense against microbial challenge. Ann Intern Med 1988, 108 (4): 595-608.
16. Werner ER, Werner-Felmayer G, Fuchs D et al. Tetrahydrobiopterin biosynthetic activities in human macrophages, fibroblasts, THP-1 and T24 cells. J Biol Chem 1990, 265: 3189-3192.
17. Werner ER, Werner-Felmayer G, Fuchs D et al. Pteridine synthesis in cytokine treated cells. In: Pterins and Biogenic Amines in Neurology, Pediatrics and Immunology (Blau N, Curtius HC, Levine RA, Cotton RGH eds) Lakeshore Publishing Company, Grosse Pointe, 1991, pp 213-224.
18. Werner ER, Werner-Felmayer G, Fuchs D et al. Parallel induction of tetrahydrobiopterin biosynthesis and indoleamine 2,3-dioxygenase activity in human cells and cell lines by interferon-γ. Biochem J 1989, 262: 861-866.
19. D'Sa, Hirayama K, West A et al. Tetrahydrobiopterin biosynthesis in C6 glioma cells: induction of GTP cyclohydrolase I gene expression by lipopolysaccharide and cytokine treatment. Mol Brain Res 1996, 41: 105-110.
20. Anastasiadis PZ, Kuhn DM, Blitz J et al. Regulation of tyrosine hydroxylase and tetrahydrobiopterin biosynthetic enzymes in PC12 cells by NGF, EGF and IFN-gamma. Brain Res 1996, 713(1-2): 125-133.
21. Anastasiadis PZ, States JC, Imerman BA et al. Mitogenic effects of tetrahydrobiopterin in PC12 cells. Mol Pharmacol 1996, 49(1): 149-155.