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Tetrahydrobiopterin Is Released from and Causes Preferential Death of Catecholaminergic Cells by Oxidative Stress

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ABSTRACT

The underlying cause of the selective death of the nigral dopaminergic neurons in Parkinson's disease is not fully understood. Tetrahydrobiopterin (BH4) is synthesized exclusively in the monoaminergic, including dopaminergic, cells and serves as an endogenous and obligatory cofactor for syntheses of dopamine and nitric oxide. Because BH4 contributes to the syntheses of these two potential oxidative stressors and also undergoes autoxidation, thereby producing reactive oxygen species, it was possible that BH4 may play a role in the selective vulnerability of dopaminergic cells. BH4 given extracellularly was cytotoxic to catecholamine cells CATH.a, SK-N-BE(2)C, and PC12, but not to noncatecholamine cells RBL-2H3, CCL-64, UMR-106-01, or TGW-nu-1. This was not caused by increased dopamine or nitric oxide, because inhibition of their syntheses did not attenuate the damage and BH4 did not raise their cellular levels. Dihydrobiopterin and biopterin were not toxic, indicating that the fully reduced form is responsible. The toxicity was caused by generation of reactive oxygen species, because catalase, superoxide dismutase, and peroxidase protected the cells from the BH4-induced demise. Furthermore, thiol agents, such as reduced glutathione, dithiothreitol, β-mercaptoethanol, and *N*-acetylcysteine were highly protective. The BH4 toxicity was initiated extracellularly, because elevation of intracellular BH4 by sepiapterin did not result in cell damage. BH4 was spontaneously released from the cells of its synthesis to a large extent, and the release was not further enhanced by calcium influx. This BH4-induced cytotoxicity may represent a mechanism by which selective degeneration of dopaminergic terminals and neurons occur.

In Parkinson's disease, dopaminergic neurons in the substantia nigra pars compacta undergo selective degeneration. Although the physiological and neurochemical consequences of this phenomenon have been extensively documented, the underlying cause of dopamine cell death or the mechanism by which these cells degenerate is still not understood. Of various mechanisms proposed thus far, oxidative stress is believed to play an important role. Decreased glutathione level (Sian et al., 1994) and increased iron concentration (Sofic et al., 1988) and lipid peroxidation (Dexter et al., 1989) have been noted in the substantia nigra of patients with Parkinson's disease and of aged individuals.

Potential candidates responsible for the selective dopaminergic cell death would be molecules that are specifically present in these cells and participate in generation of reactive oxygen species. Of these, dopamine is a likely candidate because it produces hydrogen peroxide during its metabolism by monoamine oxidase as well as during its autoxidation (Stokes et al., 1999). Iron is also thought to be a culprit, because it accumulates specifically in the substantia nigra and globus pallidus and promotes formation of the highly reactive hydroxyl radical by catalyzing the Fenton reaction (Harley et al., 1993). Furthermore, the presence of the catecholamine-synthesizing enzyme tyrosine hydroxylase may play a role, because reactive oxygen species are generated through partial uncoupling of the hydroxylation reaction (Haavik et al., 1997).

Another molecule exclusively present in monoaminergic, including dopaminergic, neurons is tetrahydrobiopterin (BH4) (Hwang et al., 1998). Evidence suggests the possibility that BH4 may contribute to cell death via a number of mechanisms. First, as an obligatory (Kaufman, 1993) and rate-limiting cofactor for tyrosine hydroxylase reaction in the substantia nigra (Miwa et al., 1985), its intracellular concentration would determine the synthesis rate of dopamine. Therefore, an increase in the intracellular BH4 level would cause a rise in dopamine synthesis, followed by generation of oxidative stress. In addition, BH4 also serves as a cofactor for

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ABBREVIATIONS: BH4, tetrahydrobiopterin; FBS, fetal bovine serum; HS, horse serum; L-NAME, N^G-nitro-L-arginine methyl ester; L-NMMA, *N*-monomethyl-L-arginine; LDH, lactate dehydrogenase; MEM, minimum essential medium; GTPCH, GTP cyclohydrolase I.

the synthesis of nitric oxide (Kwon et al., 1989). A rise in the availability of BH4, therefore, might enhance synthesis of nitric oxide, which is further oxidized to the highly reactive peroxynitrite.

In addition to its contribution toward the syntheses of these two potentially toxic molecules, BH4 itself can directly generate oxidative stress. BH4 undergoes oxidation during the hydroxylation reaction (Davis and Kaufman, 1993) as well as nonenzymatic autoxidation (Fisher and Kaufman, 1973; Davis et al., 1988) to produce hydrogen peroxide and superoxide radical. Furthermore, BH4 is observed to stimulate oxidation of nitric oxide to peroxynitrite (Mayer et al., 1995). 6-Biopterin, the completely oxidized form of BH4, is reported to be toxic to melanocytes (Schallreuter et al., 1994). Furthermore, death of hippocampal CA1 neurons could be prevented by inhibition of BH4 synthesis in an ischemic animal model (Cho et al., 1999).

These findings led us to hypothesize that because of its selective synthesis in monoaminergic cells and potential to generate oxidative stress both directly and indirectly, BH4 may render dopamine cells preferentially vulnerable to oxidative stress. The present study was undertaken to determine whether BH4 has a role in dopaminergic cell death and, if so, to elucidate the mechanism underlying this toxicity. We show that BH4 is spontaneously released and the extracellular BH4 subsequently exerts a preferential toxic effect on catecholaminergic cells via generation of reactive oxygen species independently of dopamine or nitric oxide syntheses.

Experimental Procedures

Materials. Human neuroblastoma cell line TGW-ν-I was obtained from Japanese Cancer Research Resources Bank and CATH.a cell line was donated by Tufts University Medical School. PC-12, RBL-2H3, CCL-64, and UMR 106-01 cells were purchased from the American Type Culture Collection (Rockville, MD). All culture media, fetal bovine serum (FBS), horse serum (HS), L-glutamine, amphotericin, trypsin/EDTA, and penicilline-streptomycin were from GibcoBRL (Gaithersburg, MD). BH4, α-methyl-L-tyrosine, BayK8644, 3-iodo-L-tyrosine, N^G-nitro-L-arginine methyl ester (L-NAME) and *N*-monomethyl-L-arginine (L-NMMA) were obtained from RBI (Natick, MA). Dihydrobiopterin, biopterin, ascorbate, lactate dehydrogenase (LDH) standard, reduced and oxidized glutathione, L-2-oxothiazolidine-4-carboxylate, sepiapterin, veratridine, A23187, catalase, superoxide dismutase, and horseradish peroxidase were purchased from Sigma Chemical (St. Louis, MO). The CytoTox 96 nonradioactive cytotoxicity assay kit was purchased from Promega (Madison, WI). All other chemicals were reagent grades and were from Sigma or Merck (Rahway, NJ).

Cell Cultures. CATH.a cells were grown in RPMI 1640 medium supplemented with 8% HS and 4% FBS; SK-N-BE(2)C cells were grown in RPMI 1640 medium with 10% FBS. TGW-nu-1 cells were grown in Eagle's minimum essential medium (MEM) with 10% FBS and PC-12 cells in RPMI 1640 medium containing 10% HS and 5% FBS. RBL-2H3 cells were cultured in MEM containing Eagle's balanced salt solution and 15% FBS and UMR 106-01 cells were grown in Dulbecco's MEM containing 5% FBS. CCL-64 were grown in Eagle's MEM with Earle's balanced salt solution with 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 10% FBS. All cells were grown as monolayers in the presence of 100 I.U./l penicillin and 10 μg/ml streptomycin. When confluent, they were subcultured 1:3 to 1:5 after dispersing adherent cells using trypsin/EDTA. Cultures were maintained at 37°C in 95% air, 5% CO_2 humidified atmosphere. For experiments, cells were plated on polystyrene tissue culture dishes at a density of 2×10^5 cells/well in 24-well culture plate or 3×10^6 cells/60-mm plate. After 24 h, cells were fed with fresh media, at which time BH4 was added.

Primary cultures of bovine adrenal medullary cells were prepared as previously described (Hwang et al., 1994). The cell viability as estimated by trypan blue exclusion was greater than 95% and chromaffin cells assessed by neutral red staining over 85%. Cells were plated in Dulbecco's MEM/F12 medium containing 10% FBS, 50 μg/ml penicillin, 50 μg/ml streptomycin, 5 ng/ml amphotericin B, 250 μM ascorbate, and 5 μg/ml cytosine arabinoside. Two days (38–40 h) after the plating the medium was replaced with fresh medium, which was considered time 0.

Determination of LDH Activity. Cell toxicity was assessed by activity of LDH released into the culture medium, determined spectrophotometrically at 490 nm using the CytoTox 96R Nonradioactive Cytotoxicity Assay kit.

Morphological Studies and Cell Counting. Morphological changes of cells were examined and photomicrographs were taken using a Leica DM IRB inverted microscope. For trypan blue exclusion assay, an aliquot of the cell suspension was diluted 1:1 with 0.4% trypan blue solution and the dye-excluding viable cells and dye-staining dead cells were each counted on a hemacytometer.

Determination of Biopterin. Biopterin was determined according to the method reported previously (Cho et al., 1999). For intracellular biopterin, cells were harvested and homogenized in 1 ml of 0.1 M phosphoric acid. For extracellular biopterin, 900-μl aliquots of medium were mixed with 100 μl of 1 M phosphoric acid. Both samples were subsequently mixed with 0.2 ml of acidic iodine solution (0.5% I_2/1% potassium iodide in 0.2 M trichloroacetic acid) and incubated in the dark for 1 h at room temperature. The oxidation reaction was terminated by addition of 0.1 ml of 1% ascorbic acid. The mixture was centrifuged at 8000*g* for 15 min and the supernatant was diluted with distilled water and analyzed by HPLC coupled with fluorescence detector with 5% methanol as mobile phase. Biopterin content was calculated using a Waters 991 computerized integrator system and a standard curve was prepared every time.

Determination of Dopamine. Quantification of intracellular dopamine was performed as reported previously (Hwang et al., 1994). Briefly, cells were collected and treated with perchloric acid, and the acid soluble fraction was diluted appropriately. Catecholamines were separated by HPLC using C18 Novapak column in the mobile phase consisting of 0.1 M sodium acetate, 0.1 M citric acid, 0.5 mM sodium octyl sulfate, 0.15 mM NaEDTA, and 1 mM di-*n*-butylamine in H_2O/methanol (90:10) and detected electrochemically by a Waters 460 detector. The amounts of dopamine were calculated using Waters 991 computerized integrator system and a curve prepared every time.

Determination of Nitrite. Accumulated nitrite, an oxidative metabolite of nitric oxide, in the cell culture medium was measured by the Griess reaction (Green et al., 1982). Briefly, a 200-μl aliquot of medium from each well was mixed with 100 μl of Griess reagent consisting of 1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride and 2.5% H_3PO_4 in a 96-well microtiter plate and the absorbance was read at 540 nm using a plate reader.

Data Analyses. All data are reported as mean ± S.E. Comparisons were made using ANOVA and Newman-Keuls multiple comparison test.

Results

BH4 Causes Catecholaminergic Cell Death. To determine the effect of BH4 on catecholamine cell viability, we first tested CATH.a, a clonal cell line of the central nervous system producing primarily dopamine and, to a lesser extent, norepinephrine (Suri et al., 1993). The cells were exposed to various BH4 concentrations and durations, and degrees of cell death were assessed by LDH activity released in the medium. As shown in Fig. 1A, LDH was increased in a

manner dependent on the concentration of BH4 and duration of treatment. Significant elevation was observed as early as 2 h with 250 μM and 5 h with 50 μM. In 24 h, 50, 100, and 250 μM BH4 yielded dramatic increases in LDH activities in the medium to 213 ± 2, 297 ± 8, and 431 ± 40% of untreated control, respectively. The degree of cell death diminished with increasing cell density (not shown) and we carried out all subsequent experiments at 2.6 to 2.8 $\times$ 10^4 cells/cm^2.

Morphological analysis by inverted light microscopy (Fig. 1B) showed that within 5 h, the cells exposed to 100 μM and 250 μM BH4 have become shrunken, granular, and round. Significant detachment from the bottom of the culture plate characteristic of cell death was observed. Although 50 μM BH4 did not cause apparent morphological changes in 5 h, further incubation to 24 h resulted in the characteristic shrinking and detachment as well.

Percentages of viable cells under these conditions were assessed by counting the trypan blue-staining and -excluding cells. As shown in Fig. 1C, number of viable cells corresponded well with the morphological changes (Fig. 1B) and LDH activity increases (Fig. 1A). With 50, 100, and 250 μM BH4 exposures for 24 h, percentages of viable cells were 63 ± 3, 43 ± 2, and 16 ± 2% of total cells, respectively. Taken together, the results of LDH assay, morphological analysis, and trypan blue staining demonstrated toxic effects of the extracellularly applied BH4 on the catecholaminergic CATH.a cells.

BH4-Induced Cell Death Occurs Preferentially in Catecholaminergic Cells. To test whether this BH4 cytotoxicity is specific for catecholamine cells, we evaluated various cell lines for their susceptibility to BH4. The catecholaminergic cell lines CATH.a, human neuroblastoma SK-N-BE(2)C, and rat pheochromocytoma PC12 and the noncatecholaminergic cell lines rat basophilic leukemia RBL-2H3, mink epithelial CCL-64, rat osteosarcoma UMR-106-01, and human neuroblastoma TGW-nu-1 cells were incubated in the presence of 0, 100, and 250 μM BH4 for 24 h. As shown in Fig. 2, BH4 was cytotoxic to all three catecholamine cells under these conditions. Although SK-N-BE(2)C and PC12 cells were not as vulnerable at 100 μM, they too died at 250 μM (473 ± 28 and 281 ± 9% of untreated control, respectively). On the other hand, the serotonin-producing RBL-2H3 cells were not susceptible under these conditions (100 ± 15% at 250 μM BH4). Noncatecholamine cells CCL-64 and UMR-106-01 also did not die (97 ± 2 and 80 ± 9%, respectively). Only TGW-nu-I cells exhibited a slight increase (128 ± 2%), but not to levels comparable with the damage observed with the catecholaminergic cells. Thus, BH4 was shown to be preferentially toxic to catecholaminergic cells.

BH4-Induced Cell Death Is Not Mediated by Enhanced Synthesis of Dopamine or Nitric Oxide. Because BH4 serves as an obligatory cofactor for both dopamine and nitric oxide syntheses, it was possible that the cytotoxicity occurs via enhanced level of these potentially toxic com-

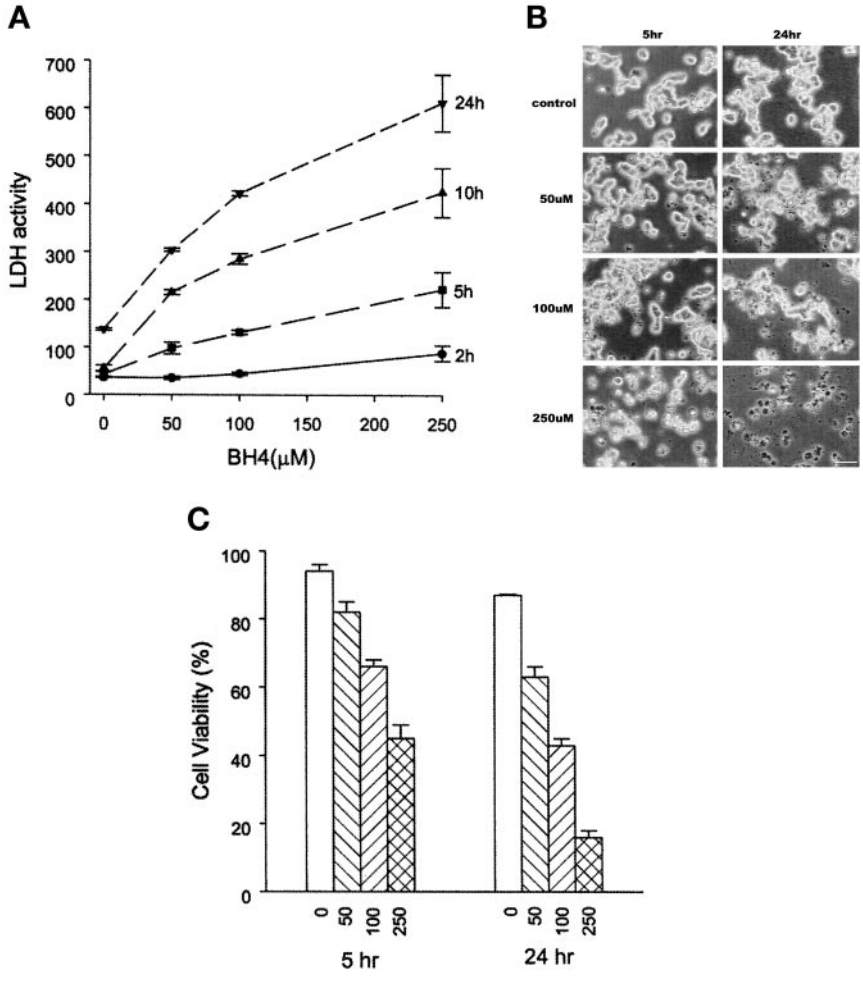

Fig. 1. Cytotoxic effect of BH4. A, CATH.a cells were incubated with 0 to 250 μM BH4 for 0 to 24 h and the degrees of cell death were assessed by LDH activity released into the medium. Data were obtained from two independent experiments (n = 4 each) and expressed as mean ± S.E. of LDH activity. ANOVA, Newman-Keuls multiple comparison test, $P < .005$ versus untreated control cells for all 5-, 10-, and 24-h values. B, representative photographs of morphological analysis. CATH.a cells were incubated with 0 to 250 μM BH4 for 5 or 24 h and their morphological changes were examined under inverted microscope. Bar, 50 μm. C, number of trypan blue staining dead cells. CATH.a cells were exposed to 0 to 250 μM BH4 for 5 or 24 h, after which they were subjected to trypan blue staining. The trypan blue excluding viable cells and total cells were counted from which percentages of viable cells were calculated. Data were obtained from two independent experiments (n = 4 each) and are expressed as mean ± S.E. of percentage of total cells. $P < .005$ versus untreated control cells for all values, by Newman-Keuls multiple comparison test.

pounds. To test this, we asked whether inhibition of their syntheses might attenuate the damage by BH4. CATH.a cells were treated with tyrosine hydroxylase inhibitors α-methyl-L-tyrosine and 3-iodo-L-tyrosine, or nitric oxide synthase inhibitors L-NAME or L-NMMA along with BH4. As shown in Fig. 3A, extent of cell death after cotreatment with α-methyl-L-tyrosine (315 ± 4% of untreated control) or 3-iodo-L-tyrosine (308 ± 4%) did not differ significantly from that with BH4 alone (297 ± 8%). Cotreatment with L-NAME and L-NMMA resulted in LDH activities 301 ± 4 and 306 ± 6% of untreated control, respectively. The inhibitors of dopamine and nitric oxide alone did not have any effect on cell death. Thus, concomitant synthesis of dopamine or nitric oxide was demonstrated to be not required for the BH4 toxicity.

To further confirm this, dopamine and nitric oxide levels were directly measured after the BH4 treatment. Cells were treated with 0, 10, 50, and 100 μM for up to 6 h, as longer exposure would damage the cells (Fig. 1). As shown in Fig. 3B, no changes were observed in the intracellular dopamine contents under these experimental conditions ($P > .6$). Nitric oxide accumulated in the medium by these cells is very low (0.68 ± 0.08 nM in the form of nitrite; $n = 3$) and 100 μM BH4 did not significantly change the level (0.56 ± 0.06; $P > .2$). Because the exposure to 100 μM BH4 for 5 h was sufficient to produce morphological and biochemical changes of cell death (Fig. 1, A–C), these results demonstrated that the BH4-induced cytotoxicity is not mediated by increases in these molecules and that BH4 may have a direct role.

Biopterin Is Not Toxic. Because 6-biopterin, the completely oxidized form of BH4, was reported to be toxic to melanocytes (Schallreuter et al., 1994), it was possible that the BH4 toxicity on catecholamine cells occurred after BH4 was oxidized. However, dihydrobiopterin resulted in only marginal degree of cell death (115 ± 7% of untreated control) and biopterin seemed to have some protective effect (82 ± 5%) (Fig. 4). Thus, unlike melanocytes, the catecholaminergic CATH.a cells were not susceptible to the oxidized form of BH4 and only the fully reduced form seemed mediate the toxicity.

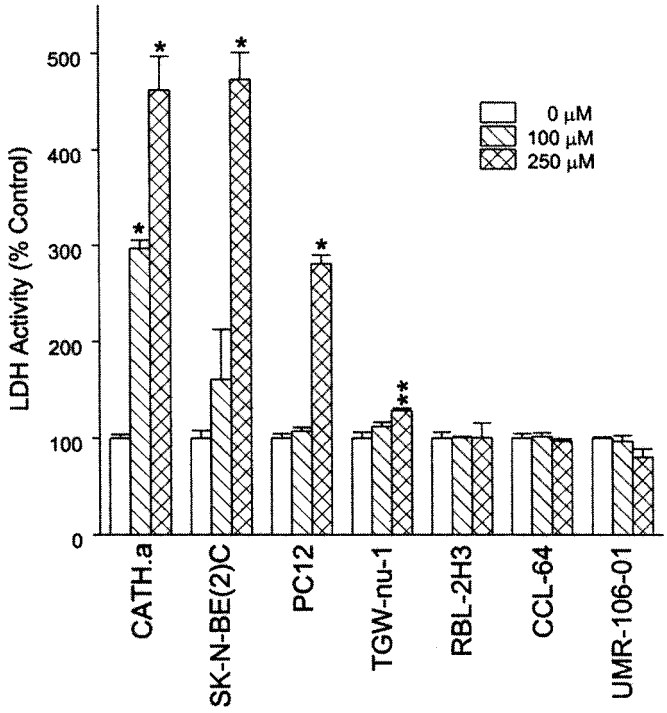

Fig. 2. Specificity of BH4-induced cytotoxicity to catecholaminergic cells. Various cell lines were incubated in the presence of 0, 100, or 250 μM BH4 for 24 h, after which degrees of cell death were assessed by LDH activity released into the medium. Data were obtained from two independent experiments ($n = 4$ each) and are expressed as mean ± S.E. of LDH activity in percentage of untreated control cells. ANOVA, Newman-Keuls multiple comparison test; *$P < .001$; **$P < .005$ versus respective untreated control cells.

BH4 Toxicity Is Produced by Generation of Oxygen Radicals. Because BH4 can be autoxidized to produce hydrogen peroxide and superoxide radical (Fisher and Kaufman, 1973; Davis et al., 1988), we tested whether reactive oxygen species are involved. CATH.a cells were treated with BH4 in the presence of the free radical scavenging enzymes catalase, superoxide dismutase, or peroxidase, and tested whether the enzymes might attenuate the BH4-induced cell death. As shown in Fig. 5, catalase and peroxidase, the enzymes responsible for scavenging hydrogen peroxide, caused reduction of LDH activity to levels comparable with those of the untreated control (122 ± 7 and 105 ± 4%, respectively). Superoxide dismutase, catalyzing the conversion of superoxide to hydrogen peroxide, was somewhat protective (220 ± 22%). The enzymes themselves had no significant effect on cell viability. Taken together, the BH4-induced cell damage seemed to be produced primarily by hydrogen peroxide generated during oxidation of BH4.

As hydrogen peroxide can freely cross cell membrane, a number of cellular reactions inside, outside, or on the membranes of the cell may occur. To determine the site most affected by BH4, various antioxidants were tested at their sublethal concentrations normally effective in scavenging oxygen species. As shown in Fig. 6, ascorbate, a hydrophilic compound that primarily acts outside the cell, could not protect the cells against BH4 (290 ± 33% of untreated control). Vitamin E, a potent inhibitor of membrane peroxidation, was also ineffective (285 ± 24%). Butylated hydroxyanisole and butylated hydroxytoluene, lipophilic agents that freely cross the membrane and scavenge intracellular free radicals, caused even further cell death (463 ± 50 and 462 ± 17%, respectively). These phenolic agents, however, caused significant cell death when administered alone at concentrations as low as 25 and 50 μM, respectively (data not shown), as has been demonstrated to occur in some conditions (reviewed by Stich, 1991). Melatonin, which is effective in removing intracellular reactive oxygen species, was also not protective (265 ± 11%). On the other hand, reduced glutathione, which does not freely cross the membrane, completely abolished the BH4 toxicity (101 ± 3%).

The finding that only reduced glutathione was an effective protectant suggested that molecules with sulfhydryl groups may be a primary target. To test this notion further, we tested a battery of sulfhydryl agents for their ability to prevent the BH4-induced cytotoxicity. As shown in Fig. 7, reduced glutathione, dithiothreitol, β-mercaptoethanol, and *N*-acetylcysteine abolished the toxic effect of BH4, resulting in LDH activity of 101 ± 3, 99 ± 11, 105 ± 10, and 129 ± 25% of untreated control, respectively, whereas the oxidized glutathione was not effective (261 ± 14%). L-2-Oxothiazolidine-4-carboxylate, a nonthiol compound used in glutathione synthesis only after it enters the cell (Meister et al., 1986) was not protective (290 ± 9%). The various agents used in the experiments did not have any effect on cell death when they

were administered alone (data not shown). Taken together, only the extracellularly acting agents with a reduced sulfhydryl group were effective in attenuating the BH4-induced cell death.

Intracellular BH4 Is Not Toxic. To exclude the possibility of involvement of intracellular BH4, we tested whether elevation of intracellular BH4 would influence cell viability. The cells were treated with the BH4 precursor sepiapterin, after which changes BH4 levels and cell death were assessed. As shown in Table 1, sepiapterin caused intracellular biopterin level to rise dramatically, but no significant amount of cell death was observed. Assuming that a cell is a sphere with a diameter of 12 μm (Fig. 1), the concentration of BH4 in the untreated cells was calculated to be approximately 112 μM. The intracellular BH4 of the 100 and 200 μM sepiapterin-treated cells were calculated to be 2422 and 2879 μM, respectively. Because cell death did not occur under this condition, intracellular BH4 is probably not involved in the toxicity.

BH4 Is Spontaneously Released. Having established that the BH4-induced damage occurs on the outside of the cell, it was necessary to show that intracellular BH4 can be exported into the extracellular space. To test this, CATH.a cells were placed in fresh medium and amounts of intracellular and extracellular BH4 of unstimulated cells were measured at various time points. As shown in Fig. 8, BH4 accumulated in the medium in a time-dependent manner, whereas its intracellular level remained relatively constant. This indicated that the cells produce and continuously release BH4, suggesting that the release is probably constitutive.

To ensure that this release is not an anomalous phenomenon pertaining to the immortalized cell line, we tested whether BH4 release is similarly observed in primary cultured catecholamine cells. For this, we used adrenal medulla because it expresses its synthesis enzyme GTP cyclohydrolase I (GTPCH) (Hwang et al., 1998) and its primary cultured cells can be obtained in a large number and to high homogeneity from the bovine tissue (Hwang and Choi, 1995; Hwang et al., 1997). In addition, because these cells can be cultured in the absence of serum, we could further eliminate potential effector molecules fortuitously present in the serum. The cells were cultured for 48 h in the absence of serum and the amounts of intracellular and released BH4 were measured and compared with CATH.a. As shown in Table 2, large accumulation of BH4 in the medium was observed with the primary cultured cells as well. The ratio of the released to intracellular BH4 was similar between the primary cultured catecholamine cells and the cell line, at 14- and 11-fold, respectively. The data indicated that spontaneous BH4 release is a common phenomenon in both immortalized and primary cultured catecholamine cells.

We then tested whether the release can be further enhanced by short-term induction of calcium influx. After changing into fresh medium, CATH.a cells were incubated in the presence of potassium (50 mM KCl), veratridine (4 μM), BayK 8644 (10 μM), and calcium ionophore A23187 (100 nM) for 1 h at 37°C and the amounts of released BH4 were determined. The various stimulators did not cause changes in the amount of released BH4, which were 99.5 ± 1.6, 98.3 ± 1.5, 97.2 ± 1.5, and 97.7 ± 2.5% of the untreated control (n = 4; mean ± S.E., $P > .2$ for all drugs), respectively. This indicated that the release mechanism is independent of increased intracellular calcium.

Discussion

The present study demonstrates a novel role of BH4 in catecholaminergic cell death. BH4 synthesized in these cells is spontaneously released and subsequently exerts a selective toxic effect independently of dopamine or nitric oxide syntheses.

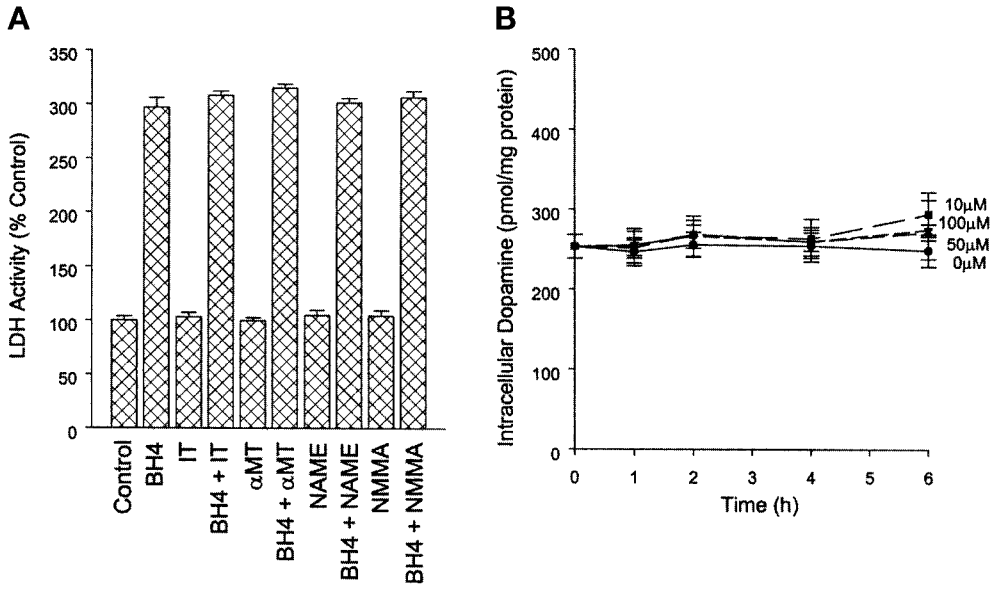

Fig. 3. BH4 toxicity is not mediated by dopamine or nitric oxide synthesis. A, effects of inhibition of dopamine and nitric oxide syntheses on BH4-induced cell death. CATH.a cells were treated with 3-iodo-L-tyrosine (IT; 100 μM), α-methyl-L-tyrosine (αMT; 10 μM), L-NAME (300 μM), or L-NMMA (300 μM) along with BH4 (100 μM) and cultured for 24 h. Cell death was assessed by LDH activity in the medium. Data were obtained from two independent experiments (n = 4 each) and are expressed as mean ± S.E. of LDH activity in percent of untreated control cells. ANOVA, Newman-Keuls multiple comparison test; $P < .001$ versus untreated control cells and $P > .2$ versus BH4 alone for each cotreatment. B, absence of dopamine synthesis increases by extracellularly given BH4. Cells were exposed to 0, 10, 50, or 100 μM BH4 for 0, 1, 2, 4, or 6 h after which intracellular dopamine was measured. Data were obtained from two independent experiments (n = 4 each) and are expressed as mean ± S.E. No changes in dopamine ($P > .6$) were observed.

This toxicity is abolished by catalase and peroxidase as well as by thiol antioxidants, suggesting the involvement of reactive oxygen species generated during autoxidation of BH4.

Once released into the extracellular space, BH4 may be autoxidized to generate hydrogen peroxide and superoxide radicals (Fisher and Kaufman, 1973; Davis et al., 1988). In addition, the autoxidation is accelerated with time because of further reduction of the partially reduced radicals by BH4 (Fisher and Kaufman, 1973). The reactive oxygen species thus produced can potentially participate in a number of cellular reactions from which cellular demise would ensue. As sulfhydryl groups present on cysteine residues of proteins and peptides are the strongest cellular nucleophiles at physiological pH, they may be the primary targets of the oxygen species. This is supported by the present finding that the thiol reagents abolished the BH4 toxicity. That catecholamine cells were more vulnerable suggests possible involvement of proteins expressed specifically to this phenotype. Conceivably, sulfhydryl groups located on proteins specific to dopaminergic/catecholaminergic phenotype, such as transporter proteins, receptors, or enzymes involved in catecholamine synthesis/metabolism pathways, may be the site whose modification produces the preferential cell death of this phenotype. In addition, BH4 itself may attack free protein sulfhydryls, as previously shown for tryptophan hydroxylase (Kuhn and Arthur, 1997) and tyrosine hydroxylase (Roskoski et al., 1990).

The fact that BH4 is produced exclusively in the monoaminergic neurons and terminals (Hwang et al., 1998) and that the short-lived oxygen species would be most reactive within near vicinity suggests that in vivo, these cells and terminals would be the primary target of BH4 toxicity. Among monoaminergic cells, cells producing dopamine may be most vulnerable to BH4 in vivo. Dopamine has a higher rate of oxidation and higher toxic potential compared with other catecholamines (Graham et al., 1978) and the dopamine cells of the substantia nigra accumulate iron, which renders oxidative damage by catalyzing the Fenton reaction. Thus, the dopamine cells are already under a substantial level of oxidative stress, and the generation of oxygen species by BH4 would further exacerbate their vulnerability.

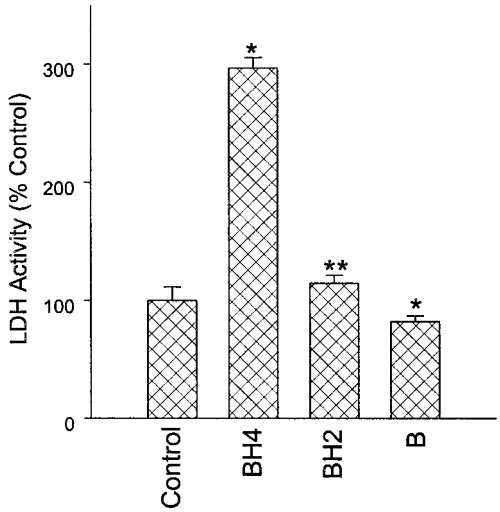

Fig. 4. Effects of oxidized forms of biopterin on CATH.a cell viability. CATH.a cells were incubated in the presence of 100 μM BH4, dihydrobiopterin (BH2) or biopterin (B) for 24 h and cell death was assessed by LDH activity in the medium. Data were obtained from two independent experiments (n = 4 each) and are expressed as mean ± S.E. of LDH activity in percentage of untreated control cells. ANOVA, Newman-Keuls multiple comparison test; *P < .01; **P > .05 compared with untreated control cells.

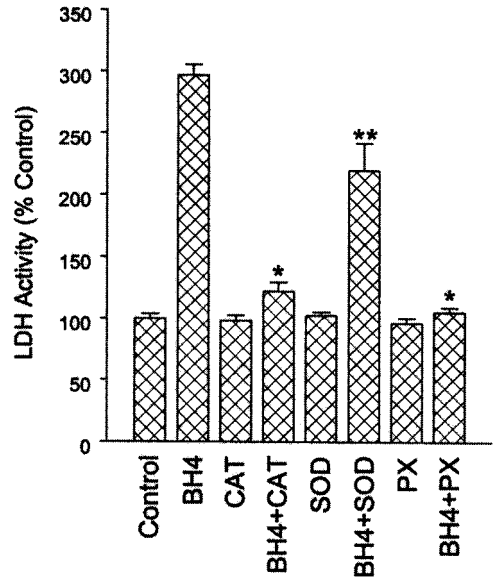

Fig. 5. Effects of various antioxidant enzymes on BH4-induced cell death. CATH.a cells were incubated with 100 μM BH4 in the absence or presence of enzymes catalase (CAT; 1,000 units/ml), superoxide dismutase (SOD; 1,000 units/ml), or peroxidase (PX; 10,000 units/ml) for 24 h. Cell death was assessed by LDH activity in the medium. Data were obtained from three independent experiments (n = 4 each) and are expressed as mean ± S.E. of LDH activity in percentage of untreated control cells. ANOVA, Newman-Keuls multiple comparison test; for cotreatments, *P < .001 versus BH4 treated and P > .2 versus untreated control cells; **P < .005 compared with BH4 treated and untreated control cells; enzymes alone had no effect (P > .5 versus untreated control cells).

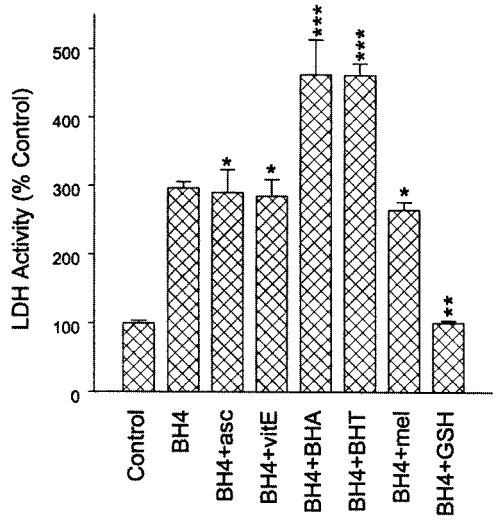

Fig. 6. Effects of various antioxidants on BH4-induced cell death of CATH.a. CATH.a cells were incubated with 100 μM BH4 in the absence or presence of ascorbate (asc; 250 μM), vitamin E (vitE; 500 μM), butylated hydroxyanisole (BHA; 100 μM), butylated hydroxytoluene (BHT; 500 μM), melatonin (mel; 1 mM), and reduced glutathione (GSH; 1 mM) for 24 h. Cell death was assessed by LDH activity in the medium. Data were obtained from three independent experiments (n = 4 each) and are expressed as mean ± S.E. of LDH activity in percentage of untreated control cells. ANOVA, Newman-Keuls multiple comparison test; *P > .01 versus BH4 treated and P < .001 versus untreated control cells; **P < .001 versus BH4 treated and P > .2 versus untreated control cells; ***P < .001 versus BH4 treated and untreated control cells.

Ninety percent of total biopterin in catecholaminergic cells is reported to be in the tetrahydro form (Brautigam et al., 1984). Intracellular concentrations of BH4 in the catecholaminergic cells are estimated to be 18 μM in adrenal medullary cells (Abou-Donia et al., 1986), 25 μM in PC12 cells (Brautigam et al., 1984), 40 μM (Brautigam et al., 1984) and 115 μM (Kapatos and Kaufman, 1983) in N1E115 neuroblastoma cells, and 112 μM in CATH.a cells (the present data). In vivo, intraneuronal BH4 concentration in the striatal dopaminergic terminals has been estimated to be 100 μM (Levine et al., 1981). Although BH4 concentration in the extracellular space of the BH4 synthesizing neurons is not known, the present study shows that BH4 is released to a large extent. In 24 h, CATH.a and adrenal medullary cells release 6 times their intracellular BH4 content to the extracellular space. Considering the relatively small extracellular space in vivo, the effective concentration of extracellular BH4 would be substantially high.

It is conceivable that a clearance mechanism exists so that BH4 does not accumulate to a toxic level in the extracellular space. We have observed that the noncatecholaminergic TGW-nu-I cells take up extracellular BH4 rather efficiently but CATH.a cells do not (H. J. Choi and O. Hwang, unpublished observations). A previous study reported that BH4 is taken up into PC12 cells by passive diffusion (Anastasiadis et al., 1995). It is possible that PC12, having relatively low intracellular BH4 (Brautigam et al., 1984), takes up extracellularly BH4 applied at a higher concentration, whereas CATH.a with its already high intracellular BH4 (the present data) does not. Conceivably, BH4 in the extracellular space may be removed in vivo by uptake into the adjacent cells with low or no intracellular BH4 by passive diffusion.

The fact that the release is not enhanced by short-term stimulation suggests that BH4 synthesis would primarily determine the rate of BH4 release. Because the rate-limiting enzyme GTPCH largely controls BH4 synthesis, agents that render changes in the activity of this enzyme would influence the amount of released BH4. The fact that the dopaminergic

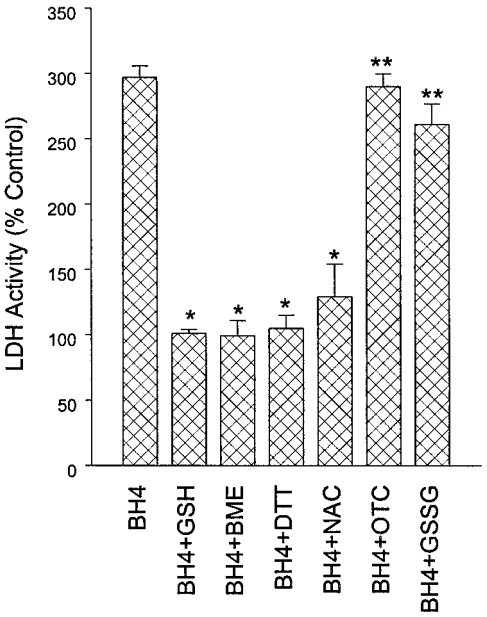

Fig. 7. Effects of various thiol agents on BH4-induced cell death of CATH.a. CATH.a cells were incubated with 100 μM BH4 in the absence or presence of reduced glutathione (GSH; 1 mM), β-mercaptoethanol (βME; 1 mM), dithiothreitol (DTT; 1 mM), *N*-acetylcysteine (NAC; 1 mM), L-oxothiazolidine 4-carboxylate (OTC, 1 mM), or oxidized gluthathione (GSSG; 1 mM) for 24 h. Cell death was assessed by LDH activity in the medium. Data were obtained from three independent experiments (n = 4 each) and are expressed as mean ± S.E. of LDH activity in percentage of untreated control cells. ANOVA, Newman-Keuls multiple comparison test; *P < .001 versus BH4 treated and P > .2 versus untreated control cells; **P > .05 versus BH4 treated and P < .001 versus untreated control cells.

TABLE 1

Effect of sepiapterin on BH4 levels and CATH.a cell death

CATH.a cells were incubated in the presence of 100 μM or 200 μM sepiapterin. After 24 h, medium was taken and assayed for LDH activity as an index of cell death and for biopterin concentration. The cells were harvested and intracellular biopterin levels were determined. Data were obtained from two independent experiments (n = 4 each) and are expressed as mean ± S.E. ANOVA and Newman-Keuls multiple comparison test.

	Intracellular BH4	Extracellular BH4	Cell Death
	ng/mg protein	*nM*	*% Control*
Control	179 ± 4	25 ± 6	100
100 μM Sep	3906 ± 195*	2903 ± 122*	116 ± 8**
200 μM Sep	4629 ± 213*	6715 ± 201*	106 ± 8**

Sep, sepiapterin.
* P < .001 compared with untreated control; ** P > .05.

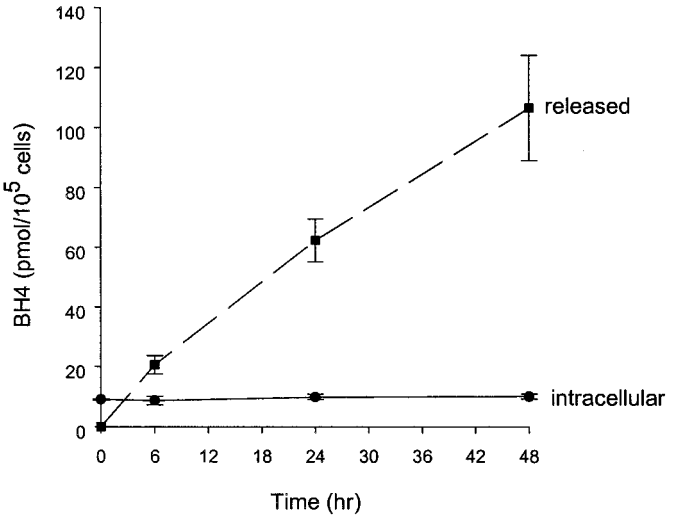

Fig. 8. Release of BH4 from unstimulated CATH.a cells. CATH.a cells growing in culture were changed into fresh medium and were further incubated for 0, 6, 24, and 48 h. At each time point, the media and cells were collected and amounts of intracellular and released biopterin were determined by HPLC coupled with fluorescence detector. Data were obtained from three independent experiments (n = 4 each) and are expressed as mean ± S.E. of LDH activity in percentage of untreated control cells. ANOVA, Newman-Keuls multiple comparison test; P < .001 versus time 0 for released BH4 at each time point; P > .5 for intracellular BH4.

TABLE 2

Intracellular and released BH4

Primary culture of adrenal medullary cells were prepared from bovine adrenal glands and CATH.a cells were cultured as described under *Experimental Procedures*. After changing into fresh medium, the cells were further incubated for 48 h and the media were collected and the cells were harvested after two Dulbecco's PBS washes. Amounts of BH4 were determined by HPLC coupled with fluorescence detector. Data were obtained from three independent experiments (n = 4 each) and are expressed as mean ± S.E.

	CATH.a	Primary Cultured Adrenal Medullary Cells
	pmol/10^6 cells	
Intracellular	100 ± 9	36 ± 1
Released	1065 ± 174	487 ± 58

cells of the substantia nigra contain the lowest amount of GTPCH and BH4 among monoaminergic cells in the brain (Hirayama and Kapatos, 1998) suggests the possibility that BH4 availability is closely monitored in vivo to avoid damage. In aberrant conditions in which such mechanism is no longer effective and released BH4 is increased to a toxic level and/or clearance mechanisms do not properly function, however, the consequences would be detrimental. We have previously reported that long-term calcium influx increases GTPCH gene expression, BH4 synthesis, and released BH4 (Hwang et al., 1999). This implies that in the case of sustained and excessive calcium influx, such as during activation of postsynaptic NMDA receptors in the dopamine cells of substantia nigra (Loopuijt and Schmidt, 1998), a rise in released BH4 might contribute to cell death.

Why extracellular, but not intracellular, BH4 is toxic is not clear at present. Interestingly, elevation of intracellular BH4 has been reported to enhance proliferation (Tanaka et al., 1989; Anastasiadis et al., 1996) and to protect cells against oxidative stressors (Ishii et al., 1999; Nakamura et al., 2000) in various cell types. We have also observed in the present study that elevation of intracellular BH4 with sepiapterin was not toxic. It is possible that, under normal conditions, an efficient scavenging system exists in dopaminergic neurons that effectively removes reactive oxygen species and that the intracellular BH4 action is mediated by a mechanism not involving oxidation of BH4. Consistent with this, dopamine cells are reported to contain higher levels of glutathione than nondopaminergic cells (Nakamura et al., 1997).

In conclusion, we propose that the ability to synthesize BH4 exclusively in the monoaminergic cells and its spontaneous release into the extracellular space in combination with the vulnerability of catecholamine/dopamine cells to BH4 contribute to the selective dopamine cell death. This may represent an additional mechanism by which selective degeneration of dopaminergic terminals and neurons occur in such neurodegenerative diseases as Parkinson's disease.

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