Tetrahydrobiopterin Enhances Apoptotic PC12 Cell Death following Withdrawal of Trophic Support*

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Panos Z. Anastasiadis‡§, Hao Jiang‡, Laurent Bezin‡, Donald M. Kuhn¶, and Robert A. Levine†‡**

From the ‡William T. Gossett Neurology Laboratories of Henry Ford Hospital, Detroit, Michigan 48202, §Cellular and Clinical Neurobiology, Department of Psychiatry and Behavioral Neurosciences, Wayne State University, Detroit, Michigan 48201, and ¶John D. Dingle Veterans Administration Medical Center, Detroit, Michigan 48201

(6R)-Tetrahydro-L-biopterin (BH4) is the rate-limiting cofactor in the production of catecholamine and indoleamine neurotransmitters and is also essential for the synthesis of nitric oxide by nitric-oxide synthase. We have previously reported that BH4 administration induces PC12 cell proliferation and that nerve growth factor- or epidermal growth factor-induced PC12 cell proliferation requires the elevation of intracellular BH4 levels. We show here that BH4 accelerates apoptosis in undifferentiated PC12 cells deprived of serum and in differentiated neuron-like PC12 cells that are deprived of nerve growth factor withdrawal. Increased production of catecholamines or nitric oxide cannot account for the enhancement of apoptosis by BH4. Furthermore, increased calcium influx by exogenous BH4 administration is not involved in the BH4 proapoptotic effect. Our data also argue against the possibility that increased oxidative stress, due to BH4 autoxidation, is responsible for the observed BH4 effects. Instead, they are consistent with the hypothesis that BH4 induces apoptosis by increasing cell cycle progression. Elevation of intracellular BH4 during serum withdrawal increased c-Myc (and especially Myc S) expression earlier than serum withdrawal alone. Furthermore, N-acetylcysteine and the cyclin-dependent kinase inhibitor olomoucine ameliorated the BH4 proapoptotic effect. These data suggest that BH4 affects c-Myc expression and cell cycle-dependent events, possibly accounting for its effects on promoting cell cycle progression or apoptosis.

Apoptotic cell death is important for normal nervous system development, where neurons that make proper connections and receive sufficient trophic support survive, whereas neurons that are deprived of trophic support die via apoptosis (for a review, see Ref. 1). The requirement for neurotrophic support is thought to continue in mature neurons (for a review, see Ref. 2). During normal aging and in Parkinson’s disease (PD), nigrostriatal dopamine neurons preferentially degenerate. The etiology of this selective cell loss remains unknown. Local availability of trophic factors is able to protect cells from degeneration in animal models of PD (3–6), suggesting that lack of trophic support could contribute to the observed neurodegeneration in normal aging and in PD. Furthermore, morphological characteristics of apoptotic cell death have been reported in brain sections from PD patients (7, 8), but the involvement of apoptosis in PD is still controversial.

PC12 cells have been extensively used as a model of catecholaminergic neurons in culture as well as for the study of neuronal apoptotic death. Naive (undifferentiated) PC12 cells grown in the presence of serum undergo apoptosis upon serum withdrawal (9–11). Furthermore, PC12 cells that become neuronally differentiated and postmitotic following prolonged incubation with NGF undergo apoptosis upon NGF and serum withdrawal (9, 10). Undifferentiated PC12 cell death upon serum withdrawal has been linked to an inappropriate progression through the cell cycle, while apoptotic death of “neuronal” PC12 cells following NGF withdrawal was linked to illegitimate cell cycle reentry (12–15). This interpretation favors the hypothesis that during normal cell growth trophic factors are required for proper cell cycle progression, and in their absence cells die as they try to progress through the cell cycle.

We have previously reported that (6R)-tetrahydro-L-biopterin (BH4) enhances PC12 cell growth by inducing cell cycle progression rather than cell survival (16, 17). BH4 is known for its role as an essential and rate-limiting cofactor in the synthesis of catecholamine and indoleamine neurotransmitters (18, 19) and nitric oxide (20). Nevertheless, the induction of PC12 cell proliferation by BH4 was not mediated by catecholamine or nitric oxide synthesis (16, 21). In addition, the induction of PC12 cell proliferation after a 24-h exposure to epidermal growth factor or nerve growth factor (NGF) required the elevation of intracellular BH4 and activation of its initial biosynthetic enzyme, GTP cyclohydrolase (17). Since BH4 enhanced PC12 cell cycle progression and mediated the proliferative effect of NGF, we tested its effects on apoptotic death of naive and neuronally differentiated PC12 cells and the effects on NGF-mediated PC12 cell survival.

EXPERIMENTAL PROCEDURES

Cell Culture

Rat PC12 cells were maintained in tissue culture flasks as previously described (16). Logarithmically growing cells were harvested by mechanical dislodging, and after centrifugation the pellet was resuspended in Dulbecco’s modified Eagle’s medium (DMEM). Cell viability was determined by trypan blue (0.01%) exclusion, and cells were resuspended to the desired final density in the appropriate medium and replated. 24 h later, test conditions were added to the medium.

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‡ To whom correspondence should be addressed: Dept. of Cell Biology, Vanderbilt Univ, 1161 21st Ave. S., MCN #C-2310, Nashville, TN 37232-2175.

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1 The abbreviations used are: PD, Parkinson’s disease; BH4, tetrahydrobiopterin; NGF, nerve growth factor; NAS, N-acetylsertotonin; DAHP, diaminohydroxyprrinidn; kilobase pair; LDH, lactate dehydrogenase; DTT, dithiothreitol; NAC, N-acetylcysteine; zVAD-fmk, benzyloxy carbonyl-Val-Ala-Asp-fluoromethylketone.

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Models of PC12 Apoptotic Cell Death

Model A: Undifferentiated, Dividing PC12 Cells—Apoptosis was induced by serum withdrawal. 24 h following plating of nonsynchronized, logarithmically growing PC12 cells in 75-cm² tissue culture flasks (7 × 10⁶ cells/flask), cells were washed twice with serum-free DMEM and incubated for 24 h in serum-free medium containing 1% bovine serum albumin. Cells were also treated for 24 h with test conditions in serum-free medium.

Model B: Differentiated, Neuron-like PC12 Cells—PC12 cells were plated in 75-cm² tissue culture flasks (1 × 10⁶ cells/flask) and incubated for 8 days in reduced serum medium (DMEM (−); without fetal bovine serum and containing only 1% heat-inactivated horse serum) containing 50 ng/ml 2.5 S NGF. After 8 days, apoptotic death was induced by NGF withdrawal. Cells were washed twice with DMEM (−) (without NGF) and incubated for 24 h in 75-cm² tissue culture flasks (without NGF) containing a 1:2000 dilution of anti-NGF antibody (Sigma catalog no. N5142). At this concentration, the antibody completely blocked neurite outgrowth following a 3-day NGF treatment. The effect of various treatments on apoptotic death was tested by incubating cells for 24 h in the presence of test compounds in DMEM (−) containing anti-NGF antibody.

Cell Counting

Cells were plated in 75-cm² flasks (7 × 10⁶ cells/flask), equilibrated for 24 h, incubated in test conditions (10.36 × 10⁶ cells/flask) for 24 h, and dislodged with trypsin. Cells were then pelleted and resuspended in Dulbecco’s phosphate-buffered saline without calcium and magnesium; cell number was determined by direct counting using a hemacytometer. In some cases, cell number was estimated by the use of the CellTiter 96-assay were verified by direct cell counting.

DNA Fragmentation

PC12 cells were trypsinized and counted using a hemacytometer as described earlier. Soluble, cytoplasmic DNA was extracted routinely from 4 × 10⁶ cells or in certain cases (to better resolve DNA laddering) from 8 × 10⁶ cells, run on agarose gels, and visualized as previously described (22). Control experiments excluded the possibility that genomic DNA fragmentation occurred during cell counting. Similarly, trypsinization did not adversely affect laddering, since identical results were obtained when cytoplasmic DNA was directly extracted from plated cells and controlled later for total protein levels.

Thymidine Incorporation

PC12 cells were cultured in 60-mm dishes (2 × 10⁶ cells/dish), and 24 h later, cells were treated with test conditions for an additional 24 h. Tritiated methyl thymidine incorporation was measured as previously described (23).

Lactate Dehydrogenase Assay

PC12 cells were plated at 15 × 10⁶ cells/well. After 24 h, cells were washed twice with serum-free DMEM and test conditions were added. Following a 24-h incubation, the activity of lactate dehydrogenase (LDH) was measured in the medium as a measure of total dead, burst cells using a kit from Promega (Cytotox 96). The results represent extracellular LDH activity and were expressed as a percentage of the total LDH activity in the well.

Quantitation of Soluble DNA

In some cases, cytotoxicity was also measured by the total amount of double-stranded DNA released in the culture medium during incubation with test conditions. PicoGreen (Molecular Probes, Inc., Eugene, OR) was used for double-stranded DNA quantitation as previously described (17).

Western Blot Analysis of c-Myc Expression

PC12 cells were cultured in test conditions for appropriate incubation times and harvested at indicated times (1.5–24 h) by trypsinization. After cell counting, lysates were obtained by sonication at 4 °C in 20 mM Tris (pH 7.5), 50 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.5% SDS, 1 mM EDTA, and 0.5% aprotinin. Proteins (50 µl equivalent to 625,000 cells) were electrophoretically separated on a SDS-polyacrylamide gel (7.5% separating, 4% stacking) and electrophoresed onto a nitrocellulose membrane (BA 85; Schleicher & Schuell). Immunodetection was performed by blocking in TBST (20 mM Tris (pH 7.5), 150 mM NaCl, 0.2% Tween 20) containing 3% nonfat dry milk, incubating with affinity-purified anti-c-Myc rabbit polyclonal antibody raised against the full-length murine c-Myc (provided by Dr. S. R. Hann) and peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch). Control experiments established that trypsinization did not adversely affect c-Myc protein levels or cleavage, since identical results were obtained when cell lysates were controlled for total protein content (without trypsinization) rather than cell number.

RESULTS

BH₄ and DNA Fragmentation during Serum Withdrawal—We initially tested the effect of BH₄ on serum withdrawal-induced PC12 apoptotic cell death. To selectively enhance intracellular BH₄ levels and minimize degradation of BH₄ in the culture medium, we routinely treated PC12 cells with sepiapterin. Sepiapterin enters the cells readily and is converted to BH₄ intracellularly through the sequential actions of sepiapterin reductase and dihydrofolate reductase (salvage BH₄ biosynthetic pathway) (16, 25). Incubation of PC12 cells for 24 h in the absence of serum induces DNA fragmentation (Fig. 1) as reported previously (9–11). Elevation of intracellular BH₄ levels with sepiapterin enhances DNA fragmentation (soluble DNA was extracted from an equal number of cells in all cases), resulting in characteristic DNA laddering, one of the hallmarks of apoptotic cell death (Fig. 1A). A range of sepiapterin concentrations (0, 25, 50, and 100 µM) were then tested for their effect on DNA fragmentation, and, as depicted in Fig. 1B, increasing concentrations of sepiapterin resulted in proportional increases in DNA fragmentation.

We next tested whether inhibition of BH₄ biosynthesis could block fragmentation following serum withdrawal. Diaminohydroxyprymidine (DAHP), an inhibitor of GTP cyclohydrolase (26, 27), the first enzyme in the salvage BH₄ biosynthetic pathway (25, 28, 29), did not alter the serum withdrawal effect on DNA fragmentation (Fig. 1C). Similar results were obtained with N-acetylsperotonin (NAS) (28), an inhibitor of sepiapterin reductase, which is the last enzyme in the salvage BH₄ biosynthesis (25, 28, 29) (data not shown). To explain why inhibitors of BH₄ synthesis did not alter DNA fragmentation following serum withdrawal, we considered the possibility that inhibition of endogenous BH₄ prevents the generation of nitric oxide (NO) by nitric-oxide synthase, which can protect PC12 cells from apoptosis (30–32). Indeed, we observed an increase in DNA fragmentation by inhibiting NO synthesis (discussed in detail below). In preliminary studies, the minimum dose of sodium nitroprusside (an NO donor) able to protect against apoptotic DNA fragmentation following serum withdrawal was reduced 10-fold from 100 to 10 µM in the presence of 1 mM DAHP. These data suggest that inhibition of endogenous BH₄, affects PC12 cell survival by at least two pathways; it inhibits the proapoptotic BH₄ effects but at the same time blocks the antiapoptotic effects of NO. Thus, it is possible that the lack of an effect on DNA fragmentation with BH₄ biosynthetic inhibitors is due to the concomitant inhibition of NO synthesis.

We then tested whether BH₄ enhances serum withdrawal-induced cell death via a caspase-dependent pathway. Previous studies have established a role for cysteine proteases (caspases) in the apoptotic death of PC12 cells (32–35). Fig. 1D shows that...
diation with prior DNA fragmentation results (Fig. 1C). 24 h later, soluble DNA was extracted and resolved on a 1.2% agarose gel. Each lane was loaded with DNA extracted from $8 \times 10^6$ cells. B, serum withdrawal-induced DNA fragmentation was blocked by 50 ng/ml NGF, while increasing concentrations of sepiapterin (25, 50, and 100 $\mu$M) induced a dose-dependent increase in serum withdrawal-induced DNA fragmentation. Soluble DNA was extracted from $4 \times 10^6$ cells per condition and resolved on a 1.2% agarose gel. C, after washing with serum-free medium, cells were incubated for 24 h in either serum-containing medium (control), serum-free medium alone, or serum-free medium supplemented with either 50 ng/ml NGF, 1 mM DAHP, 100 $\mu$M sepiapterin, NGF plus DAHP, or NGF plus sepiapterin. Soluble DNA was extracted from $4 \times 10^6$ cells per condition and resolved on a 1.2% agarose gel. D, cells were serum-starved as before and incubated in the presence of sepiapterin alone, zVAD-fmk (50 $\mu$M) alone, or a combination of sepiapterin and zVAD-fmk for 24 h. Soluble DNA was extracted from an equal number of cells per condition and resolved on a 1.2% agarose gel. Comparative results for all figures were obtained in at least three independent experiments. bp, base pair.

Fig. 1. Effect of intracellular BH$_4$ levels on PC12 DNA fragmentation upon serum withdrawal. A, 24 h after plating PC12 cells were washed free of serum and cultured in DMEM with serum (serum) or without serum (− serum) or in serum-free medium containing 100 $\mu$M sepiapterin (−SEP). 24 h later, soluble DNA was extracted and resolved on a 1.2% agarose gel. Each lane was loaded with DNA extracted from $8 \times 10^6$ cells. B, serum withdrawal-induced DNA fragmentation was blocked by 50 ng/ml NGF, while increasing concentrations of sepiapterin (25, 50, and 100 $\mu$M) induced a dose-dependent increase in serum withdrawal-induced DNA fragmentation. Soluble DNA was extracted from $4 \times 10^6$ cells per condition and resolved on a 1.2% agarose gel. C, after washing with serum-free medium, cells were incubated for 24 h in either serum-containing medium (control), serum-free medium alone, or serum-free medium supplemented with either 50 ng/ml NGF, 1 mM DAHP, 100 $\mu$M sepiapterin, NGF plus DAHP, or NGF plus sepiapterin. Soluble DNA was extracted from $4 \times 10^6$ cells per condition and resolved on a 1.2% agarose gel. D, cells were serum-starved as before and incubated in the presence of sepiapterin alone, zVAD-fmk (50 $\mu$M) alone, or a combination of sepiapterin and zVAD-fmk for 24 h. Soluble DNA was extracted from an equal number of cells per condition and resolved on a 1.2% agarose gel. Comparative results for all figures were obtained in at least three independent experiments. bp, base pair.

The sepiapterin-induced enhancement of DNA fragmentation is completely blocked by zVAD-fmk, a caspase inhibitor, which is thought to inhibit apoptotic cell death induced by either withdrawal of trophic support or oxidative stress in PC12 cells (36). Trophic factors also reverse the BH$_4$ effect, since NGF (50 ng/ml) treatment eliminated DNA fragmentation induced by serum withdrawal in the presence or absence of sepiapterin (Fig. 1C and D).

Effect of Intracellular BH$_4$ on PC12 Cell Number and Extracellular LDH Activity During Serum Withdrawal—Incubation of PC12 cells for 24 h in serum-free medium caused a reduction of cell number to $\approx 50\%$ of control (Fig. 2A). Sepiapterin did not significantly affect cell counts compared with serum-deprived medium alone, whereas NGF treatment partially reversed the effect of serum withdrawal. Both NAS and DAHP, inhibitors of de novo BH$_4$ biosynthesis (26–28), also increased PC12 cell survival after 24 h of serum withdrawal in an apparent contradiction with prior DNA fragmentation results (Fig. 1C). Finally, N-ω-nitroarginine, an inhibitor of nitric-oxide synthase (37), did not affect death by serum withdrawal at a concentration that totally blocks NO production and NGF-induced neurite formation (38). These results suggest that inhibition of BH$_4$ biosynthesis can protect from or delay apoptotic death but fail to demonstrate an elevation in cell death following sepiapterin treatment.

Since enhancement of DNA fragmentation is likely to be an irreversible effect and has been shown to be a relatively late event in the progression of apoptotic PC12 cell death (10), we expected that elevation of intracellular BH$_4$ would also increase cell death. To further explore this possibility, we tested the activity of extracellular LDH as a measure of total cell death during the 24-h incubation period. Extracellular LDH activity was expressed as percentage of total activity (both in the medium and in living cells), which represents the death of cells that have released LDH following loss of membrane integrity. In control experiments, the presence of serum (but not NGF or other treatments) altered LDH activity, and for this reason the effects of test compounds on extracellular LDH accumulation were controlled against the NGF protective effect in the absence of serum. At 24 h, extracellular LDH activity was increased to 28% following serum withdrawal as compared with 17% of control (50 ng/ml NGF in serum-free medium; Fig. 2B). LDH activity was further increased to 56% by sepiapterin treatment, indicating that increased intracellular BH$_4$ indeed enhances PC12 cell death. Incubation with NAS protected PC12 cells from the serum withdrawal effects on cell death, while in combination with NGF, NAS further potentiated the NGF protective effect. These results were verified by measuring total DNA in the medium as another indication of cytotoxicity (data not shown) and are in agreement with the previous results on DNA fragmentation.

The fact that sepiapterin significantly increased cell death (LDH results) without decreasing the number of intact cells when compared with serum deprivation alone (Fig. 2A) indicates that total cell number (intact and burst) increased upon BH$_4$ treatment, suggesting that BH$_4$ can induce cell proliferation in the absence of serum. To test whether BH$_4$ increases the number of dying PC12 cells following serum withdrawal or accelerates the death of already committed or sensitive cells, we tested LDH accumulation following 48 h in test conditions (Fig 2B). Sepiapterin treatment of serum-deprived PC12 cells for 48 h did not alter extracellular LDH activity when compared with serum deprivation alone, suggesting that BH$_4$ accelerates the death of cells that are already predisposed to death under conditions of serum withdrawal. NAS still protected cells from death and potentiated the NGF trophic effect at 48 h.

Incubation of PC12 cells for 3 days in serum-free medium results in the selection of growth-arrested, apoptosis-resistant PC12 cells (40). Subsequent treatment of these cells with sepiapterin for an additional 24 h caused a dose-dependent increase in cell number evidenced by the use of a colorimetric cell
proliferation/cytotoxicity assay (Fig. 2C). This result strongly suggests that BH₄ can induce cell proliferation in the absence of serum.

Effect of Altered Intracellular BH₄ on NGF Withdrawal-induced DNA Fragmentation—PC12 cells that have acquired a neuron-like phenotype and are postmitotic following a prolonged treatment with NGF undergo apoptotic cell death following NGF withdrawal (9, 10). As shown in Fig. 3, DNA cell number and extracellular LDH activity. A, cells were plated in 75-cm² flasks at 7 × 10⁶ cells/flask. 24 h later, cells were washed free of serum and then placed in fresh serum-containing medium (DMEM) or in serum-free DMEM in the absence or presence of additional test compounds. Test compounds included NGF, sepiapterin, DAHP, NAS, and Nω-nitro-arginine (NNA). After a 24-h incubation, total cells were counted using a hemacytometer as described under "Experimental Procedures." Values are expressed as percentage of control and represent the mean ± S.E. of 3–6 independent determinations performed in duplicate. *, p < 0.05; **, p < 0.01; ***, p < 0.001; analysis of variance as compared with DMEM.

B, extracellular LDH activity in the culture medium was measured using the Cytotox 96 kit from Promega as suggested by the manufacturer. Since the addition of serum significantly increased the assay background, results with test conditions were compared with the protective effect of NGF on serum withdrawal-induced cytotoxicity. Cells were incubated for 24 or 48 h in serum-free DMEM alone or containing either NGF, sepiapterin, NAS, or a combination of NGF and NAS. Extracellular LDH activity is expressed as a percentage of total cellular LDH activity.

C, color formation at 490 nm was measured after incubation of cells in CellTiter 96 Aqueous reagent as described under "Experimental Procedures." Color formation linearly correlated with PC12 cell number under these conditions. Values represent the mean ± S.E. of three independent determinations performed in triplicate. *, p < 0.05; **, p < 0.01; Student’s t test as compared with results without serum in the presence of sepiapterin 100 μM.
fragmentation is enhanced 24 h after NGF withdrawal, and this effect is markedly enhanced by elevation of intracellular BH₄ with sepiapterin. In addition, inhibition of the intracellular conversion of sepiapterin to BH₄ by NAS blocked the sepiapterin effect, while DAHP, which inhibits only de novo BH₄ synthesis and does not block conversion of sepiapterin to BH₄, had no effect on sepiapterin's proapoptotic effect. DAHP and, to a lesser extent, NAS protected neuronal PC12 cells from DNA fragmentation following NGF withdrawal and further potentiated the NGF protective effect. The effect of sepiapterin on DNA fragmentation was blocked by continued NGF treatment. Fig. 3 also shows the effects of actinomycin D (which inhibits DNA transcription) on neuron-like PC12 cell DNA fragmentation. Actinomycin D protected neuron-like PC12 cells both from NGF withdrawal-induced and sepiapterin-potentiated DNA fragmentation, indicating that active DNA transcription is required for the sepiapterin effect on neuron-like PC12 cell death. Similar experiments under serum withdrawal-induced apoptosis failed to show a protective effect and rather resulted in enhanced cytotoxicity both for control (as reported previously) (41) and for sepiapterin treated cells² in the presence of actinomycin D.

Total cell counts following NGF withdrawal-induced apoptotic death were also tested. After 24 h of NGF withdrawal, the PC12 cell number dropped to 60% of control (NGF-treated cells), whereas the addition of sepiapterin, in the absence of NGF, only produced a decrease to 80% of control (not shown). Despite the increased cell number in sepiapterin-treated cells when compared with cells subjected to NGF withdrawal alone, extracellular soluble DNA accumulation during the 24-h incubation was significantly elevated in sepiapterin-treated cells (data not shown). These data are also consistent with an increase in cellular proliferation following sepiapterin treatment (16), accompanied by an acceleration of apoptotic cell death in sensitive cells. Finally, both cell counts and total DNA levels verified that NAS and DAHP fully protect neuron-like PC12 cells from NGF withdrawal-induced apoptotic death.

**Effect of Nitric Oxide, Catecholamine, or Hydrogen Peroxide Metabolism and Effect of Calcium Influx on Serum Withdrawal-induced DNA Fragmentation**—Since toxic effects resulting from NO production and catecholamine accumulation have been reported (42–45) and since BH₄ enhances their synthesis (46, 47), we examined whether elevation of intracellular BH₄ enhances apoptotic death of PC12 cells through these mechanisms. Treatment of serum-deprived PC12 cells with 5 mM α-nitro-L-arginine methyl ester (NAME), which totally blocks NO production in PC12 cells (38), failed to reduce and instead potentiated control and sepiapterin-induced DNA fragmentation (Fig. 4A). N-ω-Nitro-L-arginine, another nitric-oxide synthase inhibitor, also failed to alter serum withdrawal-induced cell death (Fig. 2A), indicating that the effect of BH₄ on the potentiation of apoptotic PC12 cell death is not mediated by increased NO metabolism.

To test whether BH₄ induces apoptotic cell death by increasing intracellular catecholamine levels, we treated PC12 cells with 20 μM α-methyl-para-L-tyrosine, which blocks catecholamine production (16) by inhibiting tyrosine hydroxylase, the BH₄-requiring and rate-limiting enzyme in catecholamine synthesis (18). α-Methyl-para-L-tyrosine (α-MPT) failed to reduce DNA fragmentation following serum withdrawal in the absence or presence of sepiapterin, suggesting that this BH₄ effect is independent from the production of PC12 cell catecholamines (Fig. 4B).

Using a spectrophotometric assay to quantify hydrogen peroxide (24), we have verified that BH₄ autoxidation in medium can lead to hydrogen peroxide formation (48, 49). Hydrogen peroxide formation is as high as 30% of the initial BH₄ concentration within 30 min of incubation in cell-free medium or PBS and was blocked by 1500 units/ml of catalase or 1 mM dithiothreitol (DTT). However, DTT treatment does not inhibit the sepiapterin-induced enhancement of apoptotic death (Fig. 5A), although it prevents BH₄ degradation to hydrogen peroxide in vitro. Treatment of PC12 cells for 24 h with catalase (1500 units/ml) or superoxide dismutase (1000 units/ml) also fails to block the sepiapterin-induced enhancement in PC12 cell DNA fragmentation following serum deprivation (Fig. 5B). In contrast, catalase inhibits BH₄ degradation in vitro and hydrogen peroxide-induced cytotoxicity (Fig. 5C) and DNA fragmentation in PC12 cells. Superoxide dismutase and catalase also inhibit the cytotoxic effect of BH₄ in serum-maintained PC12 cells treated in the absence of DTT (data not shown). As reported previously, in the presence of DTT (it has no effect by itself), oxidative degradation is minimized, and BH₄ induces cell growth like sepiapterin (16), while hydrogen peroxide induces cell death under the same conditions. These results suggest that hydrogen peroxide production cannot account for the enhancement of apoptotic death by elevating intracellular BH₄ levels during withdrawal of trophic support.

Since calcium influx can induce apoptotic PC12 cell death (10, 50, 51) and previous reports suggested that BH₄ induces

² P. Z. Anastasiadis, H. Jiang, L. Bezin, D. M. Kuhn, and R. A. Levine, unpublished results.
³ P. Z. Anastasiadis, H. Jiang, L. Bezin, D. M. Kuhn, and R. A. Levine, unpublished data.
calcium influx (52, 53), we also tested the possibility that calcium influx is responsible for the enhancement of apoptotic PC12 cell death following sepiapterin treatment. Treatment with 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid or with 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid/acetoxymethylester (54) to chelate extracellular or intracellular calcium, respectively, failed to show a reversal of the sepiapterin-induced enhancement of DNA fragmentation and cell death (data not shown).

Effect of BH4 on c-Myc Expression under Conditions of Serum Deprivation—We then tested the effect of BH4 on c-Myc expression under conditions of serum withdrawal, since c-Myc expression has been linked to apoptosis in several model systems (55–57). Fig. 6 shows that incubation of serum-starved PC12 cells in the presence of sepiapterin (100 μM) significantly induced the expression of c-Myc S proteins at early time points when compared with control (minus serum). At later time points (9–24 h) c-Myc S expression was inhibited by sepiapterin treatment. Similar results were evident for c-Myc 1 (68 kDa) and c-Myc 2 (65 kDa) expression under longer exposures (data not shown). Nonetheless, sepiapterin treatment preferentially induced the expression of c-Myc S proteins (predicted sizes between 45 and 50 kDa), which are amino-terminally truncated versions of full-length c-Myc arising through the use of downstream AUG codons (58). Thus, alterations in c-Myc expression may be responsible for the enhancement of PC12 cell apoptosis by elevated intracellular BH4 during withdrawal of trophic support.

Effect of N-Acetylcysteine or Inhibition of Cyclin-dependent Kinases on BH4-induced DNA Fragmentation—Previous reports have established the ability of N-acetylcysteine (NAC) to prevent apoptotic death of PC12 cells (59–61). This protective effect is not mediated by the ability of NAC to affect glutathione metabolism (60) or to inhibit cell cycle progression (61) but rather by a mechanism that involves the activation of the MAP kinase pathway (61). We found that NAC at moderate concentrations (5 mM) is able to fully inhibit the sepiapterin-induced DNA fragmentation under conditions of serum withdrawal (Fig. 7A).

We also tested the effect of the cyclin-dependent kinase inhibitor, olomoucine, on the proapoptotic effect of sepiapterin. Olomoucine is reportedly capable of blocking apoptotic cell death of differentiated, neuron-like PC12 cells following NGF withdrawal (14, 36, 63), while it increases cell death of serum-deprived PC12 cells. Indeed, in repeated experiments, olomoucine moderately increased DNA fragmentation in serum-deprived PC12 cells (Fig. 7B). Interestingly, while the addition of sepiapterin alone potently enhanced DNA fragmentation, upon the addition of 200 μM olomoucine, the sepiapterin-induced DNA fragmentation was reduced to levels observed with the addition of olomoucine alone (Fig. 7B). This result suggests that olomoucine blocks sepiapterin-induced apoptosis even under conditions of serum withdrawal.

DISCUSSION

We have previously reported that enhanced proliferation of PC12 cells by epidermal growth factor or NGF requires an increase in intracellular BH4 levels, which are raised ~3-fold (17). To achieve the same proliferative effect by exogenous administration, intracellular BH4 levels need to be increased 12-fold (16). Doses of sepiapterin up to 100 μM raise intracellular BH4 levels in a dose-dependent manner up to ~12-fold, and we have seen enhancement of apoptosis throughout the range of sepiapterin concentrations up to 100 μM. Thus, the concentrations of sepiapterin used previously and in the current study are likely to generate BH4 levels that are physiologically relevant. The relevance of endogenous, intracellular BH4 to apoptotic cell death is also supported by our observations that BH4 biosynthetic inhibitors prevent apoptosis in neurotally differentiated PC12 cells and potentiate the antiapoptotic effects of NO and NGF.

Elevation of intracellular BH4 was shown to increase PC12 cell number in a manner consistent with enhancing cell cycle progression rather than increasing cell survival (16). We now
show that elevated intracellular BH₄ enhances apoptotic death of undifferentiated PC12 cells in a dose-dependent manner following serum withdrawal and of neuron-like PC12 cells in a transcription-dependent manner following NGF withdrawal. We also show that BH₄ biosynthetic inhibitors protect differentiated neuron-like PC12 cells from apoptotic death. We found no evidence implicating catecholamine or NO metabolism (which are known to be induced by elevated BH₄) in mediating the BH₄ effect, since inhibitors of their synthesis did not alter the apoptotic profile.

Generation of superoxide radicals or hydrogen peroxide during BH₄ autooxidation and induction of calcium influx are also not likely to be involved in the BH₄ effect. Our results are consistent with the hypothesis that BH₄ enhances the rate of death in cells that are already responsive to apoptotic death following withdrawal of trophic support. Cells that escape apoptotic death under these conditions respond to BH₄ with increased cell growth (Fig. 2C). Increased cell proliferation is also the likely explanation for increased cell counts in sepiapterin-treated neuron-like PC12 cells after NGF withdrawal (when compared with NGF withdrawal alone), despite increased cytotoxicity and DNA fragmentation. Indeed, treatment of neuron-like PC12 cells with BH₄ in the absence of NGF and serum results in increased cell number after 5 days in culture (64). One possibility, in view of our data, is that increased cell number is the result of increased proliferation of apoptosis-resistant PC12 cells in the presence of BH₄.

Elevation of intracellular BH₄ in PC12 cells with sepiapterin increased c-Myc expression (especially c-Myc S) at early time points compared with serum deprivation alone. Interestingly, c-Myc S proteins are often found constitutively expressed in tumors or transiently expressed during rapid cell growth (58). It is possible that the early increase in c-Myc proteins is not relevant to the enhancement of apoptotic cell death by BH₄, but prior data indicate that both c-Myc and c-Myc S can induce apoptosis in several cell types via the illegitimate progression of the cell cycle (65, 66). In PC12 cells, exogenous c-Myc expression can block NGF-induced growth arrest and differentiation (67, 68). It is thus possible that the effects of BH₄ both on PC12 cell proliferation and apoptosis are mediated by c-Myc (or c-Myc S).

The BH₄-induced enhancement of cell death was completely abrogated by the cysteine protease inhibitor zVAD-fmk, indicating that caspases are involved in the BH₄ pro-apoptotic effect. Interestingly, NAC also completely blocked the BH₄ effect (Fig. 7A). Previous studies have demonstrated that the anti-apoptotic effects of NAC in PC12 cells are not due to its antioxidant capacity (60) but are instead dependent on new transcription and the activation of the Ras-ERK signaling pathway (61). Given our data with inhibitors of oxidative stress, we feel that NAC prevents BH₄-induced DNA fragmentation by a mechanism that does not involve its antioxidant potential. In support of this, administration of reduced gluta-
Tetrahydrobiopterin Effects on Apoptotic Cell Death

In the presence of sufficient trophic support, BH4 promotes PC12 cell growth by a mechanism that involves increased cell cycle progression. BH4 biosynthetic inhibitors inhibited growth factor-induced proliferation, suggesting that BH4 inhibition affects a growth factor-mediated signaling event. In the absence of trophic factors, BH4 accelerates DNA fragmentation and PC12 cell death. This BH4 effect can be blocked by NAC, olomoucine, and zVAD-fmk, agents that were previously shown to inhibit PC12 apoptotic death due to loss of trophic support. Together with data showing that BH4 affects the expression of Myc-related proteins and indicates that BH4 may promote cell cycle progression even under conditions of serum withdrawal, our results suggest that the effects of BH4 on PC12 cell growth or death, are mediated by the same mechanism.

Fig. 8. Schematic diagram depicting our current understanding of the effects of BH4 on PC12 cell growth or death. In the presence of sufficient trophic support, BH4 promotes PC12 cell growth by a mechanism that involves increased cell cycle progression. BH4 biosynthetic inhibitors inhibited growth factor-induced proliferation, suggesting that BH4 inhibition affects a growth factor-mediated signaling event. In the absence of trophic factors, BH4 accelerates DNA fragmentation and PC12 cell death. This BH4 effect can be blocked by NAC, olomoucine, and zVAD-fmk, agents that were previously shown to inhibit PC12 apoptotic death due to loss of trophic support. Together with data showing that BH4 affects the expression of Myc-related proteins and indicates that BH4 may promote cell cycle progression even under conditions of serum withdrawal, our results suggest that the effects of BH4 on PC12 cell growth or death, are mediated by the same mechanism.

An interesting possibility not addressed by our data is whether the BH4 effect following serum withdrawal (not shown). Although not tested, it is likely that the BH4 effect is blocked by the activation of the Ras-ERK signaling pathway. Finally, olomoucine, an inhibitor of cyclin-dependent kinases, also reversed the BH4 effect on DNA fragmentation. By itself, olomoucine is able to block NGF withdrawal but not serum deprivation-induced cell death (14).

The above results also allow us to postulate that BH4 affects one of three major pathways involved in PC12 cell apoptotic death. Previous studies have demonstrated that PC12 cell death by superoxide dismutase 1 depletion (leading to oxidative stress) can be reversed by zVAD-fmk but not by olomoucine (36). Furthermore, apoptosis induced by DNA-damaging agents is reversed by olomoucine but not by zVAD-fmk or NAC (69, 70). The only pathway of apoptotic cell death that is blocked by either zVAD-fmk, olomoucine, or NAC is that of trophic factor deprivation (36, 60). Previous reports suggested that BH4 can induce cell proliferation (16, 71) and that growth factor-induced proliferation correlates with endogenous BH4 levels in PC12 cells (17). In view of these data, we postulate that BH4 affects a growth factor-dependent signaling event, which leads to increased proliferation in the presence of sufficient trophic support or enhances cell death in its absence. Fig. 8 summarizes our current understanding of the effects of BH4 on the proliferation and survival of PC12 cells.

An interesting possibility not addressed by our data is whether treatment of serum-deprived cells with BH4 enhances NGF withdrawal. Our results suggest that BH4 affects a growth factor-dependent signaling event, which leads to increased proliferation in the presence of sufficient trophic support or enhances cell death in its absence. Fig. 8 summarizes our current understanding of the effects of BH4 on the proliferation and survival of PC12 cells.

Whether a shift in the balance of BH4 from being supportive of neuronal function to becoming destructive for neuron viability occurs in neurodegenerative disorders warrants further investigation. The validity of this hypothesis is relevant to patient management, since BH4 has been used as a therapeutic agent for the treatment of neurodegenerative disorders where monoaminergic deficits are evident, including Parkinson’s and Alzheimer’s disease. Our data may also be relevant to the development of novel experimental approaches including the engineering of cells expressing high levels of BH4 and catecholamines for transplantation therapy in Parkinson’s disease.

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