Tetrahydrobiopterin deficiency increases neuronal vulnerability to hypoxia

María Delgado-Esteban,* Angeles Almeida*,† and Jose M. Medina*

*Departamento de Bioquímica y Biología Molecular, Universidad de Salamanca, Salamanca, Spain

†Unidad de Investigación, Hospital Universitario de Salamanca, Salamanca, Spain

Abstract

Tetrahydrobiopterin (BH_4) is an essential co-factor for nitric oxide synthases (NOS). The aim of the present work was to study whether BH_4 deficiency affects the vulnerability of neurones in primary culture to hypoxia. Intracellular BH_4 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, BH_4 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 BH_4-deficient neurones. All these effects were counteracted when neuronal BH_4 levels were restored by incubating cells with exogenous BH_4 during the hypoxic period. Moreover, hypoxia-induced damage in BH_4-deficient neurones was prevented when N^{ω}-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, BH_4 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 BH_4, NAME, haemoglobin or superoxide dismutase plus catalase during hypoxia. Our results suggest that BH_4 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.

J. Neurochem. (2002) **82**, 1148–1159.

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 (6*R*)-5,6,7,8-tetrahydrobiopterin (BH_4) is an essential co-factor in NOS-catalysed NO biosynthesis (Knowles and Moncada 1994; Marletta 1993). BH_4 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 BH_4 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, BH_4 is thought to be required to maintain NOS quarternary 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 ($^{\bullet}BH_3$) is formed during the NOS-catalysed reaction, BH_4 being involved in electron transfer in the first cycle of the NOS reaction. Particularly, BH_4 transfers an electron to the ferrous-dioxy intermediate to enable the formation of a heme-based oxidant that rapidly hydroxylates arginine, and the resulting $^{\bullet}BH_3$ would subsequently be reduced by the NOS flavins (Hurshman *et al.* 1999; Schmidt *et al.* 2001; Wei *et al.* 2001).

Received December 13, 2001; revised manuscript received April 15, 2002; accepted May 20, 2002.

Address correspondence and reprint requests to Angeles Almeida, Departamento de Bioquímica y Biología Molecular, Universidad de Salamanca, Edificio Departamental, Plaza Doctores de la Reina, s/n, 37007 Salamanca, Spain. E-mail: aaparra@gugu.usal.es

Abbreviations used: BH_4, tetrahydrobiopterin; CAT, catalase; CoQ_1, coenzyme Q_1; DAHP, 2,4-diamino-6-hydroxypyrimidine; DAPI, 4′,6-diamino-2-phenulindole; DMEM, Dulbecco's modified Eagle's Medium; FCS, fetal calf serum; GSH/GSSH, reduced/oxidized glutathione; GSx, total glutathione; NAME, N^{ω}-nitro-L-arginine monomethyl ester; NOS, nitric oxide synthase; PBS, phosphate-buffered saline; SOD, superoxide dismutase.

 © 2002 International Society for Neurochemistry, *Journal of Neurochemistry*, **82**, 1148–1159

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 L-arginine and BH_4 (Heinzel *et al.* 1992; Mayer *et al.* 1992; Pou *et al.* 1992; Vásquez-Vivar *et al.* 1999). BH_4 deficiency results in neuronal NOS mediated-generation of superoxide ($O_2^{\bullet-}$) *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, $O_2^{\bullet-}$ and $ONOO^-$, is thought to be responsible for the neurotoxicity of NO (Beckman *et al.* 1990).

In mammalian cells, *de novo* biosynthesis of 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 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 BH_4 deficiency on neuronal vulnerability to hypoxia. Neuronal BH_4 concentrations were depleted by treatment with 2,4-diamino-6-hydroxypyrimidine (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 *de novo*-synthesis of BH_4 (Gal *et al.* 1978; Nichol *et al.* 1985). Our results show that 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^{ω}-nitro-L-arginine monomethyl ester (NAME), tetrahydrobiopterin (BH_4), 2,4-diamino-6-hydroxypyrimidine (DAHP), 4,6-diamino-2-phenylindole (DAPI), coenzyme Q_1, CoQ_1, 2-vinylpyridine, 1-isobutyl-3-methylxanthine, superoxide dismutase (SOD), catalase (CAT), mannitol and haemoglobin were obtained from Sigma Chemical Co. (St Louis, MO, USA). DMEM was always supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL) and amphotericin B (0.25 μg/mL; Sigma). Fetal calf serum and cytochrome *c* were purchased from Boëhringer 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, Boëhringer or Merck (Darmstadt, Germany). Plastic tissue culture dishes were purchased from Nunc (Denmark) or Linbro (McLean, VA, USA). Peroxynitrite ($ONOO^-$) 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°C for at least 3–4 months.

Animals

Albino Wistar rats fed *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 2.5×10^5 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% CO_2/95% 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 μM in order to prevent non-neuronal proliferation and neurones were used on day 9.

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 *de novo* synthesis of BH_4 (Gal *et al.* 1978; Nichol *et al.* 1985).

Model of hypoxia *in vitro*

The culture medium was removed and cells were washed once with pre-warmed (37°C) buffered Hanks' solution (5.26 mM KCl, 0.43 mM KH_2PO_4, 132.4 mM NaCl, 4.09 mM $NaHCO_3$, 0.33 mM Na_2HPO_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% N_2/5% CO_2 in buffered Hanks' solution, previously gassed with 95% N_2/5% 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% CO_2 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 BH_4 (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 Na_2HPO_4, 1.7 mM KH_2PO_4, 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-cm^2 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-CoQ_1 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 BH_4 measurements, neurones plated in 2-cm^2 wells were rapidly washed with ice-cold PBS, scraped off with 0.1 M perchloric acid containing 6.5 mM dithioerithritol and 2.5 mM diethyltriaminepenta-acetic 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 persormance liuid chromatography (HPLC) with electrochemical detection (Howells *et al*. 1986; Barker *et al*. 1998).

For ATP determinations, neurones plated in 2-cm^2 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 $HClO_4$, and neutralized with 0.5 mL of 2 M $KHCO_2$ 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 radioimmunoassay kit (Amersham Pharmacia Biotech) and following the manufacturer's instructions.

For glutathione determinations, neurones plated in 9.6-cm^2-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 antipapain, 50 μg/mL pepstatin, 50 μg/mL amastatina, 50 μg/mL leupeptine, 50 μg/mL bestatin and 50 μg/mL soybean tripsin inhibitor. Cell suspensions were centrifuged at 14 000 *g* for 10 min, and pelleted cells were frozen at − 80°C. Protein concentrations were determined and samples were normalized for total protein. Proteins were transferred by electroelution 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 peroxynitrite (1 mM) 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 BH_4 depletion and NOS dysfunction

BH_4 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 BH_4 measurements, as previously described (Howells *et al.* 1986; Barker *et al.* 1998). Treatment with DAHP dose-dependently decreased intracellular BH_4 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

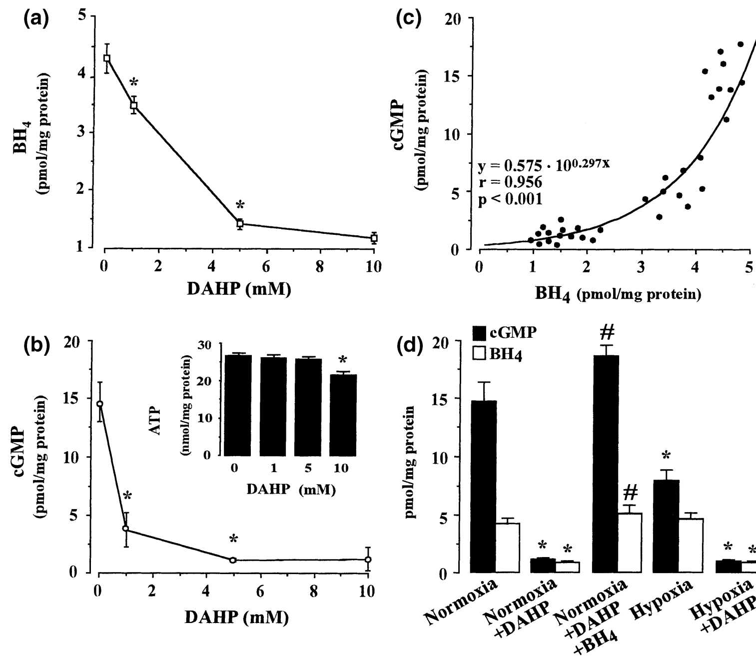

Fig. 1 Effect of DAHP treatment on BH_4 and cGMP concentrations in neurones in primary culture. Neurones were pre-incubated in culture medium containing increasing concentrations of DAHP (0, 1, 5 or 10 mM) at 37°C for 18 h. Cells were then washed and incubated under normoxia for 1 h and BH_4 concentrations were measured by HPLC with electrochemical detection (a). For cyclic GMP determinations, neurones were exposed to 1 h of normoxia or hypoxia in the presence of the phosphodiesterase inhibitor 1-isobutyl-3-methylxanthine (1 mM) (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 BH_4 (normoxia + DAHP + BH_4 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 BH_4 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% CO_2 (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 BH_4 concentrations in neurones (Fig. 1c), strongly suggesting that BH_4 availability limits ˙NO synthesis in neurones. Indeed, a supplement of 5 μM BH_4 during the incubation of DAHP-treated neurones under normoxic conditions (normoxia + DAHP + BH_4 group) increased both intracellular BH_4 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% CO_2) significantly (50%, $p < 0.05$) decreased ˙NO synthesis, as showed by cGMP concentrations, which was totally blocked when BH_4 synthesis was inhibited by DAHP treatment to neurones, as occurred under normoxic conditions (Fig. 1d).

BH_4 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

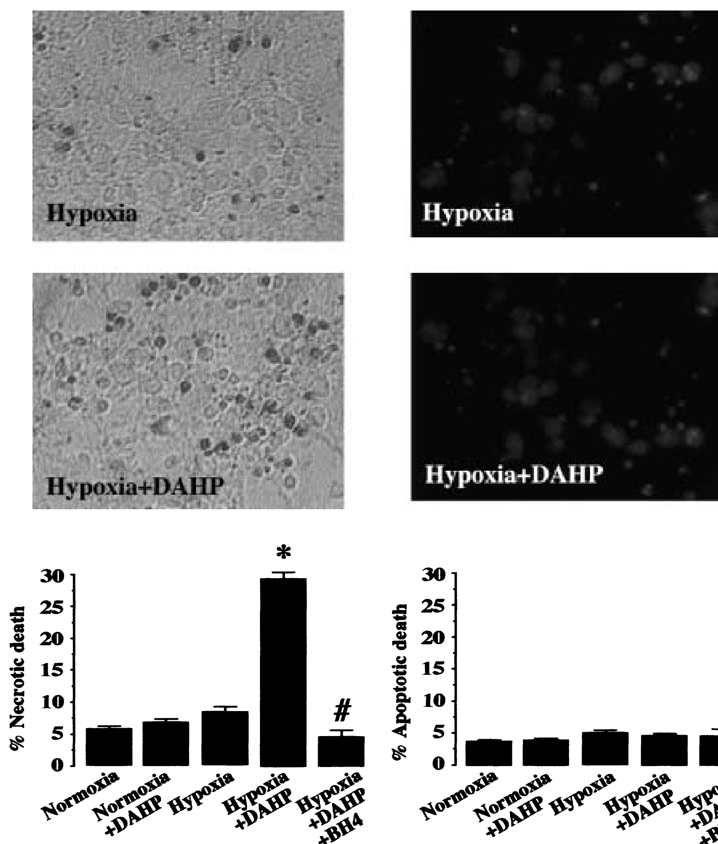

Fig. 2 Effect of 5 mM DAHP treatment on cell death in neurones in primary culture exposed to hypoxia. Neurones were pre-incubated in culture medium containing or lacking 5 mM DAPH at 37°C for 18 h. Cells were then washed and incubated under normoxia (normoxia group or normoxia + DAHP group) or hypoxia, either in the absence (hypoxia group or hypoxia + DAHP group) or in the presence of 5 μM BH_4 (hypoxia + DAHP + BH_4 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 BH_4 deficiency, neurones were exposed to hypoxia in the presence of 5 μM BH_4, which returned intracellular BH_4 levels in neurones to normal levels (Fig. 1d). The BH_4 supplement to DAHP-treated neurones totally prevented hypoxia + DAHP-induced necrosis (Fig. 2), suggesting that BH_4 deficiency increases neuronal vulnerability at 1 h of hypoxia.

Hypoxia causes ATP depletion and mitochondrial dysfunction in BH_4-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 BH_4 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 ($\triangle\Psi_m$), i.e. the central parameter that controls mitochondrial respiration and ATP synthesis (for review see Nicholls and Ward 2000). $\triangle\Psi_m$ 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 $\triangle\Psi_m$ (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 BH_4 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 $\triangle\Psi_m$ 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

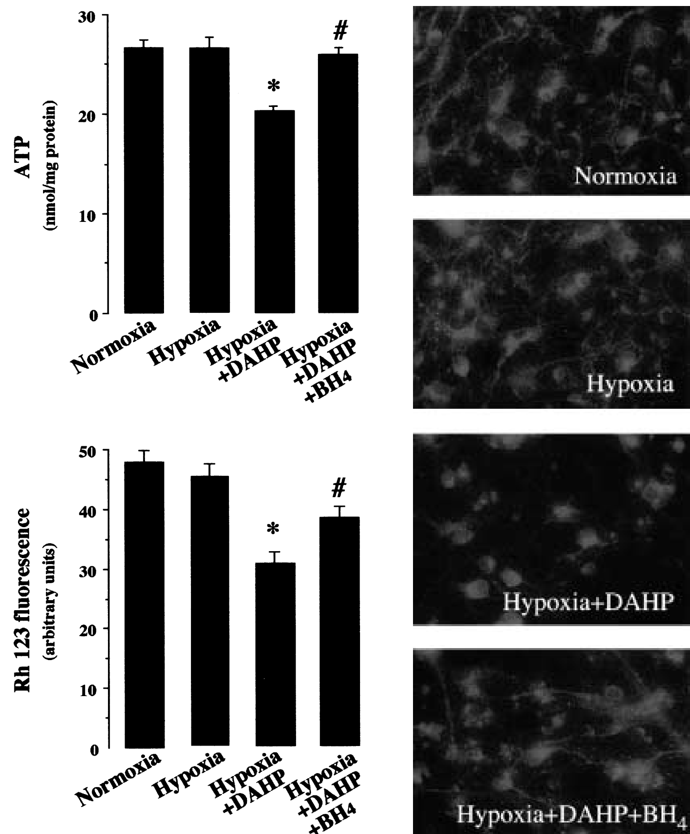

Fig. 3 Effect of 5 mM DAHP treatment on ATP concentrations and mitochondrial membrane potential in neurones in primary culture exposed to hypoxia. Neurones were pre-incubated in culture medium containing or lacking 5 mM DAPH at 37°C for 18 h. Cells were then washed and incubated under normoxia (normoxia group) or hypoxia, either in the absence (hypoxia group or hypoxia + DAHP group) or in the presence of 5 μM BH_4 (hypoxia + DAHP + BH_4 group). After 1 h, neurones were -washed and ATP concentrations were determined. For mitochondrial membrane potential measurement, cells were stained with 1 μg/mL Rh 123 during normoxia or hypoxia and Rh 123 fluorescence was observed with an inverted microscope using a fluorescein filter (excitation wavelength 488 nm). Microphotographs were taken and the intensity of Rh 123 fluorescence was quantified with an image analyser system (NIH image). Results are means ± SEM from four to eight different cell cultures. *$p < 0.05$ as compared with the normoxia group. #$p < 0.05$ as compared with the hypoxia + DAHP group.

Table 1 Effect of DAHP, an inhibitor of BH_4 biosynthesis, on the activity of mitochondrial respiratory chain complexes in neurones exposed to hypoxia

	NADH-CoQ_1 reductase (nmol/min/mg protein)	Succinate-cytochrome *c* reductase (nmol/min/mg protein)	Cytochrome *c* oxidase (k/min/mg protein)
Normoxia	18.81 ± 1.15	6.81 ± 0.24	1.46 ± 0.08
Normoxia + DAHP	18.38 ± 1.76	6.93 ± 0.59	1.39 ± 0.09
Hypoxia	17.87 ± 1.90	6.15 ± 0.97	1.33 ± 0.07
Hypoxia + DAHP	17.43 ± 2.50	6.43 ± 0.71	0.98 ± 0.08[a]
Hypoxia + DAHP + BH_4	17.52 ± 2.50	6.32 ± 0.70	1.31 ± 0.09[b]

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 BH_4 (hypoxia + DAHP + BH_4 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. [a]Significantly different when compared with the hypoxia group. [b]Significantly different when compared with the hypoxia + DAHP group.

(hypoxia + DAHP group), as compared with normoxic conditions (Fig. 3). As found with ATP concentrations, the supplement with BH_4 during the hypoxic period prevented hypoxia + DAHP-induced $\triangle\Psi_m$ collapse (Fig. 3), suggesting that hypoxia-mediated ATP depletion in BH_4-deficient neurones (hypoxia + DAHP group) might be due to mitochondrial dysfunction.

The activities of the mitochondrial respiratory-chain complexes are shown in Table 1. According to results shown in Fig. 2, DAHP treatment (normoxia + DAHP) or hypoxia did not affect mitochondrial respiratory-chain complexes activities, the observed values being very similar to those found under normoxia (Table 1). By contrast, hypoxia specifically inhibited complex IV activity by 33% in DAHP-treated neurones, which was counteracted by the BH_4 supplement during the hypoxic period. The activities of complex I and complex II-III remained unchanged under all experimental conditions studied (Table 1).

BH_4 deficiency causes oxidative stress in neurones exposed to hypoxia

Because mitochondria appear to be a subcellular target for the free radicals generated during hypoxic insult (recently reviewed in Bolaños and Almeida 1999; Chan 2001; Mattson *et al*. 2001), we studied the possible effect of hypoxia on the intracellular concentrations of reduced (GSH) and oxidized (GSSG) glutathione in neurones pre-incubated either in the absence or in the presence of 5 mM DAHP. As shown in Table 2, whereas DAHP treatment (normoxia + DAHP) or hypoxia did not affect the concentrations of GSH and GSSG in neurones, GSSG concentrations significantly increased (35%, $p < 0.05$) in DAHP-treated neurones after 1 h of hypoxia, resulting in an

Table 2 Effect of DAHP, an inhibitor of BH_4 biosynthesis, on the antioxidant system of glutathione in neurones exposed to hypoxia

	Total glutathione (nmol/mg protein)	GSH (nmol/mg protein)	GSSG (nmol/mg protein)	Oxidized state (GSSG/GSx X100)
Normoxia	13.9 ± 0.6	13.6 ± 0.5	0.27 ± 0.02	1.9
Normoxia + DAHP	14.0 ± 0.8	13.4 ± 0.7	0.30 ± 0.03	2.1
Hypoxia	14.1 ± 0.7	13.7 ± 0.7	0.30 ± 0.03	2.1
Hypoxia + DAHP	14.0 ± 0.5	13.1 ± 0.5	0.41 ± 0.03[a]	3.1
Hypoxia + DAHP + BH_4	14.3 ± 1.0	13.5 ± 0.9	0.32 ± 0.03[b]	2.2
Hypoxia + DAHP + NAME	14.1 ± 0.9	13.5 ± 0.5	0.28 ± 0.02[b]	2.0
Hypoxia + DAHP + SOD + CAT	14.2 ± 0.7	13.6 ± 0.5	0.31 ± 0.03[b]	2.2

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 BH_4 (hypoxia + DAHP + BH_4 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. [a]Significantly different when compared with the normoxia group. [b]Significantly different when compared with the hypoxia + DAHP group.

enhancement of 39% in the cellular oxidized state. These effects were prevented by the BH_4 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 H_2O_2) 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 BH_4-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 ($O2^{\bullet -}$), hydroxyl radical ($^{\bullet}$OH) or $^{\bullet}$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 (Bolaños 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 $^{\bullet}$NO and $O_2^{\bullet -}$ might be involved in the mitochondrial damage associated with BH_4 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 either in the absence or in the presence of 5 mM BH_4, 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.

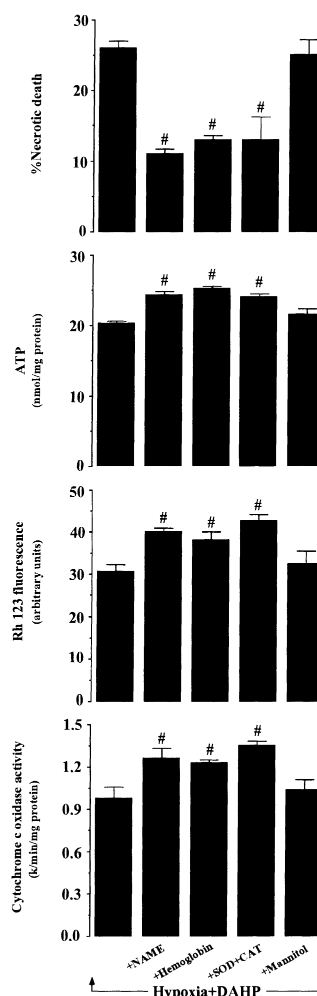

Fig. 4 Effect of NAME, haemoglobin, SOD + CAT or mannitol on necrosis, ATP concentrations, mitochondrial membrane potential and cytochrome c oxidase activity in 5 mM DAHP-treated neurones exposed to hypoxia. Neurones were pre-incubated in culture medium containing 5 mM DAPH at 37°C for 18 h. Cells were then washed and incubated under hypoxia, either in the absence or in the presence of NAME (1 mM), haemoglobin (2 μM), SOD (100 U/mL) plus CAT (100 U/mL), or mannitol (50 mM). After 1 h, cells were washed and necrotic death, ATP concentrations, Rh 123 fluorescence and cytochrome c oxidase activity were determined as described in Materials and Methods. Results are means ± SEM from three to eight different cell cultures. #$p < 0.05$ as compared with the hypoxia + DAHP group.

When DAHP-treated neurones were exposed to hypoxia in the presence of 5 μM BH_4, 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

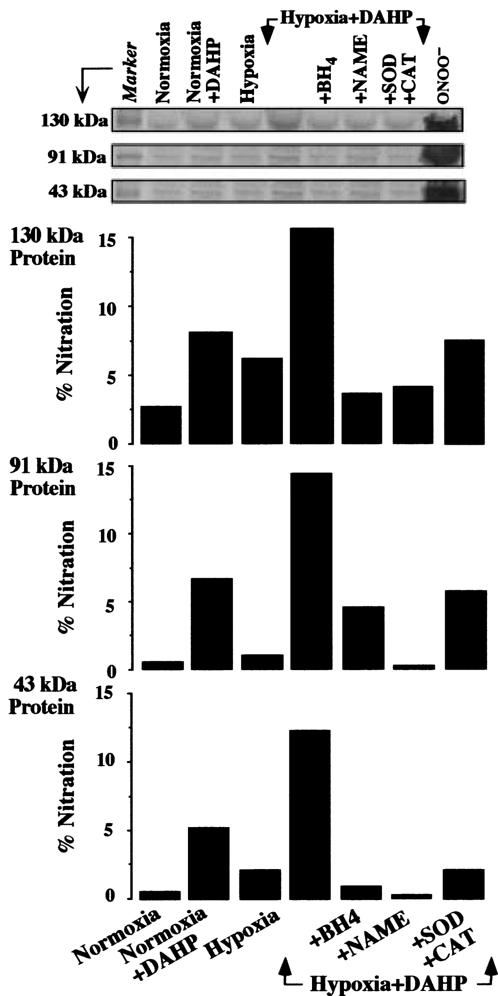

Fig. 5 Effect of 5 mM DAHP treatment on nitrotyrosine expression by Western blotting analysis in neurones exposed to hypoxia. Neurones were pre-incubated in culture medium containing or lacking 5 mM DAPH at 37°C for 18 h. Cells were then washed and incubated under normoxia or hypoxia, either in the absence or in the presence of 5 μM BH_4, 1 mM NAME or 100 U/mL SOD plus 100 U/mL CAT. After 1 h, proteins were extracted and subjected to Western blotting analysis, using an antibody against 3-nitrotyrosine. Band intensity was quantified with an image analyser system (NIH Image). Results are expressed as percentages of nitration, considering the intensity of each band from a sample of neurones treated with pure peroxynitrite (1 mM) for 5 min, as 100% nitration.

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, BH_4, NAME or SOD + CAT prevented hypoxia-mediated protein nitration in DAHP-treated cells (Fig. 5). All these results suggest that peroxynitrite is generated in BH_4-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 BH_4 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 BH_4 levels and ˙NO production in neurones (Fig. 1c), suggesting that a deficiency in the co-factor would trigger NOS dysfunction. Indeed, when BH_4 concentrations were restored by incubating neurones with a supplement of BH_4 for 1 h, nitric oxide biosynthesis sharply increased to normoxia (control) values (Fig. 1d), confirming the notion that BH_4 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 BH_4 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 BH_4-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 BH_4-deficient cells during hypoxia prevented both the necrosis and ATP depletion found under hypoxic conditions. These results suggest that BH_4 deficiency increases neuronal vulnerability to hypoxia.

Hypoxia-induced ATP depletion and necrosis in BH_4-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 BH_4-deficient neurones, without affecting complex I or II–III activities (Table 1). In addition, hypoxia triggered $\Delta\Psi_m$ collapse in BH_4-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 BH_4 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

Robinson 2000). We were therefore prompted to investigate whether oxidative stress was involved in hypoxia-induced mitochondrial damage in BH_4-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 BH_4-deficient neurones was prevented by BH_4 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 BH_4-deficient neurones after hypoxia was prevented by NAME or by SOD + CAT, indicating that nitric oxide synthase and $O_2^{\bullet -}$ generation are involved in the proccess. Indeed, NAME, haemoglobin and SOD + CAT, but not mannitol, abolished hypoxia-induced mitochondrial damage, ATP depletion and necrosis in BH_4-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 $^{\bullet}$NO and/or $O_2^{\bullet -}$, but not $^{\bullet}$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 BH_4 deficiency results in neuronal NOS mediated-generation of $O_2^{\bullet -}$ *in vitro* (Pou *et al*. 1992; Abu-Soud *et al*. 1997; Vásquez-Vivar *et al*. 1999), which is known to react rapidly with $^{\bullet}$NO to form peroxynitrite ($ONOO^-$; Beckman *et al*. 1990). The generation of these species, $O_2^{\bullet -}$, and more convincingly $ONOO^-$, are thought to be responsible for $^{\bullet}$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 BH_4-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 BH_4-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 BH_4 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 BH_4 deficiency-mediated nitrotyrosine formation during hypoxia (Fig. 5), strongly suggesting that at low concentrations of BH_4 neuronal NOS is converted into an efficient peroxynitrite synthase. It is important to note that this work is the first evidence that BH_4 deficiency causes NOS dysfunction leading to $ONOO^-$ generation in cultured neurones. Although Pfeiffer *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 $ONOO^-$ is been synthesized under our experimental conditions.

In conclusion, our results suggest that BH_4 availability is essential for NOS to synthesize $^{\bullet}$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-resposive dystonia, inborn errors of BH_4 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 contex, 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).

Acknowledgements

This work was funded by grants from the FIS (AA, JMM) and FEDER-DGCYT (JMM).

References

Abu-Soud H., Gachhui R., Raushel F. and Stuehr D. (1997) The ferrous-dioxy complex of neuronal nitric oxide synthase. Divergent effects of 1-arginine and tetrahydrobiopterin on its stability. *J. Biol. Chem.* **272**, 17349–17353.

Alderton W. K., Cooper C. E. and Knowles R. G. (2001) Nitric oxide synthases: structure, function and inhibition. *Biochem. J.* **357**, 593–615.

Almeida A. and Bolaños J. P. (2001) A transient inhibition of mitochondrial ATP synthesis by nitric oxide synthase activation triggered apoptosis in primary cortical neurons. *J. Neurochem.* **77**, 676–690.

Almeida A. and Medina J. M. (1998) A rapid method for the isolation of metabolically active mitochondria from rat neurons and astrocytes in primary culture. *Brain Res. Prot.* **2**, 209–214.

Almeida A., Heales S. J. R., Bolaños J. P. and Medina J. M. (1998) Glutamate neurotoxicity is associated with nitric oxide-mediated mitochondrial dysfunction and glutathione depletion. *Brain Res.* **790**, 209–216.

Almeida A., Bolaños J. P. and Medina J. M. (1999) Nitric oxide mediates glutamate-induced mitochondrial depolarization in rat cortical neurons. *Brain Res.* **816**, 580–586.

Almeida A., Delgado-Esteban M., Bolaños J. P. and Medina J. M. (2002) Oxygen and glucose deprivation induces mitochondrial dysfunction

and oxidative stress in neurones but not in astrocytes in primary culture. *J. Neurochem.* **81**, 207–217.

Barford P., Blair J., Eggar C., Hamon C., Morar C. and Whitburn S. (1984) Tetrahydrobiopterin metabolism in the temporal lobe of patients dying with senile dementia of Alzheimer type. *J. Neurol. Neurosrg. Psychiatry* **47**, 736–738.

Barker J. E., Strangward H. M., Brand M. P., Hurst R. D., Land J. M., Clark J. B. and J. H. S. (1998) Increased inducible nitric oxide synthase protein but limited nitric oxide formation occurs in astrocytes of the hph-1 (tetrahydrobiopterin deficiency) mouse. *Brain Res.* **804**, 1–6.

Beckman J. S., Beckman T. W., Chen J., Marshall P. A. and Freeman B. A. (1990) Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. USA* **87**, 1620–1624.

Bogdan C., Werner E., Stenger S., Wachter H., Rollinghoff M. and Werner-Felmayer G. (1995) 2,4-Diamino-6-hydroxypirimidine, an inhibitor of tetrahydrobiopterin synthesis, down-regulates the expression of iNOS protein and mRNA in primary murine macrophages. *FEBS Lett.* **363**, 69–74.

Bolaños J. P. and Almeida A. (1999) Roles of nitric oxide in brain hypoxia-ischemia. *Biochim. Biophys. Acta* **1411**, 415–436.

Bolaños J. P. and Medina J. M. (1996) Induction of nitric oxide synthase inhibits gap junction permeability in cultured rat astrocytes. *J. Neurochem.* **66**, 2091–2099.

Bolaños J. P., Heales S. J. R., Land J. M. and Clark J. B. (1995) Effect of peroxynitrite on the mitochondrial respiratory chain: differential susceptibility of neurones and astrocytes in primary cultures. *J. Neurochem.* **64**, 1965–1972.

Bolaños J. P., Almeida A., Stewart V., Peuchen S., Land J. M., Clark J. B. and Heales S. J. R. (1997) Nitric oxide-mediated mitochondrial damage in the brain: mechanisms and implications for neurodegenerative diseases. *J. Neurochem.* **68**, 2227–2240.

Bonfoco E., Krainc C., Ankarcrona M., Nicotera P. and Lipton S. A. (1995) Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with *N*-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proc. Natl. Acad. Sci. USA* **92**, 7162–7166.

Bredt D. S. and Snyder S. H. (1994) Nitric oxide: a physiologic messenger molecule. *Annu. Rev. Biochem.* **63**, 175–195.

Brown G. C. and Cooper C. E. (1994) Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal respiration by competing with oxygen at cytochrome oxidase. *FEBS Lett.* **356**, 295–298.

Budd S. L. and Nicholls D. G. (1996) A re-evaluation of the role of mitochondria in neuronal Ca^{2+} homeostasis. *J. Neurochem.* **66**, 403–411.

Chan P. H. (2001) Reactive oxygen radicals in signaling and damage in the ischemic brain. *J. Cereb. Blood Flow Metab.* **21**, 2–14.

Curtius H. C., Niederwieser A., Levine R. and Muldner H. (1984) Therapeutic efficacy of tetrahydrobiopterin in Parkinson's disease. *Adv. Neurol.* **40**, 463–466.

Dawson V. L. and Dawson T. M. (1996) Nitric oxide neurotoxicity. *J. Chem. Neuroanatom.* **10**, 179–190.

Dawson V. L., Dawson T. M., Bartley D. A., Uhl G. R. and Snyder S. H. (1993) Mechanisms of nitric oxide-mediated neurotoxicity in primary brain cultures. *J. Neurosci.* **13**, 2651–2661.

Delgado-Esteban M., Almeida A. and Bolaños J. P. (2000) D-Glucose prevents glutathione oxidation and mitochondrial damage after glutamate receptor stimulation in rat cortical primary neurons. *J. Neurochem.* **75**, 1618–1624.

Dringen R. and Hamprecht B. (1996) Glutathione content as an indicator for the presence of metabolic pathways of amino acids in astroglial cultures. *J. Neurochem.* **67**, 1375–1382.

Duchen M. R. (1990) Effects of metabolic inhibition of the membrane properties of isolated mouse primary sensory neurones. *J. Physiol.* **424**, 387–409.

Fernell E., Watanabe Y., Adolfsson I., Tani Y., Bergstrom M., Hartvig P., Lilja A., von Knorring A. L., Gillberg C. and Langstrom B. (1997) Possible effects of tetrahydrobiopterin treatment in six children with autism-clinical and positron emission tomography data: a pilot study. *Dev. Med. Child Neurol.* **39**, 313–318.

Furukawa Y., Kish S. J., Bebin E. M., Jacobson R. D., Fryburg J. S., Wilson W. G., Shimadzu M., Hyland K. and Trugman J. M. (1998) Dystonia with motor delay in compound heterozygotes for GTP-cyclohydrolase I gene mutations. *Ann. Neurol.* **44**, 10–16.

Gal E. M., Nelson J. M. and Sherman A. D. (1978) Biopterin. III. Purification and characterization of enzymes involved in the cerebral synthesis of 7,8-dihydrobiopterin. *Neurochem. Res.* **3**, 69–88.

García-Nogales P., Almeida A., Fernández E., Medina J. M. and Bolaños J. P. (1999) Induction of glucose-6-phosphate dehydrogenase by lipopolysaccharide contributes to preventing nitric oxide-mediated glutathione depletion in cultured rat astrocytes. *J. Neurochem.* **72**, 1750–1758.

Garthwaite J., Charles S. L. and Chess-Williams R. (1988) Endothelium-derived relaxing factor release on activation of NMDA receptors suggests a role as intercellular messenger in the brain. *Nature* **336**, 385–387.

Heinzel B., John M., Klatt P., Böhme E. and Mayer B. (1992) Ca^{2+}/calmodulin-dependent formation of hydrogen peroxide by brain nitric oxide synthase. *Biochem. J.* **281**, 627–630.

Howells D. W., Smith I. and Hyland K. (1986) Estimation of tetrahydrobiopterin and other pterins in cerebrospinal fluid using reversed-phase high-performance liquid chromatography with electrochemical and fluorescence detection. *J. Chromatogr.* **381**, 285–294.

Hughes M. N. and Nicklin H. G. (1968) The chemistry of pernitrites. Part I. Kinetics of decomposition of pernitrous acid. *J. Chem. Soc. Abstract* 450–452.

Hurshman A. R., Krebs C., Edmondson D. E., Huynh B. H. and Marletta M. A. (1999) Formation of a pterin radical in the reaction of the heme domain of inducible nitric oxide synthase with oxygen. *Biochemistry* **38**, 15689–15696.

Ishii M., Shimuzu S., Yamamoto T., Momose K. and Kuroiwa Y. (1997) Acceleration of oxidative stress-induced endothelial cell death by nitric oxide synthase dysfunction accompanied with decrease in tetrahydrobiopterin content. *Life Sci.* **61**, 739–747.

Johnson L. V., Walsh M. L. and Chen L. B. (1980) Localization of mitochondria in living cells with rhodamine 123. *Proc. Natl. Acad. Sci. USA* **77**, 990–994.

Kaufman S. (1993) New tetrahydrobiopterin-dependent systems. *Annu. Rev. Nutr.* **13**, 261–286.

Khodorov B., Pinelis V., Vergun O., Storozhevykh T. and Vinskaya N. (1996) Mitochondrial deenergization underlies neuronal calcium overload following a prolonged glutamate challenge. *FEBS Lett.* **397**, 230–234.

King T. E. (1967) Preparation of succinate cytochrome c reductase and the cytochrome b-c1 particle, and reconstitution of succinate cytochrome c reductase. *Methods Enzymol.* **10**, 216–225.

Knowles R. G. and Moncada S. (1994) Nitric oxide synthases in mammals. *Biochem. J.* **298**, 249–258.

Koshimura K., Murakami Y., Tanaka J. and Kato Y. (1998) Self-protection of PC12 cells by 6R-tetrahydrobiopterin from nitric oxide toxicity. *J. Neurosci. Res.* **54**, 664–672.

Kumura E., Yshimine T., Tanaka S., Hayakawa T., Shiga T. and Kosaka H. (1994) Nitrosyl hemoglobin production during reperfusion after focal cerebral-ischemia in rats. *Neurosci. Lett.* **177**, 165–167.

Lowry O. H., Rosebrough N. J., Lewis-Farr A. and Randall R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275.

MacMillan-Crow L. A., Crow J. P., Kerby J. K., Beckman J. S. and Thompson J. A. (1996) Nitration and inactivation of manganese superoxide dismutase in chronic rejection of human renal allografts. *Proc. Natl. Acad. Sci. USA* **93**, 11853–11858.

Marletta M. A. (1993) Nitric oxide synthase structure and mechanism. *J. Biol. Chem.* **268**, 12231–12234.

Mattson M. P., Duan W., Pedersen W. A. and Culmsee C. (2001) neurodegenerative disorders and ischemic brain diseases. *Apoptosis* **6**, 69–81.

Mayer B., Heinzel B., Klatt P., John M., Schmidt K. and Böhme E. (1992) Nitric oxide synthase-catalyzed activation of oxygen and reduction of citochromes: reaction mechanisms and possible physiological implications. *J. Cardiovasc. Pharmacol.* **20**, 54–56.

Moncada S., Palmer R. M. J. and Higgs E. A. (1991) Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol. Rev.* **43**, 109–142.

Nichol C. A., Smith G. K. and Duch D. S. (1985) Biosynthesis and metabolism of tetrahydrobiopterin and molybdopterin. *Annu. Rev. Biochem.* **54**, 729–764.

Nicholls D. G. and Ward M. W. (2000) Mitochondrial membrane potential and neuronal glutamate excitotoxicity: mortality and millivolts. *Trends Neurosci.* **23**, 166–174.

Niederwieser A., Curtius H. C., Wanc M. and Leupold D. (1982) Atypical phenylketonuria with defective biopterin metabolism. Monotherapy with tetrahydrobiopterin or sepiapterin, screening and study of biosynthesis in man. *Eur. J. Pediatr.* **138**, 110–112.

Pfeiffer S., Lass A., Schmidt K. and Mayer B. (2001) Protein tyrosine nitration in mouse peritoneal macrophages activated *in vitro* and *in vivo*: evidence against an essential role of peroxynitrite. *FASEB J.* **15**, 2355–2364.

Pou S., Pou W. S., Bredt D. S., Snyder S. H. and Rosen G. M. (1992) Generation of superoxide by purified brain nitric oxide synthase. *J. Biol. Chem.* **267**, 24173–24176.

Ragan C. I., Wilson M. T., Darley-Usmar V. M. and Lowe P. N. (1987) Subfractionation of mitochondria and isolation of the proteins of oxidative phosphorylation. In: *Mitochondria. A Practical Approach, Volume* (Darley-Usmar V. M., Rickwood D. and Wilson M. T., eds), pp. 79–112. IRL Press, London.

Raha S. and Robinson B. H. (2000) Mitochondria, oxygen free radicals, disease and aging. *Trends Biochem. Sci.* **25**, 502–508.

Sastry P. S. and Rao K. S. (2000) Apoptosis and the nervous system. *J. Neurochem.* **74**, 1–20.

Saura M., Perez-Sala D., Canada F. J. and Lamas S. (1996) Role of tetrahydrobiopterin availability in the regulation of nitric oxide synthase expression in human mesangial cells. *J. Biol. Chem.* **271**, 14290–14295.

Schmidt P. P., Lange R., Gorren A. C., Werner E. R., Mayer B. and Andersson K. K. (2001) Formation of a protonated trihydrobiopterin radical cation in the first reaction cycle of neuronal and endothelial nitric oxide synthase detected by electron paramagnetic resonance spectroscopy. *J. Biol. Inorg. Chem.* **6**, 151–158.

Thöny B., Auerbach G. and Blau N. (2000) Tetrahydrobiopterin biosynthesis, regeneration and functions. *Biochem. J.* **347**, 1–16.

Vásquez-Vivar J., Hogg N., Martásek P., Karoui H., Pritchard K. A. and Kalyanaraman B. (1999) Tetrahydrobiopterin-dependent inhibition of superoxide generation from neuronal nitric oxide synthase. *J. Biol. Chem.* **274**, 26736–26742.

Wei C., Wang Z. Q., Wang Q., hemann C., Hille R. and Stuehr R. J. (2001) Rapid kinetic studies link tetrahydrobiopterin radical formation to heme-dioxy reduction and arginine hydroxylation in inducible nitric-oxide synthase. *J. Biol. Chem.* **276**, 315–319.

Wharton D. C. and Tzagoloff A. (1967) Cytochrome oxidase from beef heart mitochondria. *Methods Enzymol.* **10**, 245–250.