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## Utilization of exogenous tetrahydrobiopterin in nitric oxide synthesis in human neuroblastoma cell line

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### Abstract

We and others have previously reported that neurons expressing neuronal nitric oxide synthase (nNOS) do not co-express GTP cyclohydrolase I, the enzyme that synthesizes its cofactor tetrahydrobiopterin (BH4). BH4 is released from catecholaminergic cells and nNOS-expressing cells are located close to BH4-producing catecholaminergic nerve terminals. We show that BH4 is taken up into the nNOS-expressing human neuroblastoma cells TGW-I-nu in a linear, dose-dependent manner and elevates NO production. Direct exposure to BH4, dihydrobiopterin or biopterin, or coculture with catecholaminergic CATH.a cells increases NO production by TGW-I-nu. Thus, BH4-requiring nNOS cells may obtain BH4 from neighboring catecholaminergic cells or terminals and an intercellular crosstalk may exist between the two cells in vivo.

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Nitric oxide (NO) is a highly reactive gas whose role as a neurotransmitter involved in synaptic plasticity, learning/memory, and modulation of neurotransmission has been recognized. NO is synthesized from L-arginine by three classes of nitric oxide synthase (NOS): neuronal (nNOS), endothelial, and inducible forms, all of which require tetrahydrobiopterin (BH4) for their activity.

Previous immunocytochemical and in situ hybridization studies including ours have reported that GTP cyclohydrolase I (GTPCH), the first and rate-limiting enzyme in BH4 biosynthesis, is detected in aromatic amino acid hydroxylase-expressing neurons, but not in nNOS-expressing neurons [7,12,13], which led to the hypothesis that BH4 might not be produced in the nNOS-expressing neurons but may be obtained from elsewhere.

One such source may be monoaminergic neurons, which require BH4 for monoamine synthesis. We have previously observed by double labeling that tyrosine hydroxylase-positive dopaminergic nerve terminals are in close contact

with nNOS-positive cell bodies in the brain [7]. This was also corroborated by electron microscopic analysis showing close association of dopaminergic terminals and NADPH diaphorase-staining neurons in the striatum [4]. We have subsequently observed that BH4 synthesized in catecholaminergic cells such as primary cultured adrenal medullary cells and CATH.a cells is spontaneously released [2], suggesting that BH4 would be extracellularly available for the nearby nNOS neurons. These findings led us to hypothesize that nNOS neurons may obtain BH4 that is released from nearby monoaminergic cells or terminals. In this study we tested whether NO synthesis in nNOS-expressing cells is indeed promoted by exogenously supplied BH4 or coculture with BH4-producing cells.

Culture conditions for various cell lines used in this study are described elsewhere [2]. For uptake experiments, cells were plated at a density of  $2 \times 10^5$  cells/well in 24 well culture plates. After 24 h, cells were fed with fresh media at which time BH4 was added. For coculture experiments, CATH.a cells, separately cultured in the presence or absence of 100  $\mu$ M sepiapterin for 24 h in RPMI 1640 medium, were added to the culture plates containing TGW-

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I-nu cells. Intracellular and extracellular biopterin were measured by the method using HPLC coupled with fluorescence detector as described previously [9,10]. Accumulated nitrite, an oxidative metabolite of NO, in the cell culture medium was measured by the Griess reaction [6]. Statistical analyses were performed by ANOVA and Newman–Keuls comparison test. Data were considered significant if  $P < 0.05$ .

TGW-I-nu, isolated from human neuroblastoma, has been reported to contain high levels of nNOS mRNA and protein, comparable to that expressed in the nNOS-rich cerebellum [5]. Our initial study showed that the cells have the lowest amount of BH4 among various cell lines tested (see below, Fig. 1B), while immortalized cells are known to produce BH4 to some degree unlike normal cells. We also observed that although NOS activity in the cell lysate assayed in the presence of excess BH4 was determined to be 110 nmol citrulline/mg protein/h, NO actually produced by these cells in the absence of exogenously supplied BH4 was 100-fold lower (1.05 nmol NO/mg protein/h). This suggested that although TGW-I-nu cells express catalyti-

cally active NOS, limiting amount of its cofactor BH4 might hamper generation of NO.

Whether extracellular BH4 could be taken up into TGW-I-nu was first tested. Because radiolabelled BH4 was not available, we exposed the cells to various concentrations of BH4 and measured intracellular BH4. As shown in Fig. 1A, BH4 was increased in a dose-dependent manner upon exposure to BH4. The increase was also time-dependent until 6 h, after which no further augmentation was observed. No cell death occurred under these conditions. Therefore, the increased level of intracellular BH4 seemed to be equilibrated by 6 h. In order to determine if there exists selectivity for this apparent uptake, various cells were then tested. As shown in Fig. 1B, regardless of their initial BH4 content, exposure to 50 and 100  $\mu\text{M}$  BH4 led to approximately 200 and 400 ng BH4/mg protein, respectively, in all cells tested. Monoaminergic cells with already high BH4 content (CATH.a, PC12 and RBL-2H3) were less affected than those with low BH4 content (TGW-I-nu and CCL-64) by 50  $\mu\text{M}$  BH4, but they all reached similar levels upon exposure to 100  $\mu\text{M}$  BH4. (BH4 content in CATH.a and PC12 could not be measured with 100  $\mu\text{M}$  BH4 because the cells died under this condition; see ref. [2]). Thus, the BH4 uptake seemed to be not affected by the cell line but to occur down the BH4 concentration gradient, until intracellular BH4 content reaches equilibrium with the extracellular concentration. In addition, the uptake was not affected by varying temperature (Fig. 2A) or glucose concentrations (Fig. 2B). Therefore, the BH4 uptake appeared to be a passive, rather than an active, process.

To test whether the elevated intracellular BH4 might be utilized in NO synthesis, NO in the form of nitrite was measured after TGW-I-nu cells were exposed to BH4. As shown in Fig. 3A, nitrite was elevated with increasing level of extracellular BH4, with the highest rate of increment at 10  $\mu\text{M}$ . Dihydrobiopterin and biopterin were observed to be as effective as BH4 in eliciting NO synthesis. We also asked whether NO production would be stimulated when TGW-I-nu cells were cocultured with the BH4-producing CATH.a cells. As shown in Fig. 3B, nitrite level was increased by 2-fold in the presence of CATH.a. Extracellular BH4 concentration under this condition was determined to be  $0.64 \pm 0.16 \mu\text{M}$ , compared to  $0.05 \pm 0.02 \mu\text{M}$  in TGW-I-nu alone. When CATH.a cells were induced to produce more BH4 by pretreatment with its precursor sepiapterin, extracellularly released BH4 reached  $5.35 \pm 0.31 \mu\text{M}$ . At this concentration, NO produced by TGW-I-nu increased by 4.1-fold. Taken together, BH4 produced by the catecholaminergic CATH.a cells were able to induce NO production in TGW-I-nu cells.

In this study, we provide evidence that extracellular BH4 released from BH4-producing cells can be readily taken up into NOS-expressing cells containing a low level of BH4 and is utilized in NO synthesis. Thus, in addition to the physical proximity observed between the BH4-producing catecholaminergic nerve terminals and nNOS neurons in

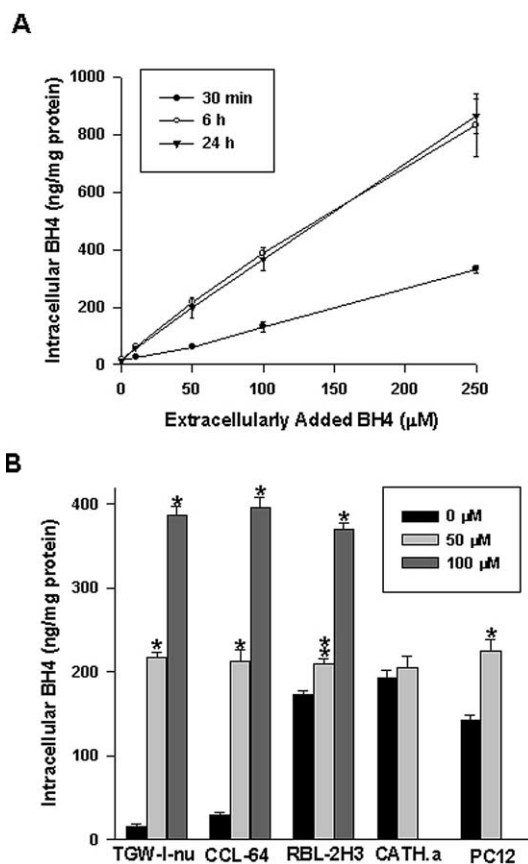


Fig. 1. Uptake of BH4 in a dose- and time-dependent and non-specific manner. (A) TGW-I-nu cells were incubated with varying concentrations of BH4 for 30 min, 6 h, or 24 h at 37°C and amounts of intracellular BH4 were determined, mean  $\pm$  SEM ( $P < 0.001$  vs untreated control for all points); and (B) various cell lines were exposed to 50 and 100  $\mu\text{M}$  BH4 for 6 h at 37°C and amounts of intracellular BH4 were measured, mean  $\pm$  SEM (\*,  $P < 0.001$ ; \*\*,  $P < 0.01$  vs respective untreated control).

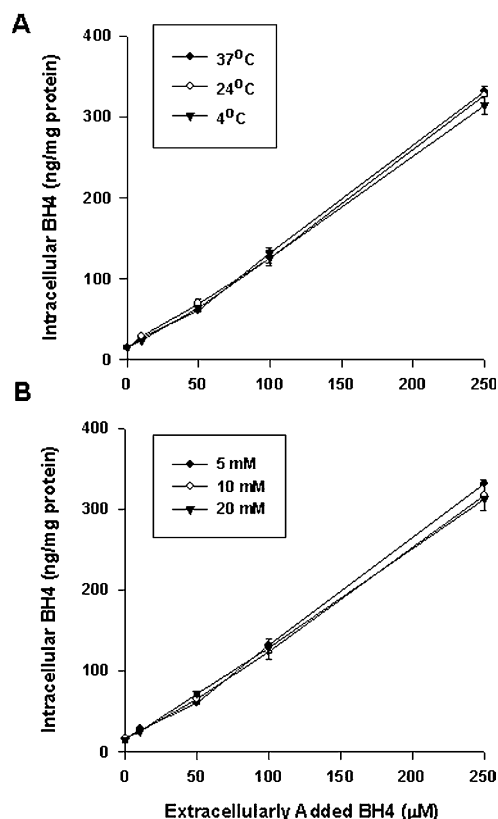


Fig. 2. BH4 is taken up into TGW-I-nu cells by diffusion. Cells were exposed to 100 μM BH4 for 6 h; (A) at 4, 24, or 37°C; and (B) in the presence of varying concentrations of glucose at 37°C for 6 h and amounts of intracellular BH4 were determined, mean ± SEM ( $P < 0.001$  vs untreated control).

vivo [4,7], there also may exist a functional relationship. In non-neuronal systems where BH4 and NO syntheses occur in the same cell, intracellular availability of BH4 is rate-limiting and thus regulates NO synthesis [15]. In neurons where NO is an important neurotransmitter as well as a neurotoxin when present in excess, an additional control mechanism for NO synthesis may exist, namely, sequestration of its cofactor BH4 in another cell. Interestingly, liver and kidney, which require BH4 for phenylalanine hydroxylase activity, also obtain BH4 largely by uptake from extracellular source [10].

We have shown that BH4 is constitutively released [2], which suggests that BH4 synthesis would determine the rate at which BH4 is released. As BH4 synthesis is largely determined by the rate-limiting enzyme GTPCH, intracellular signaling pathways and agents that cause changes in GTPCH activity would regulate the amount of released BH4. Indeed, we have previously shown that calcium influx [8] and melatonin [9] cause induction and reduction, respectively, of GTPCH, and lead to corresponding changes in the released BH4.

BH4 is involved in electron transfer in the first cycle of the NOS reaction. NOS can efficiently utilize molecular oxygen only in the presence of saturating concentrations of

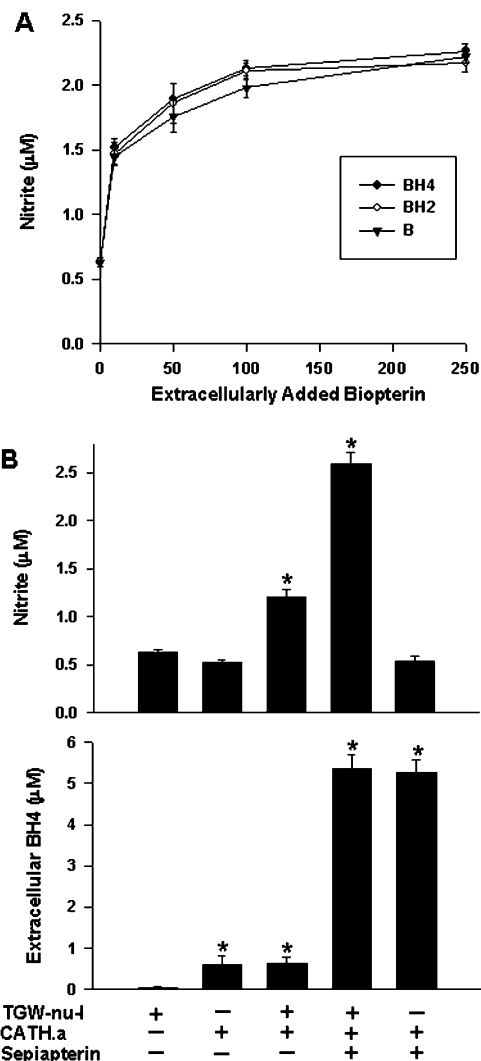


Fig. 3. NO synthesis is increased by extracellularly added BH4 or in the presence of BH4-producing cells. (A) TGW-I-nu cells were treated with various concentrations of BH4, dihydrobiopterin (BH2) or biopterin (B) for 24 h at 37°C and nitrite in the medium was measured, mean ± SEM ( $P < 0.001$  vs untreated control;  $P > 0.05$  among BH4, BH2, and B under the same conditions); and (B) TGW-I-nu cells were incubated for 24 h at 37°C in the presence of CATH.a cells and levels of nitrite and BH4 in the medium were measured. Some CATH.a cells were pretreated for 24 h with 100 μM sepiapterin as indicated, mean ± SEM (\*,  $P < 0.001$  vs TGW-I-nu alone).

BH4 [16]. BH4 deficiency results in nNOS mediated-generation of superoxide [17], which reacts rapidly with NO to form peroxynitrite. nNOS also generates the nitroxy anion (NO<sup>-</sup>) in the absence of BH4 [1]. In addition, newly synthesized NOS requires BH4 for its dimerization into active form [14]. Thus, BH4 synthesized in the neighboring cells might regulate various aspects of the enzyme in the nNOS-expressing cells. Interestingly, we have also observed recently that NO can induce gene expression of catecholamine biosynthesis enzymes including tyrosine hydroxylase [11], which also requires BH4. Taken together, it can be speculated that intercellular communication

between the catecholamine and NO producing neurons might exist through BH4 and NO.

Whether there exists a transport system for BH4 is unknown. Our results suggest that BH4 uptake into nNOS-expressing cells occurs by passive diffusion rather than by active cellular uptake, based on the finding that there was no increase in the uptake with increased temperature or glucose. Although biopterin transporter has been described in the protozoa *Leishmania* [3], no such protein with similar homology or function has been found in the mammalian cells. Further study would be necessary to understand the mode of BH4 uptake into nNOS cells.

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