Tetrahydrobiopterin deficiency increases neuronal vulnerability to hypoxia

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Abstract
Tetrahydrobiopterin (BH₄) is an essential co-factor for nitric oxide synthases (NOS). The aim of the present work was to study whether BH₄ deficiency affects the vulnerability of neurones in primary culture to hypoxia. Intracellular BH₄ levels were depleted by pre-incubating neurones with 5 mM 2,4-diamino-6-hydroxypyrimidine (DAHP) for 18 h, after which cells were exposed for 1 h to normoxic or hypoxic conditions. Our results showed that whereas neurones were resistant to hypoxia-induced cellular damage, BH₄ deficiency in neurones led to oxidative stress, mitochondrial depolarization, ATP depletion and necrosis after 1 h of hypoxia. Indeed, hypoxia specifically inhibited mitochondrial complex IV activity in BH₄-deficient neurones. All these effects were counteracted when neuronal BH₄ levels were restored by incubating cells with exogenous BH₄ during the hypoxic period. Moreover, hypoxia-induced damage in BH₄-deficient neurones was prevented when N⁶-nitro-L-arginine monomethyl ester (NAME), haemoglobin or superoxide dismutase plus catalase were present during the hypoxic period, suggesting that peroxynitrite might be involved in the process. In fact, BH₄ deficiency elicited neuronal NO dysfunction, resulting in an increase in peroxynitrite generation by cells, as shown by the enhancement in tyrosine nitration; this was prevented by supplements of BH₄, NAME, haemoglobin or superoxide dismutase plus catalase during hypoxia. Our results suggest that BH₄ deficiency converts neuronal NOS into an efficient peroxynitrite synthase, which is responsible for the increase in neuronal vulnerability to hypoxia-induced mitochondrial damage and necrosis.

Keywords: glutathione, neurones, nitric oxide, mitochondria, tetrahydrobiopterin.


Nitric oxide (NO) is a free radical gas that is synthesized in a wide variety of species and tissues (Garthwaite et al. 1988; Moncada et al. 1991; Bredt and Snyder 1994). Mammalian NO-producing enzymes, the NO synthases (NOS), exhibit unique complexity and requirement for co-factors. In addition to NADPH, reduced flavins and heme iron, pteridine (6R)-5,6,7,8-tetrahydrobiopterin (BH₄) is an essential co-factor in NOS-catalysed NO biosynthesis (Knowles and Moncada 1994; Marletta 1993). BH₄ is also a well known co-factor of aromatic amino acid hydroxylases, which are key enzymes in the biosynthesis of several neurotransmitters, including catecholamines and serotonin (Kaufman 1993). However, the role of BH₄ in NO synthesis is different from its function as a co-factor of aromatic amino acid hydroxylation (recently reviewed in Thöny et al. 2000). In fact, BH₄ is thought to be required to maintain NOS quaternary structure, preserving the enzyme as a catalytically active dimer (Kaufman 1993; Thöny et al. 2000). Moreover, it has been recently reported that a pterin radical (BH₃) is formed during the NOS-catalysed reaction, BH₄ being involved in electron transfer in the first cycle of the NOS reaction. Particularly, BH₄ transfers an electron to the ferrous-dioxy intermediate to enable the formation of a heme-based oxidant that rapidly hydroxylates arginine, and the resulting BH₃ would subsequently be reduced by the NOS flavins (Hurshman et al. 1999; Schmidt et al. 2001; Wei et al. 2001).
Neurones synthesize NO through the activation of Ca\(^{2+}\)-dependent NOS, which is constitutively expressed in these cells, after glutamate receptor stimulation (Garthwaite et al. 1988). NO is involved in many important neurological functions through its ability to increase cGMP concentrations (Moncada et al. 1991). However, under certain pathological circumstances, NO biosynthesis may be enhanced, becoming neurotoxic (Dawson et al. 1993; Dawson and Dawson 1996). Thus, in recent years, a large body of evidence has emerged suggesting that nitric oxide biosynthesis is a key factor in the pathophysiological response of the brain to hypoxia, possibly mediated by excitatory amino acids (reviewed in Bolaños and Almeida 1999).

NOS efficiently utilizes molecular oxygen for NO synthesis only in the presence of saturating concentrations of both \(\text{L-arginine}\) and \(\text{BH}_4\) (Heinzel et al. 1992; Mayer et al. 1992; Pou et al. 1992; Vásquez-Vivar et al. 1999). \(\text{BH}_4\) deficiency results in neuronal NOS mediated-generation of superoxide \((\text{O}_2^-)\) in vitro (Pou et al. 1992; Abu-Soud et al. 1997; Vásquez-Vivar et al. 1999), which is known to react rapidly with NO to form peroxynitrite \((\text{ONOO}^-)\); Beckman et al. 1990). The generation of these species, \(\text{O}_2^-\) and \(\text{ONOO}^-\), is thought to be responsible for the neurotoxicity of NO (Beckman et al. 1990).

In mammalian cells, \textit{de novo} biosynthesis of \(\text{BH}_4\) originates from guanosine \(5^-\)triphosphate (GTP) via the sequential action of three enzymes; the first and rate-limiting enzyme in this pathway is GTP cyclohydrolase I (Nichol et al. 1985). Mutations in the gene responsible for production of this enzyme cause severe neurological diseases including DOPA-responsive dystonia and cases of atypical phenylketonuria (Furukawa et al. 1998). Moreover, decreased levels of \(\text{BH}_4\) in the cerebrospinal fluid have been documented in certain neurodegenerative diseases, such as Alzheimer’s disease (Barford et al. 1984) and Parkinson’s disease (Curtius et al. 1984).

The aim of the present work was to study the possible effect of \(\text{BH}_4\) deficiency on neuronal vulnerability to hypoxia. Neuronal \(\text{BH}_4\) concentrations were depleted by treatment with 2,4-diamino-6-hydroxypropyrimidine (DAHP); Bogdan et al. 1995; Saura et al. 1996; Ishii et al. 1997; Koshimura et al. 1998), an inhibitor of GTP cyclohydrolase I, which is the rate-limiting enzyme for the \textit{de novo} synthesis of \(\text{BH}_4\) (Gal et al. 1978; Nichol et al. 1985). Our results show that \(\text{BH}_4\) deficiency increases neuronal vulnerability to hypoxia, possibly due to NOS-catalysed peroxynitrite formation followed by mitochondrial damage.

Materials and methods

Chemicals

Dulbecco’s modified Eagle’s medium (DMEM), horse serum, cytosine arabinoside, \(N^-\text{nитro-L-arginine}\) monomethyl ester (NAME), tetrahydrobiopterin (\(\text{BH}_4\)), 2,4-diamino-6-hydroxypropyrimidine (DAHP), 4,6-diamino-2-phenylindole (DAPI), coenzyme \(Q_1\), \(Q_2\), 2-vinylpyridin, 1-isobutyl-3-methylxanthine, superoxide dismutase (SOD), catalase (CAT), manitol and haemoglobin were obtained from Sigma Chemical Co. (St Louis, MO, USA). DMEM was always supplemented with penicillin (100 U/mL), streptomycin (100 \(\mu\text{g/mL}\)) and amphotericin B (0.25 \(\mu\text{g/mL}\); Sigma). Fetal calf serum and cytochrome \(c\) were purchased from Boehringer Mannheim (Heidelberg, Germany). Cytochrome \(c\) was reduced with sodium ascorbate (Sigma) immediately before use and passed through Sephadex G-25 \(M\) (PD-10 columns, Amersham Pharmacia Biotech, Uppsala, Sweden) to remove excess ascorbate. Primary and secondary antibodies were obtained from Sigma. Other substrates, enzymes and coenzymes were purchased from Sigma, Boehringer or Merck (Darmstadt, Germany). Plastic tissue culture dishes were purchased from Nunc (Denmark) or Linbro (McLean, VA, USA). Peroxynitrite \((\text{ONOO}^-)\) was generated and quantified spectrophotometrically as previously described (Hughes and Nicklin 1968).

Alkaline stock solutions, with an approximate concentration of 0.3–0.4 mM, were stable at \(-70^\circ\text{C}\) for at least 3–4 months.

Animals

Albino Wistar rats fed \textit{ad libitum} on a stock laboratory diet were used for the experiments. Rats were maintained at 22°C with a 12-h light-dark cycle. Virgin females weighing 210–250 g were caged overnight with males and conception was confirmed the next morning by the presence of spermatozoa in vaginal smears.

Cell culture

Cerebral cortex neurones in primary culture were prepared from fetal rats at 16–17 days of gestation (Almeida and Medina 1998). Dissociated cell suspensions were plated at a density of \(2.5\times10^5\) cells/cm\(^2\) in either 2-cm\(^2\), 4-cm\(^2\), 9.6-cm\(^2\) or 28.2-cm\(^2\) plastic Petri dishes coated with poly-D-lysine in DMEM supplemented with 10% fetal calf serum (FCS). Cells were incubated at 37°C in a humidified atmosphere containing 5% \(\text{CO}_2/95%\text{ air}\). Forty-eight hours after plating, the medium was replaced with DMEM supplemented with 5% horse serum and 20 mM d-glucose. On day 4 of culture, cytosine arabinoside was added at a final concentration of 10 \(\mu\text{M}\) in order to prevent non-neuronal proliferation and neurones were used on day 9.

\(\text{BH}_4\) deficiency model

Eighteen hours before the experiments were performed, cells were pre-incubated in culture medium containing increasing concentrations of DAPH (1, 5 or 10 mM; Bogdan et al. 1995; Saura et al. 1996; Ishii et al. 1997; Koshimura et al. 1998), a specific inhibitor of GTP cyclohydrolase I, the rate-limiting enzyme for the \textit{de novo} synthesis of \(\text{BH}_4\) (Gal et al. 1978; Nichol et al. 1985).

Model of hypoxia \textit{in vitro}

The culture medium was removed and cells were washed once with pre-warmed \((37^\circ\text{C})\) buffered Hanks’ solution (5.26 mM KCl, 0.43 mM KH\(_2\)PO\(_4\), 132.4 mM NaCl, 4.09 mM NaHCO\(_3\), 0.33 mM Na\(_2\)HPO\(_4\), 5.44 mM glucose, 2 mM CaCl\(_2\), and 20 mM HEPES, pH 7.4). Hypoxia was induced by incubating cells at 37°C for 1 h in a humidified atmosphere of 95% \(\text{N}_2/5%\text{ CO}_2\) in buffered Hanks’ solution, previously gassed with 95% \(\text{N}_2/5%\text{ CO}_2\) for 30 min. Under these conditions, the concentrations of oxygen in the incubation
medium were 6.7 ± 0.5 μM as measured with a Clark-type oxygen electrode. In parallel, a group of cells (normoxia group) were incubated at 37°C for 1 h in a humidified atmosphere of 95% air/5% CO₂ in buffered Hanks’ solution. Under these conditions, oxygen concentrations in the incubation medium were 190 ± 15 μM. In some experiments, cells were exposed to hypoxia either in the absence or the presence of BH₄ (5 μM), NAME (1 mM), haemoglobin (2 μM), SOD (100 U/mL) plus CAT (100 U/mL), or mannitol (50 mM) in buffered Hanks’ solution (Bolaños and Medina 1996; Almeida et al. 1998).

**Determination of necrosis and apoptosis**
Necrotic and apoptotic cell death was determined after 1 h hypoxia as previously described (Delgado-Esteban et al. 2000). Necrosis was assessed by examination of trypan blue-stained cells. Briefly, neuronal and astrocytic cultures were carefully washed with warm (37°C) phosphate-buffered saline (PBS, 136 mM NaCl, 2.7 mM KCl, 7.8 mM Na₂HPO₄, 1.7 mM KH₂PO₄, pH 7.4) and incubated with 0.2% trypan blue in PBS for 2 min at room temperature. Microphotographs (20× magnification; Diaphot, Nikon) were taken for each experimental condition and viable plus necrotic (stained) cells were counted. Apoptosis was assessed by staining the nuclei of cells with propidium iodide, a fluorescent dye that binds DNA and allows quantification of apoptotic neurones, i.e. neurones displaying fragmented or condensed nuclei. Briefly, neuronal and astrocytic cultures were washed with warm PBS (37°C) and fixed with 4% (wt/vol) paraformaldehyde in PBS for 30 min at room temperature. After being washed with PBS, cells were exposed to 1 μg/mL propidium iodide for 10 min at room temperature in the dark and were then washed twice with PBS. Cells were scored for chromatin condensation by fluorescence microscopy, using a fluorescein filter (330–380 nm excitation; 30× magnification). Total and apoptotic nuclei were counted. In all cases, approximately 600–1000 cells were counted per well by an author blind to the protocol design. At least three different cell cultures utilizing four separate wells were employed, such that a minimum of 7200–12 000 neurones was counted for each data point.

**Rhodamine 123 staining and fluorescence measurements**
Cells were stained with Rhodamine 123 (Rh 123) as previously reported (Almeida et al. 1999). In brief, neurones were incubated in buffered Hanks’ solution containing 1 μg/mL of Rh 123 for 15 min at 37°C (Budd and Nicholls 1996; Khodorov et al. 1996); unbound dye was removed by washing cells twice with pre-warmed (37°C) buffered Hanks’ solution, and fluorescence microphotographs were taken with an inverted microscope using a fluorescein filter (excitation filter 480–490 nm; emission filter 510–530 nm). Pictures were scanned and the intensity of Rh 123 fluorescence was quantified and the values were subtracted from the background intensity values using an image analyser system (NIH Image), kindly supplied by Wayne Rasband (National Institutes of Health, Bethesda, MD, USA).

**Enzyme activity determinations**
For the determination of mitochondrial respiratory chain complex activities, neurones plated on 28.2-cm² Petri dishes were used. Following normoxia or hypoxia, neuronal cultures were washed with ice-cold PBS and the surviving cells were collected by trypsinization and resuspended in 300 μL of 0.1 M potassium phosphate buffer (pH 7.0). Cell suspensions (containing about 3–4 mg of protein/mL) were frozen and thawed three times to ensure cell lysis. Enzyme activities were determined in the cell lysates using a Hitachi U2000 spectrophotometer (Hitachi Ltd, Tokyo, Japan). NADH-CoQ₁ reductase (complex I; EC 1.6.99.3) activity was measured as described in Ragan et al. (1987). The activity of succinate-cytochrome c reductase (complex II-III; EC 1.8.3.1) was determined following the method of King (1967). Cytochrome c oxidase (complex IV; EC 1.9.3.1) activity was determined as described by Wharton and Tzagoloff (1967). All enzyme activities were expressed as nanomoles per minute per milligram of protein, except for cytochrome c oxidase, which was expressed as the first-order rate constant (k/min/mg of protein).

**Metabolite determinations**
For BH₄ measurements, neurones plated in 2-cm² wells were rapidly washed with ice-cold PBS, scraped off with 0.1 M perchloric acid containing 6.5 mM dithioerythritol and 2.5 mM diethyltriaminepentaacetic acid. Samples were centrifuged at 15 000 g for 2 min, and 20 μL of the clear supernatant was used for the analysis. The assay was carried out by high performance liquid chromatography (HPLC) with electrochemical detection (Howells et al. 1986; Barker et al. 1998).

For ATP determinations, neurones plated in 2-cm² wells were thoroughly washed with ice-cold PBS, in order to remove dead cells, scraped off with 2 × 0.5 mL of 0.3 M HClO₄, and neutralized with 0.5 mL of 2 M KHCO₃ at pH 6.5. The perchlorate precipitate was removed by centrifugation and ATP was determined in the supernatants by chemiluminescence, using a commercially available kit (Sigma) and following the manufacturer’s instructions.

For cyclic GMP determinations, neurones were exposed to the normoxic or hypoxic conditions described above, except that the phosphodiesterase inhibitor 1-isobutyl-3-methylxanthine (1 mM) was included throughout the experiments. After 1 h, the buffer was aspirated, cells were scraped off in 2 × 0.5 mL of ice-cold ethanol, the ethanol was dried, and the cell extracts were used for cyclic GMP determinations employing a commercially available radiolmmunoassay kit (Amersham Pharmacia Biotech) and following the manufacturer’s instructions.

For glutathione determinations, neurones plated in 9.6-cm²-well plates were washed with ice-cold PBS and immediately collected by scraping off with 0.5 mL of 1% (wt/vol) sulfosalicylic acid. Cell lysates were centrifuged at 13 000 g for 5 min at 4°C and the supernatants were used for glutathione determinations on the same day, as previously described (Dringen and Hamprecht 1996; García-Nogales et al. 1999).

**Western blotting analysis**
Neurones were washed with PBS and scraped off with lysis buffer containing 2% sodium dodecyl sulfate, 2 mM EDTA, 2 mM EGTA, 5 mM Tris, 100 μM phenylmethylsulfonyl fluoride, 50 μg/mL antipain, 50 μg/mL pepstatin, 50 μg/mL amastatin, 50 μg/mL leupeptin, 50 μg/mL bestatin and 50 μg/mL soybean trypsin inhibitor. Cell suspensions were centrifuged at 14 000 g for 10 min, and pelletted cells were frozen at ~80°C. Protein concentrations were determined and samples were normalized for total protein. Proteins were transferred by electrophoresis to polyacrylamide...
membrane (0.2 μm, Trans-Blot medium, Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked with 5% (w/v) low-fat milk in 20 mM Tris, 500 mM NaCl, 0.1% (w/v) Tween-20 (pH 7.5) for 4 h. Western blot analysis was performed using 3-nitrotyrosine antibody from rabbit (1 : 500). Binding of alkaline phosphate-coupled goat anti-rabbit IgG (1 : 1000) was visualized using a chromogenic substrate (3-amino-9-ethylcarbazole) and hydrogen peroxide. Band intensity was quantified with an image analyser system (NIH Image). Results are expressed as percentage of nitration, considering the intensity of each band from a sample of neurones treated with pure peroxynitrile (1 μM) for 5 min as 100% nitration.

Protein determinations
Proteins were determined either in the cell suspensions, lysates or in parallel cell culture incubations after solubilization with 0.1 M NaOH. Protein concentrations were determined by the method of Lowry et al. (1951), using bovine serum albumin as standard.

Statistical analysis
Measurements from individual cultures were performed in triplicate and the results are expressed as the mean ± SEM values for the number of culture preparations indicated in the legends of the figures and tables. Statistical analysis of the results was determined by one-way ANOVA followed by the least significant difference multiple range test. In all cases, p < 0.05 was considered significant.

Results

Pre-incubation of neurones with DAHP, an inhibitor of GTP cyclohydrolase I, triggers BH₄ depletion and NOS dysfunction
BH₄ is an essential co-factor for nitric oxide synthases and its availability appears to limit the activity of these enzymes (Kaufman 1993). In order to modulate intracellular concentrations of this co-factor in neurones in primary culture, cells were pre-incubated in culture medium either in the absence or in the presence of increasing concentrations of DAHP, an inhibitor of GTP cyclohydrolase I. After 18 h of pre-incubation, samples were collected for BH₄ measurements, as previously described (Howells et al. 1986; Barker et al. 1998). Treatment with DAHP dose-dependently decreased intracellular BH₄ concentrations in neurones, the minimum value being reached with 5 mM DAHP treatment (Fig. 1a).

In order to assess the possible neurotoxicity of DAHP treatment, ATP concentrations were measured in neurones (b and c). In some experiments, neurones were pre-incubated in culture medium containing or lacking 5 mM DAHP at 37°C for 18 h, and cells were then exposed to normoxia (normoxia group or normoxia + DAHP group) or hypoxia (hypoxia group or hypoxia + DAHP group) either in the absence or in the presence of 5 μM BH₄ (normoxia + DAHP + BH₄ group) (d). Results are means ± SEM from four to eight different cell cultures. *p < 0.05 as compared with 0 mM DAHP (normoxia group).
pre-incubated with increasing concentrations of DAHP. We observed that DAHP treatment did not affect ATP concentrations up to a dose of 5 mM, a concentration of 10 mM DAHP being neurotoxic (Fig. 1b, insert).

Because BH₄ is an essential co-factor for NOS-catalysed reaction, we measured the concentrations of cyclic GMP (cGMP) in DAHP treated-neurones (Fig. 1b), as an index of NO synthesis (Garthwaite et al. 1988). After 18 h of pre-incubation with increasing concentrations of DAHP, neurones were incubated at 37°C for 1 h in a humidified atmosphere of 95% air/5% CO₂ (normoxia) in buffered Hanks’ solution containing the phosphodiesterase inhibitor 1-isobutyl-3-methylxanthine (1 mM) and cGMP levels were measured as described in Materials and methods. As shown in Fig. 1(b), DAHP treatment to cells dose-dependently decreased cGMP concentrations, the minimum value being reached at a concentration of 5 mM DAHP. It is important to note that there was a significant \( p < 0.001 \) correlation between cGMP and BH₄ concentrations in neurones (Fig. 1c), strongly suggesting that BH₄ availability limits NO synthesis in neurones. Indeed, a supplement of 5 \( \mu \)M BH₄ during the incubation of DAHP-treated neurones under normoxic conditions (normoxia + DAHP + BH₄ group) increased both intracellular BH₄ levels and cGMP concentrations in comparison to DAHP-treated neurones (normoxia + DAHP group), the values being very similar to those found in untreated neurones (normoxia group; Fig. 1d). All these results led us to choose a dose of 5 mM DAHP for ensuing experiments.

In agreement with previous results (Marletta 1993; Knowles and Moncada 1994) experimental hypoxia (95% air/5% CO₂) significantly (50%, \( p < 0.05 \)) decreased NO synthesis, as showed by cGMP concentrations, which was totally blocked when BH₄ synthesis was inhibited by DAHP treatment to neurones, as occurred under normoxic conditions (Fig. 1d).

BH₄ deficiency induces neuronal death after 1 h of hypoxia

Cells were pre-incubated either in the absence or in the presence of 5 mM DAHP for 18 h and were then exposed to 1 h of normoxia or hypoxia. As shown in Fig. 2, DAHP treatment per se (normoxia + DAHP) or hypoxia did not affect neuronal viability (either necrosis or apoptosis), suggesting that neurones were resistant to these experimental conditions. However, the combination of both experimental conditions, i.e. DAHP treatment and hypoxia (hypoxia + DAHP group) significantly increased the number of necrotic cells as compared with normoxia, without changing the number of apoptotic cells under this condition. In order to

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**Fig. 2** Effect of 5 mM DAHP treatment on cell death in neurones in primary culture exposed to hypoxia. Neurones were pre-incubated in culture medium containing or lacking 5 mM DAPH at 37°C for 18 h. Cells were then washed and incubated under normoxia (normoxia group or normoxia + DAHP group) or hypoxia, either in the absence (hypoxia group or hypoxia + DAHP group) or in the presence of 5 \( \mu \)M BH₄ (hypoxia + DAHP + BH₄ group). After 1 h, neurones were washed and stained with either trypan blue or propidium iodide, as described in Materials and methods. Necrotic (displaying trypan blue-stained cytoplasm) and apoptotic (displaying either condensed or fragmented DAPI-stained nuclei) cells were counted. Results are expressed as percentages of necrotic or apoptotic cells vs. total cells, and are means ± SEM from quadruplicate averages obtained from four different cell cultures. \( *p < 0.05 \) as compared with the normoxia group. \# \( p < 0.05 \) as compared with the hypoxia + DAHP group.
study whether hypoxia + DAHP-mediated necrosis was due to BH4 deficiency, neurones were exposed to hypoxia in the presence of 5 μM BH4, which returned intracellular BH4 levels in neurones to normal levels (Fig. 1d). The BH4 supplement to DAHP-treated neurones totally prevented hypoxia + DAHP-induced necrosis (Fig. 2), suggesting that BH4 deficiency increases neuronal vulnerability at 1 h of hypoxia.

**Hypoxia causes ATP depletion and mitochondrial dysfunction in BH4-deficient neurones**

Because energy deficiency has been associated with necrosis, but no apoptosis (Sastry and Rao 2000) untreated and DAHP-treated neurones were exposed to normoxia or hypoxia for 1 h and ATP concentrations were measured. As shown in Fig. 3, hypoxia did not change ATP levels in neurones. In contrast, hypoxia significantly (25%, p < 0.05) decreased ATP concentrations in DAHP-treated cells (Fig. 3). In agreement with the results shown in Fig. 2, the BH4 supplement during hypoxia prevented the ATP depletion observed in DAHP-treated cells. (Fig. 3).

We have previously shown that neuronal ATP depletion is frequently associated with an impairment of mitochondrial function (Almeida and Medina 1998; Delgado-Esteban et al. 2000; Almeida and Bolaños 2001). In order to investigate whether hypoxia-induced ATP depletion in DAHP-treated neurones was due to mitochondrial dysfunction, we measured the mitochondrial membrane potential (ΔΨm), i.e. the central parameter that controls mitochondrial respiration and ATP synthesis (for review see Nicholls and Ward 2000). ΔΨm was estimated in neurones by measuring the fluorescence intensity of Rh 123, a positively charged fluorescent dye that accumulates at the negatively charged mitochondrial matrix side as a function of ΔΨm (Johnson et al. 1980; Duchen 1990). Thus, neurones were loaded with Rh 123 as previously described (Almeida et al. 1999) and then subjected to normoxia or hypoxia. Where indicated, cells were supplemented with 5 μM BH4 during hypoxia. Microphotographs were taken 1 h after the treatment, as previously reported (Almeida et al. 1999). As shown in Fig. 3, neurones incubated under normoxic conditions incorporated and retained the dye, exhibiting a bright fluorescence; similarly, hypoxia did not affect Rh 123 retention by the cells. However, hypoxia resulted in a marked loss of Rh 123 retention by DAHP-treated neurones (hypoxia + DAHP group; Fig. 3), suggesting ΔΨm collapse (mitochondrial depolarization). Rh 123 fluorescence was quantified in the scanned microphotographs by an image analyser system (Almeida et al. 1999) and revealed a 50% decrease in Rh 123 fluorescence in DAHP-treated neurones exposed to hypoxia.
Table 1: Effect of DAHP, an inhibitor of BH₄ biosynthesis, on the activity of mitochondrial respiratory chain complexes in neurones exposed to hypoxia.

<table>
<thead>
<tr>
<th></th>
<th>NADH-CoQ₁ reductase (nmol/min/mg protein)</th>
<th>Succinate-cytochrome c reductase (nmol/min/mg protein)</th>
<th>Cytochrome c oxidase (k/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxia</td>
<td>18.81 ± 1.15</td>
<td>6.81 ± 0.24</td>
<td>1.46 ± 0.08</td>
</tr>
<tr>
<td>Normoxia + DAHP</td>
<td>18.38 ± 1.76</td>
<td>6.93 ± 0.59</td>
<td>1.39 ± 0.09</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>17.87 ± 1.90</td>
<td>6.15 ± 0.97</td>
<td>1.33 ± 0.07</td>
</tr>
<tr>
<td>Hypoxia + DAHP</td>
<td>17.43 ± 2.50</td>
<td>6.43 ± 0.71</td>
<td>0.98 ± 0.08⁶</td>
</tr>
<tr>
<td>Hypoxia + DAHP + BH₄</td>
<td>17.52 ± 2.50</td>
<td>6.32 ± 0.70</td>
<td>1.31 ± 0.09⁶</td>
</tr>
</tbody>
</table>

Neurones were pre-incubated either in the absence or in the presence of 5 mM DAHP at 37°C for 18 h. Cells were then exposed to normoxia (normoxia group and normoxia + DAHP group) or hypoxia (hypoxia group and hypoxia + DAHP group) for 1 h. When appropriate, DAHP-treated neurones were exposed to hypoxia in the presence of 5 μM BH₄ (hypoxia + DAHP + BH₄ group). The activities of mitochondrial respiratory chain complexes were determined in the cells as described in Materials and Methods. Values are means ± SEM from three different culture preparations. ⁶Significantly different when compared with the hypoxia group. ⁵Significantly different when compared with the hypoxia + DAHP group.

Table 2: Effect of DAHP, an inhibitor of BH₄ biosynthesis, on the antioxidant system of glutathione in neurones exposed to hypoxia.

<table>
<thead>
<tr>
<th></th>
<th>Total glutathione (nmol/mg protein)</th>
<th>GSH (nmol/mg protein)</th>
<th>GSSG (nmol/mg protein)</th>
<th>Oxidized state (GSSG/GS X100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxia</td>
<td>13.9 ± 0.6</td>
<td>13.6 ± 0.5</td>
<td>0.27 ± 0.02</td>
<td>1.9</td>
</tr>
<tr>
<td>Normoxia + DAHP</td>
<td>14.0 ± 0.8</td>
<td>13.4 ± 0.7</td>
<td>0.30 ± 0.03</td>
<td>2.1</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>14.1 ± 0.7</td>
<td>13.7 ± 0.7</td>
<td>0.30 ± 0.03</td>
<td>2.1</td>
</tr>
<tr>
<td>Hypoxia + DAHP</td>
<td>14.0 ± 0.5</td>
<td>13.1 ± 0.5</td>
<td>0.41 ± 0.03⁶</td>
<td>3.1</td>
</tr>
<tr>
<td>Hypoxia + DAHP + BH₄</td>
<td>14.3 ± 1.0</td>
<td>13.5 ± 0.9</td>
<td>0.32 ± 0.03⁶</td>
<td>2.2</td>
</tr>
<tr>
<td>Hypoxia + DAHP + NAME</td>
<td>14.1 ± 0.9</td>
<td>13.5 ± 0.5</td>
<td>0.28 ± 0.02⁶</td>
<td>2.0</td>
</tr>
<tr>
<td>Hypoxia + DAHP + SOD + CAT</td>
<td>14.2 ± 0.7</td>
<td>13.6 ± 0.5</td>
<td>0.31 ± 0.03⁶</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Neurones were pre-incubated either in the absence or in the presence of 5 mM DAHP at 37°C for 18 h. Cells were then exposed to normoxia (normoxia group and normoxia + DAHP group) or hypoxia (hypoxia group and hypoxia + DAHP group) for 1 h. When appropriate, DAHP-treated neurones were exposed to hypoxia in the presence of 5 μM BH₄ (hypoxia + DAHP + BH₄ group), 1 mM NAME (hypoxia + DAHP + NAME group), or 100 U/mL SOD plus 100 U/mL CAT (hypoxia + DAHP + SOD + CAT group). Glutathione concentrations were determined as described in Materials and Methods. Values are expressed as means ± SEM from three to four different culture preparations. ⁶Significantly different when compared with the normoxia group. ⁵Significantly different when compared with the hypoxia + DAHP group.
enhancement of 39% in the cellular oxidized state. These effects were prevented by the BH₄ supplement during the hypoxic insult (Table 2), suggesting that NOS co-factor deficiency triggers oxidative stress in neurones under hypoxic conditions.

The presence of either the nitric oxide synthase inhibitor NAME, or the superoxide scavenger SOD + CAT (to remove H₂O₂) during the hypoxic period also prevented the increase in GSSG concentrations and hence in the cellular oxidized state found in the hypoxia + DAHP group (Table 2).

**NOS dysfunction mediates hypoxia-induced mitochondrial damage in BH₄-deficient neurones**

Our observation that the increase in GSSG concentrations in DAHP-treated neurones due to hypoxia could be a consequence of free radical generation (Table 2) prompted us to investigate whether superoxide anion (O₂⁻), hydroxyl radical (·OH) or ·NO might be involved in hypoxia + DAHP-induced mitochondrial dysfunction (Fig. 2 and Table 1). Thus, neurones were pre-incubated in culture medium containing or lacking 5 mM DAHP for 18 h and then exposed to 1 h of hypoxia either in the absence or in the presence of NAME (1 mM), haemoglobin (2 µM), SOD + CAT (100 U/mL + 100 U/mL), and mannitol (50 mM), as previously reported (Bolanos and Medina 1996; Almeida et al. 1998). As shown in Fig. 4, NAME, haemoglobin and SOD + CAT, but not mannitol, prevented the effect of hypoxia on necrosis, ATP depletion and mitochondrial dysfunction, as shown by the Rh 123 fluorescence and cytochrome c oxidase activity values, in DAHP-treated neurones. Moreover, treatment of DAHP-treated neurones with other antioxidants agents, such as Tempol (1 µM), ascorbic acid (1%) or methionine (5 µM) also prevented hypoxia-induced neuronal necrosis (26.0 ± 1.5% in hypoxia + DAHP; 8.9 ± 0.8% in hypoxia + DAHP + Tempol; 7.5 ± 0.8% in hypoxia + DAHP + ascorbic acid; 10.0 ± 1.0% in hypoxia + DAHP + methionine). All these results suggest that ·NO and O₂⁻ might be involved in the mitochondrial damage associated with BH₄ deficiency under hypoxic conditions.

In order to study whether ONOO⁻ had been generated under our experimental conditions, neurones were pre-incubated either in the absence or in the presence of 5 mM DAHP for 18 h and then were exposed to 1 h of normoxia or hypoxia in the presence of 5 mM BH₄, 1 mM NAME or 100 U/mL + 100 U/mL SOD + CAT, and ONOO⁻ generation by the cells was quantified by determining tyrosine nitration by western blotting (MacMillan-Crow et al. 1996). As shown in Fig. 5, although an increase in protein nitration was found in DAHP-treated neurones (normoxia + DAHP), the effect was higher when cells were exposed to 1 h of hypoxia (hypoxia + DAHP), as shown by the enhancement in nitrotyrosine immunoreactivity in comparison with untreated neurones exposed to hypoxia (hypoxia group), which was very similar to that observed under normoxia.

When DAHP-treated neurones were exposed to hypoxia in the presence of 5 µM BH₄, 1 mM NAME or 100 U/mL + 100 U/mL SOD + CAT, increased protein nitration above hypoxia levels was not observed (Fig. 5).

In order to estimate the percentage of protein nitration from each experimental condition, the intensity of bands was
quantified with an image analyser system (NIH Image) and the results were expressed as percentages of protein nitration, considering the intensity of each band from a sample of neurones treated with pure peroxynitrite (1 mM) for 5 min as 100% of nitration. Thus, BH₄, NAME or SOD + CAT prevented hypoxia-mediated protein nitration in DAHP-treated cells (Fig. 5). All these results suggest that peroxynitrite is generated in BH₄-deficient neurones during hypoxia.

Discussion

Pre-incubation of neurones in primary culture with increasing concentrations of DAHP, a well-known inhibitor of GTP cyclohydrolase I (Gal et al. 1978; Bogdan et al. 1995; Saura et al. 1996; Ishii et al. 1997; Koshimura et al. 1998) dose-dependently decreased intracellular levels of BH₄ as well as NO biosynthesis, as shown by cGMP concentrations (Garthwaite et al. 1988), the minimum values being reached at a concentration of 5 mM DAHP (Fig. 1b). In fact, we found an inverse correlation \( p < 0.001 \) between BH₄ levels and NO production in neurones (Fig. 1c), suggesting that a deficiency in the co-factor would trigger NOS dysfunction. Indeed, when BH₄ concentrations were restored by incubating neurones with a supplement of BH₄ for 1 h, nitric oxide biosynthesis sharply increased to normoxia (control) values (Fig. 1d), confirming the notion that BH₄ availability modulates NOS activity (Marletta 1993; Knowles and Moncada 1994; Alderton et al. 2001). In addition, these results show that the experimental model used in this work is suitable for studying the possible effects of BH₄ availability, and hence of NOS activity, on neuronal metabolism under different pathophysiological conditions, such as hypoxia.

Whereas a short period (1 h) of hypoxia did not affect neuronal viability, the same experimental protocol led to necrosis, but not to apoptosis, in DAHP-treated neurones. Furthermore, hypoxia triggered ATP depletion in BH₄-deficient neurones, which has frequently been associated with necrosis (Almeida and Bolaños 2001; Bonfoco et al. 1995; Sastry and Rao 2000). In addition, a supplement of this co-factor to BH₄-deficient cells during hypoxia prevented both the necrosis and ATP depletion found under hypoxic conditions. These results suggest that BH₄ deficiency increases neuronal vulnerability to hypoxia.

Hypoxia-induced ATP depletion and necrosis in BH₄-deficient neurones appears to be associated with the mitochondrial dysfunction observed under these circumstances (Fig. 3 and Table 1). In fact, hypoxia caused a significant inhibition of complex IV activity in BH₄-deficient neurones, without affecting complex I or II–III activities (Table 1). In addition, hypoxia triggered \( \Delta \Psi_m \) collapse in BH₄-deficient neurones (Fig. 3), suggesting that the observed inhibition of complex IV activity may be associated with mitochondrial dysfunction, leading to neuronal energy depletion and necrosis. This is supported by the fact that the supplement of BH₄ during hypoxia prevented both complex IV activity inhibition and mitochondrial depolarization (Fig. 3 and Table 1), as occurred with ATP depletion and necrosis (Figs 2 and 3). It is important to note that the observed resistance of neurones to hypoxia-induced cellular damage (Figs 2 and 3) is also corroborated by the mitochondrial parameters shown in Fig. 3 and Table 1.

Mitochondria are well-known subcellular targets for free radical toxicity (reviewed in Bolaños et al. 1997; Raha and
We were therefore prompted to investigate whether oxidative stress was involved in hypoxia-induced mitochondrial damage in BH4-deficient neurones (Table 1 and Fig. 3). Our results revealed that hypoxia per se did not affect GSH or GSSG concentrations in neurones (hypoxia group), suggesting that, under our experimental conditions, oxygen deprivation did not lead to oxidative stress. Accordingly, we have recently shown that 1 h of experimental hypoxia did not increase oxygen radical production by neurones (Almeida et al. 2002). In contrast, hypoxia significantly enhanced the oxidation of GSH to GSSG in DAHP-treated neurones. Moreover, hypoxia-mediated GSH oxidation in BH4-deficient neurones was prevented by BH4 supplement to cells during the hypoxic period (Table 2). These results strongly suggest that a deficiency of the NOS co-factor triggers oxidative stress during hypoxia, which might be responsible for the mitochondrial damage observed under these circumstances (Fig. 3 and Table 1). Furthermore, the increase in GSSG concentrations found in BH4-deficient neurones after hypoxia was prevented by NAME or by SOD + CAT, indicating that nitric oxide synthase and O2•− generation are involved in the process. Indeed, NAME, haemoglobin and SOD + CAT, but not mannitol, abolished hypoxia-induced mitochondrial damage, ATP depletion and necrosis in BH4-deficient neurones (Fig. 4). In addition, other antioxidant agents, such as Tempol, ascorbic acid or methionine also prevented hypoxia-mediated necrosis in DAHP-treated neurones. All these results strongly suggest that ‘NO and/or O2•−, but not ‘OH, generated during hypoxia in neurones deficient in the co-factor of NOS are involved in mitochondrial damage, leading to ATP depletion and cell death.

It has previously been demonstrated that BH4 deficiency results in neuronal NOS mediated-generation of O2•− in vitro (Pou et al. 1992; Abu-Soud et al. 1997; Vásquez-Vivar et al. 1999), which is known to react rapidly with ‘NO to form peroxynitrite (ONOO•). Beckman et al. 1990). The generation of these species, O2•−, and more convincingly ONOO•, are thought to be responsible for NO-mediated mitochondrial damage, particularly at the level of complex IV (Brown and Cooper 1994; Bolaños et al. 1995, 1997). Because we also observed specific damage in mitochondrial complex IV activity in BH4-deficient neurones exposed to hypoxia, we investigated whether ONOO• was being generated under our experimental conditions by determining tyrosine nitration, using western blotting (MacMillan-Crow et al. 1996). Exposure of BH4-deficient neurones to hypoxia resulted in an increase in ONOO• generation, as shown by the enhancement in nitrotyrosine immunoreactivity as compared with non-deficient neurones (hypoxia group; Fig. 5). Moreover, this effect was counteracted by the BH4 supplement, indicating that low concentrations of NOS co-factor triggers ONOO• generation by neurones exposed to hypoxia (Fig. 5). In agreement with this, NAME or SOD + CAT also prevented BH4 deficiency-mediated nitrotyrosine formation during hypoxia (Fig. 5), strongly suggesting that at low concentrations of BH4 neuronal NOS is converted into an efficient peroxynitrite synthase. It is important to note that this work is the first evidence that BH4 deficiency causes NOS dysfunction leading to ONOO• generation in cultured neurones. Although Pfieffer et al. (2001) have recently argued against an essential role of ONOO• in protein tyrosine nitration in vivo, the specific inhibition in complex IV activity found in DAHP-treated neurones exposed to hypoxia (Table 1) appears to support that ONOO• has been synthesized under our experimental conditions.

In conclusion, our results suggest that BH4 availability is essential for NOS to synthesise ‘NO. In fact, a deficiency in this co-factor triggers ONOO• generation in the NOS-catalysed reaction, which would damage mitochondrial function, increasing neuronal vulnerability to hypoxia. These results might have important implications in the prevention of neurological disorders associated with DOPA-responsive dystonia, inborn errors of BH4 metabolism (Furukawa et al. 1998), and possibly certain neurodegenerative diseases, such as Alzheimer’s disease (Barford et al. 1984) and Parkinson’s disease (Curtius et al. 1984). In this context, administration of this co-factor has been reported to improve some of the clinical symptoms of these patients (Niederwieser et al. 1982; Kumura et al. 1994; Fernell et al. 1997).

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