Phosphodiesterase-5 Inhibition Abolishes Neuron Apoptosis Induced by Chronic Hypoxia Independently of Hypoxia-Inducible Factor-1α Signaling

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Exposure to hypoxia triggers a variety of adverse effects in the brain that arise from metabolic stress and induce neuron apoptosis. Overexpression of the hypoxia-inducible factor-1α (HIF-1α) is believed to be a major candidate in orchestrating the cell's defense against stress. To test the impact of HIF-1α on apoptosis during chronic hypoxia *in vivo*, we examined the protective effect of modulating the nitric oxide (NO)/cGMP pathway by sildenafil, a selective inhibitor of phosphodiesterase-5 (PDE-5). Male ICR/CD-1 mice were divided into 3 groups (*n* = 6/group): normoxic (21% O_2), hypoxic (9.5% O_2), and hypoxic with sildenafil (1.4-mg/kg intraperitoneal injections daily). At the end of the 8-day treatment period, the mice were euthanized and cerebral cortex biopsies were harvested for analyses. We found that sildenafil: (1) did not significantly alter the hypoxia-induced weight loss and hemoglobin increase, but did augment plasma nitrates+nitrites and the tissue content of cGMP and phosphorylated (P) NO synthase III; (2) reversed the hypoxia-induced neuron apoptosis (terminal deoxynucleotidyl transferase positivity and double-staining immunofluorescence, *P* = 0.009), presumably through increased bcl-2/Bax (*P* = 0.0005); and (3) did not affect HIF-1α, but rather blunted the hypoxia-induced increase in P-ERK1/2 (*P* = 0.0002) and P-p38 (*P* = 0.004). We conclude that upregulating the NO/cGMP pathway by PDE-5 inhibition during hypoxia reduces neuron apoptosis, regardless of HIF-1α, through an interaction involving ERK1/2 and p38. Exp Biol Med 233:1222–1230, 2008

Key words: chronic hypoxia; hypoxia signaling; HIF-1α; NO; bcl-2

Introduction

Originating from insufficient O_2 supply in response to need, hypoxia causes an array of potentially lethal adverse effects. The α subunit of the hypoxia-inducible factor 1 (HIF-1α) is believed to play a pivotal role in orchestrating the cell's response to hypoxia. In the absence of O_2, HIF-1α stabilization contributes to the formation of HIF-1, a heterodimer (1) that, in association with other proteins, binds to cognate-responsive elements on DNA, regulating the transcription of hundreds of downstream genes (2). By contrast, HIF-1α hydroxylation in the presence of O_2 targets the protein to proteasomal degradation, thereby preventing its binding to DNA (3). The degree of HIF-1α overexpression *in vivo* depends on hypoxia severity and duration (4), as well as on O_2 demand, supply, and utilization characteristics in the specific organ (5). In the case of neurons, chronic hypoxia induces sustained HIF-1α overexpression and apoptosis (5, 6). It is unclear whether HIF-1α overexpression is responsible for the onset of apoptosis or whether apoptosis arises from insufficient HIF-1α, as it appears to in the hypoxic myocardium (7). As administration of a carbamylated derivative of erythropoietin reduces hypoxia-induced apoptosis with concomitant reduction in HIF-1α expression (6), the role of HIF-1α–mediated pathways in protecting hypoxic neurons from apoptosis remains obscure.

Sildenafil, a selective inhibitor of phosphodiesterase-5 (PDE-5), the enzyme that inactivates bioactive cGMP into 5′-GMP, is thought to enhance nitric oxide (NO)–driven cGMP intracellular accumulation. In the smooth muscle, cGMP-induced vasodilation is a clinical target for treatment

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of erectile dysfunction, primary or hypoxia-induced pulmonary hypertension (8), chronic heart failure (9), coronary diseases (10, 11), and myocardial infarction (12). Furthermore, sildenafil is becoming an elective strategy to control pulmonary hypertension and improve exercise capacity at extreme altitudes (13–16), and long-term treatment is being proposed to manage patients with chronic heart failure without development of remarkable adverse effects (17). In myocardial cells, the mechanism underlying sildenafil-induced protection against hypoxia involves prevention of apoptosis, most probably via the pathways operating through p38, ERK1/2, and NO (12, 18, 19). Sildenafil was successfully used in a rat model of brain ischemia after embolic stroke (20) and was found to augment neurogenesis in the subventricular zone in aged, ischemic rats with reduced numbers of neural progenitors (21). However, there is no *in vivo* evidence that sildenafil protects neurons during hypoxia.

Here, we test the hypothesis that modulation of the NO/cGMP pathway by sildenafil alleviates neuron apoptosis in mice exposed to systemic chronic hypoxia *in vivo*, a condition that occurs in several physiologic and pathologic settings. To this aim, we used a model that rules out the potentially confounding oxidative stress associated with reoxygenation during maintenance and sacrifice (22). More specifically, we examined neuron apoptosis in relation to changes in HIF-1α, ERK1/2, p38, and NO signaling paths.

Materials and Methods

Materials. Sildenafil was obtained from 25-mg Viagra tablets (Pfizer Inc., New York, NY), which were ground into fine powder using mortar and pestle, dissolved in 0.9% NaCl (2.6 mg/ml), filtered (0.22-μm pore diameter, (Thermo Fisher Scientific-Nalgene, Rochester, NY), and stored at 4°C. All other reagents were of the maximal available degree of purity.

Animals. Male ICR/CD-1 mice (5 weeks old, 29.6 ± 0.3 g body wt at entry into the study) were divided into 3 groups ($n = 6$/group, 8-day treatments): vehicle-treated normoxic (21% O_2); vehicle-treated hypoxic (9.5 ± 0.5% O_2, as previously described [22]); and sildenafil-treated hypoxic. For daily intraperitoneal (ip) injections (0.1 ml) of sildenafil (1.4 mg/kg body wt) or vehicle (0.9% NaCl), hypoxic mice were anaerobically transferred, one by one, into the procedure chamber at the same O_2 content, avoiding exposure to room air. Mice had free access to water and conventional laboratory diet until 24 hrs before sacrifice. A 12:12-hr light:dark cycle was maintained.

At day 8, mice were transferred into the procedure chamber and anesthetized by ip Na-thiopental (10 mg/100 g body wt) plus heparin (500 units). After thoracotomy, blood was withdrawn via intracardiac puncture into a heparinized syringe; the brain was then removed (<1 min), freed of cerebellum, immersed in liquid nitrogen, and stored at –80°C until use. The investigation conformed to standards of the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health (NIH Publication No. 85–23, revised 1996).

Blood Measurements. Testing included blood hemoglobin concentration, determined by the Drabkin assay ($\varepsilon = 11.05\ mM^{-1} \cdot cm^{-1}$), and plasma nitrates+nitrites (NOx, Nitric Oxide Colorimetric Assay Kit, BioVision, Mountain View, CA).

cGMP Measurement. Frozen tissue (100 mg) was homogenized in 0.1 *M* HCl and cGMP was assayed by enzyme immunoassay (Assay Design, Inc., Ann Arbor, MI).

Western Blot. Separate extracts were prepared at ice temperature for each biopsy; the relative band intensity was evaluated with respect to β-actin and was then averaged. Whole cell and cytosolic extracts were obtained from frozen tissue (100 mg) as described elsewhere (7). The protein concentration was measured by the Coomassie Plus Protein Assay Reagent Kit (Pierce Technology, Rockford, IL).

Extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. For proteins with molecular weight <50 kDa (bcl-2, Bax, vascular endothelial growth factor [VEGF], actin, ERK1/2, phosphorylated [P]-ERK1/2, p38, and P-p38), 12% acrylamide gels were used, versus 8% gels for higher molecular weight proteins (HIF-1α, NOS II, NOS III, and P-NOS III). Fifty to seventy micrograms of protein was loaded in each lane. After separation, proteins were blotted onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) and blocked overnight with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween. The membranes were incubated (2 hrs) at room temperature with the primary antibody, followed by horseradish peroxidase–conjugated secondary antibody (1 hr, room temperature). The following primary antibodies and dilutions were used: rabbit polyclonal anti–HIF-1α (Santa Cruz, 1:2000), mouse monoclonal anti–bcl-2 (Santa Cruz, 1:500), mouse monoclonal anti-Bax (Santa Cruz, 1:300), rabbit polyclonal anti-NOS III (Santa Cruz, 1:500), rabbit polyclonal anti–P-NOS III (Ser-1177, Santa Cruz, 1:200), rabbit polyclonal anti-NOS II (Santa Cruz, 1:500), rabbit polyclonal anti-VEGF165 (Calbiochem, 1:500), rabbit polyclonal anti-ERK1 (Santa Cruz, 1:200), mouse monoclonal anti–P-ERK1/2 (Santa Cruz, 1:200), mouse monoclonal anti-p38 (Santa Cruz, 1:200), mouse monoclonal anti–P-p38 (Santa Cruz, 1:200), and rabbit polyclonal anti-actin (Santa Cruz, 1:1000). The secondary antibodies included horseradish peroxidase–conjugated anti-mouse IgG (Jackson Immuno Research, West Grove, PA, 1:10,000) or anti-rabbit IgG (Jackson Immuno Research, 1:10,000).

The chemiluminescent signal was detected by incubating the membrane with LiteAblot Chemiluminescent substrate (EuroClone, Siziano, Italy) for 1 min, followed by x-ray film exposure (Kodak X-Omat Blue XB-1 Film, Eastman Kodak Co., Rochester, NY). The resulting image

was acquired and quantified by Gel Doc (Quantity One, Bio-Rad Laboratories, Inc., Hercules, CA).

Immunofluorescence. Frozen biopsies were embedded in Optimum Cutting Temperature Compound (Leica Instruments, Nussloch, Germany); serial 5-μm thick sections were then obtained in a cryomicrotome (Leica CM1510) and placed on silanized glass slides. Five distinct sections were obtained from each biopsy. Care was taken to analyze the same brain area (cerebral cortex) in all sections. The sections were dried (2 mins at room temperature), fixed in 4% buffered formalin (45 mins), rinsed 3 times in phosphate-buffered saline (PBS) (each for 5 mins), postfixed with ethanol-acetic acid 2:1 (vol:vol) (5 mins at –20°C), rinsed twice in PBS (each for 5 mins), boiled in 10 m*M* citrate at pH 6.0 (10 mins), washed 3 times in H_2O and twice in PBS for 5 min each, and finally used for histochemistry.

To detect DNA fragmentation, we used the TUNEL ApopTag Red In Situ Apoptosis Detection Kit (Chemicon International Inc., Temecula, CA). The images were acquired and analyzed as described elsewhere (6), counting the number of terminal deoxynucleotidyl transferase (TdT)–positive nuclei by examining 5 random fields in a blind procedure. Values are expressed as number of TdT-positive nuclei/0.037 mm^2. To detect co-localization of TdT positivity in neuron cells, slides were double stained with mouse monoclonal anti-NeuN antibody (Chemicon International, diluted 1:100), using goat anti-mouse IgG fluorescein-conjugated (Santa Cruz, 1:200) as secondary antibody. The green channel was selected, digitally converted to blue to improve visibility, and the images were merged.

Statistics. All data are expressed as mean ± SEM. To assess the significance of the differences among the various groups, we first performed one-way ANOVA, followed by the Bonferroni multiple comparisons test if the result was significant ($P < 0.05$).

Results

Gross Changes. All animals survived hypoxia and were admitted to statistical analysis. Body weight in normoxic animals (29.6 ± 0.3 g at the start) increased by 1.2 ± 0.3 g, and hypoxic animals lost weight, regardless of whether they received or did not receive sildenafil (–4.4 ± 0.7 g and –4.3 ± 0.3 g, respectively, ANOVA $P < 0.0001$). Hypoxia increased blood hemoglobin levels from 13.0 ± 0.3 g/dl to 19.8 ± 1.0 g/dl (ANOVA $P < 0.006$), but treatment with sildenafil did not significantly affect this parameter (20.3 ± 2.4 g/dl).

Sildenafil Administration Increases the Anti/Propoptotic Protein Ratio to a Greater Extent than Hypoxia. The degree of apoptosis was assessed from either the cytosolic bcl-2/Bax ratio (Western blot) or TdT positivity (immunofluorescence). Hypoxia alone increased the expression of the antiapoptotic protein bcl-2 non-significantly ($P > 0.05$), as well as the bcl-2/Bax ratio ($P > 0.05$), whereas the expression of the proapoptotic protein Bax remained unchanged (Fig. 1). Treatment with sildenafil increased ($P < 0.001$) the expression of bcl-2, with a marginal decrease of Bax. Therefore, the bcl-2/Bax ratio increased with respect to hypoxic animals ($P = 0.0001$).

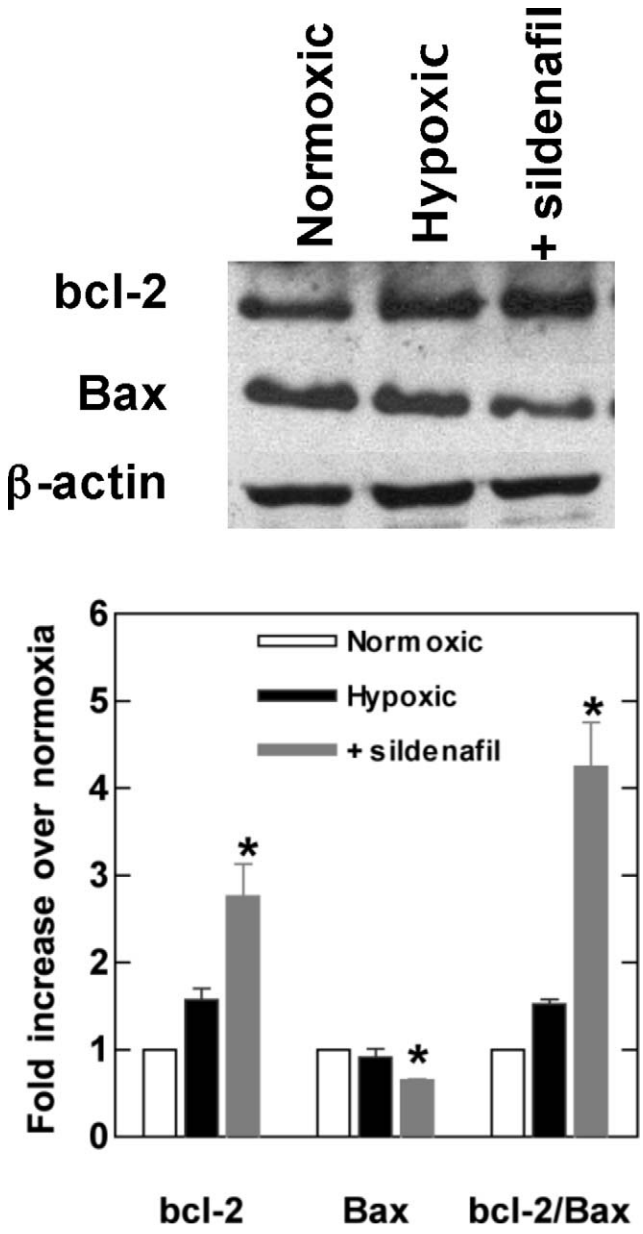


Figure 1. Sildenafil administration increases the anti/proapoptotic proteins ratio to a greater extent compared with hypoxia. Upper panel. Representative Western blots showing the expression of the anti/proapoptotic proteins bcl-2 and Bax in cytosolic extracts. Lower panel. Densitometric analysis (mean ± SEM) of the blots performed in all samples ($n = 6$/group) as referred to β-actin. The bcl-2/Bax ratio is shown on the right. Data are expressed as fold increase over normoxia. The *P* values for the one-way ANOVA test are 0.004, 0.002, and 0.0001, respectively. *, $P < 0.05$ hypoxic versus hypoxic+sildenafil (Bonferroni posttest).

Sildenafil Administration Reduces Chronic Hypoxia-Induced Neuron Apoptosis. The number of TdT-labeled nuclei per unit area, an index reflecting DNA

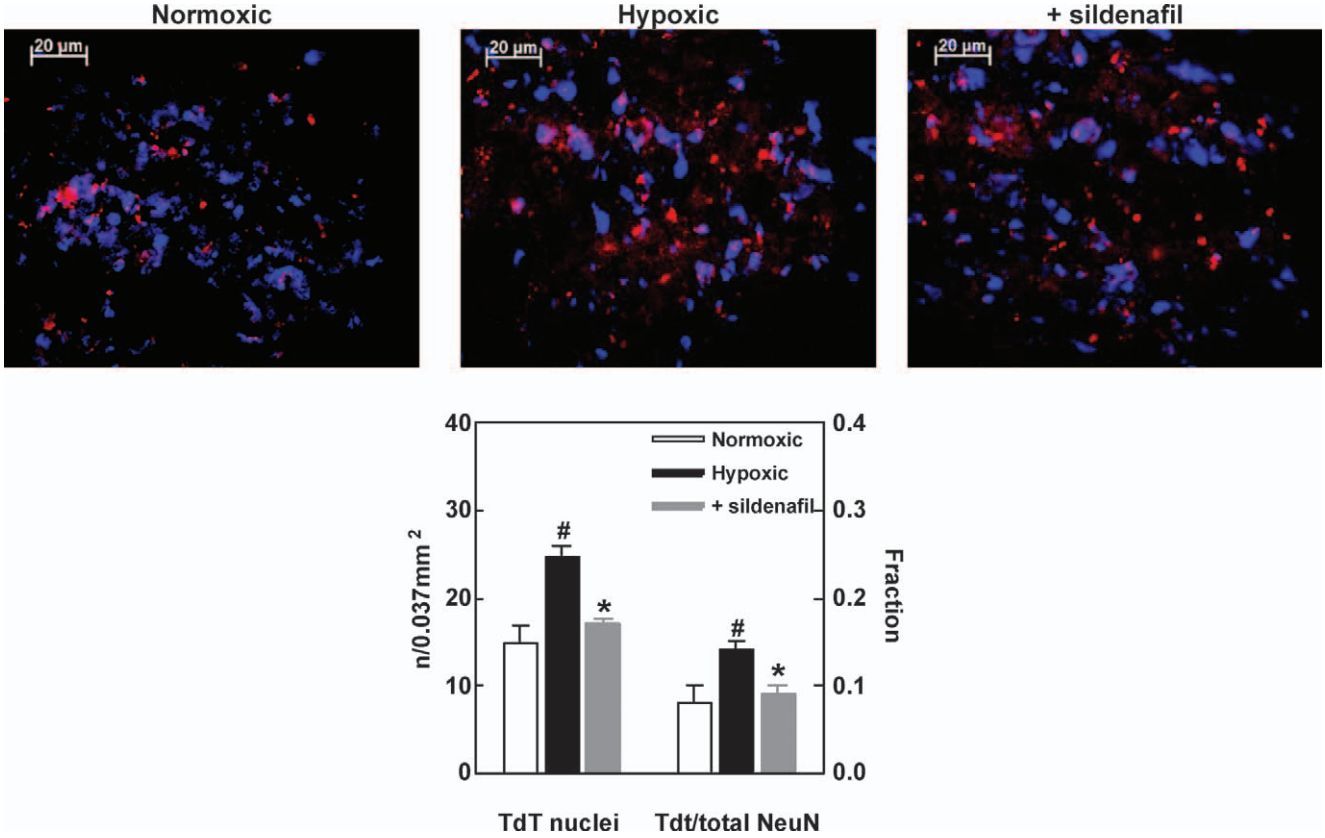


Figure 2. Sildenafil administration reduces chronic hypoxia-induced neuron apoptosis. Upper panels. Representative immunofluorescence from normoxic, hypoxic and sildenafil-treated animals. TdT-positive cells are marked in red, with NeuN-positive cells marked in blue. The same brain area (cerebral cortex) was studied in all sections. Merging of red and blue spots results in a bright purple signal. Magnification ×40; the bar represents 20 μm. Bottom panel. The left axis shows the absolute number of TdT-positive nuclei per unit area (0.037 mm^2) in all samples ($n = 6$/group, 5 sections per each biopsy, and 5 fields per each section). The right axis reports the fraction of NeuN-positive cells that display TdT positivity. The P values for the one-way ANOVA test are 0.009 and 0.004, respectively. #, $P < 0.05$ hypoxic versus normoxic; *, $P < 0.05$ hypoxic versus hypoxic+sildenafil (Bonferroni posttest). A color version of this figure is available in the online journal.

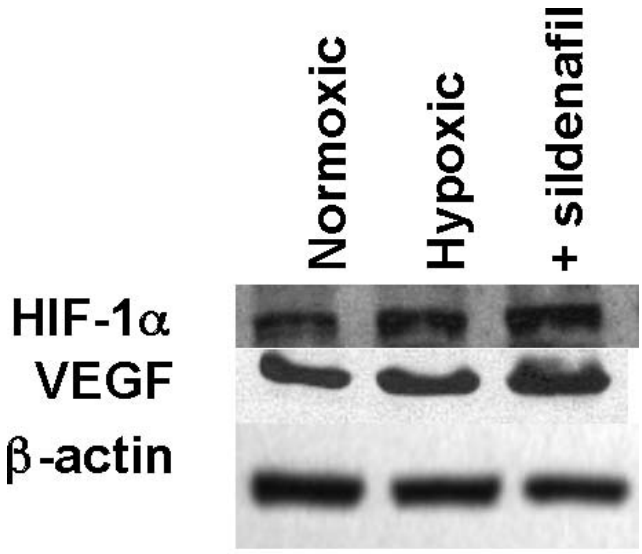
fragmentation and apoptosis, was measured by immunofluorescence (Fig. 2). TdT positivity was substantially increased in vehicle-treated hypoxic animals, but was reduced to nearly the control values in hypoxic animals treated with sildenafil.

To determine the cellular localization of TdT-labeled nuclei, we labeled the DNA fragments, which were catalytically bound to digoxigenin nucleotides, with an antidigoxigenin antibody bound to rhodamine. This action yields a red signal. Then, we stained NeuN-positive neurons with a primary antibody, which was labeled with a secondary antibody bound to fluorescein. This action yields a green signal, which was digitally converted to blue to improve visibility. After merging the images, the combination of the 2 dyes yields a bright purple color (Fig. 2). The fraction of neurons exhibiting TdT-associated immunoreactivity was 8% ± 1% in normoxic animals, 14% ± 1% in vehicle-treated hypoxic animals, and 9% ± 1% in sildenafil-treated hypoxic animals ($P = 0.004$).

Hypoxia and Sildenafil Differentially Increase the Expression of HIF-1α and VEGF. A major transcription factor activated by hypoxia, nuclear HIF-1α, increased after 8 days of hypoxia (Fig. 3). However, treatment with sildenafil did not produce further alteration of HIF-1α. VEGF, a product of one of the major genes activated by HIF-1, was overexpressed in the hypoxic animals, and sildenafil treatment further increased its expression.

Effect of Hypoxia and Sildenafil Administration on the NO/cGMP Pathways. The NO signaling pathway was assessed by measuring tissue cGMP and the level of NOx. Both cGMP and NOx were upregulated by hypoxia, with sildenafil further increasing their levels (Fig. 4).

Effect of Hypoxia and Sildenafil Administration on the Signaling Pathways. To assess the involvement of the NO, ERK1/2, and p38 signaling pathways, we measured the cytosolic level of the related proteins in both the total and phosphorylated forms by Western blot (Fig. 5). NOS III, NOS II, ERK1/2, and p38 were detectable even in normoxic controls, but their total forms did not vary during hypoxia, regardless of the presence of sildenafil. The phosphorylated form of NOS III, or P-NOS III (Ser-1177), was upregulated by hypoxia and further increased by



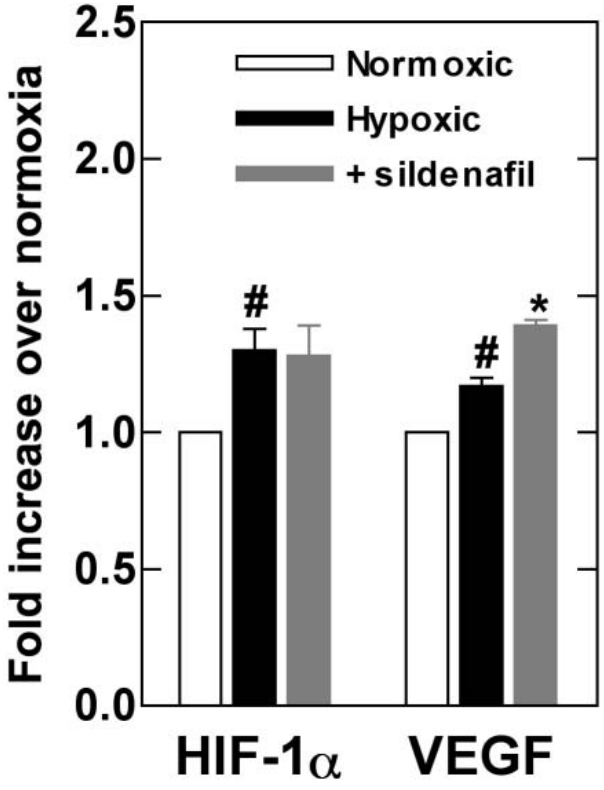


Figure 3. Hypoxia and sildenafil increase differentially HIF-1α and VEGF expression. Upper panel. Representative Western blot showing the expression of HIF-1α and VEGF in whole extracts. Lower panel. Densitometric analysis of the blots ($n = 6$/group) performed in all samples as referred to β-actin. Data are expressed as fold increase over normoxia. The *P* values of the one-way ANOVA tests are 0.01 and <0.0001, respectively; #, $P < 0.05$ hypoxic versus normoxic; *, $P < 0.05$ hypoxic versus hypoxic+sildenafil (Bonferroni posttest).

sildenafil. Both P-ERK1/2 and P-p38 were upregulated by hypoxia ($P = 0.0002$ and $P = 0.004$, respectively). By contrast, sildenafil abolished hypoxia-induced upregulation of these proteins.

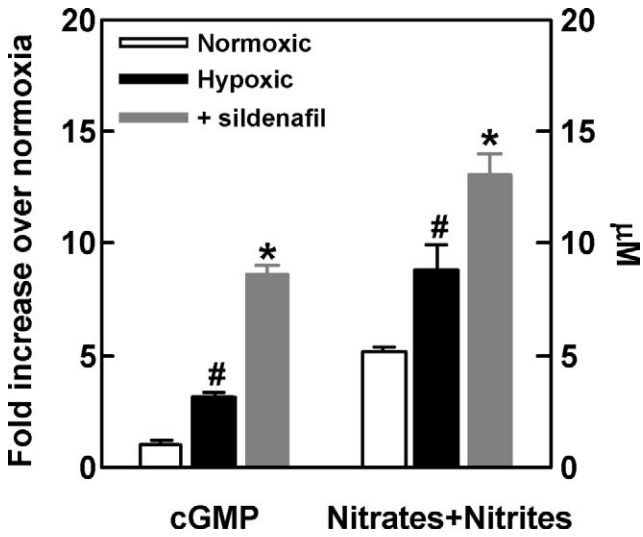


Figure 4. Effect of hypoxia and sildenafil administration on the NO/cGMP paths. The left and right axes report the tissue level of cGMP and the plasma concentration of nitrates+nitrites (NOx), respectively. The *P* values for the one-way ANOVA test are 0.0001 and 0.0002, respectively. #, $P < 0.05$ hypoxic versus normoxic; *, $P < 0.05$ hypoxic versus hypoxic+sildenafil (Bonferroni posttest).

Discussion

Exposure of mice to 9.5% O_2 for 8 days increased the number of TdT-positive apoptotic neurons in the brain cerebral cortex. Upregulation of the NO/cGMP path by sildenafil increased P-NOS III, VEGF, and NOx, while HIF-1α remained unchanged. By contrast, P-ERK1/2 and P-p38 were reduced. These changes enhanced expression of bcl-2, with antiapoptotic effects (23). Thus, *in vivo* treatment of hypoxic mice with sildenafil reduced neuron apoptosis, regardless of the HIF-1α concentration.

The sildenafil dose tested in this study, 1.4 mg/kg body wt ip, is within the *per os* doses of 0.2–1.5 mg/kg/day normally used in humans, without appreciable dose-dependent variations (24). Despite the short (4–6 hrs) expected plasma half-life of sildenafil (25), that dose doubled the cGMP concentration. The specially designed hypoxic chambers prevented any accidental exposure to room air and unwanted reoxygenation before sacrifice (26). HIF-1α was still induced after 8 days of chronic hypoxia *in vivo*; this differs from studies showing that HIF-1α expression peaks and returns to baseline values within hours after the onset of hypoxia (4), but agrees with previous work supporting sustained HIF-1α expression after a 14-day period of hypoxia (5).

Neuron apoptosis was assessed by TdT positivity followed by immunoreactivity co-localization against digoxigenin nucleotides bound to DNA fragments and anti-NeuN antibody. This method was used in a previous study where *in vivo* administration of a carbamylated derivative of erythropoietin reversed hypoxia-induced apoptosis concomitantly with reduced HIF-1α expression (6). Here, the modulation of the NO/cGMP path by sildenafil reduced

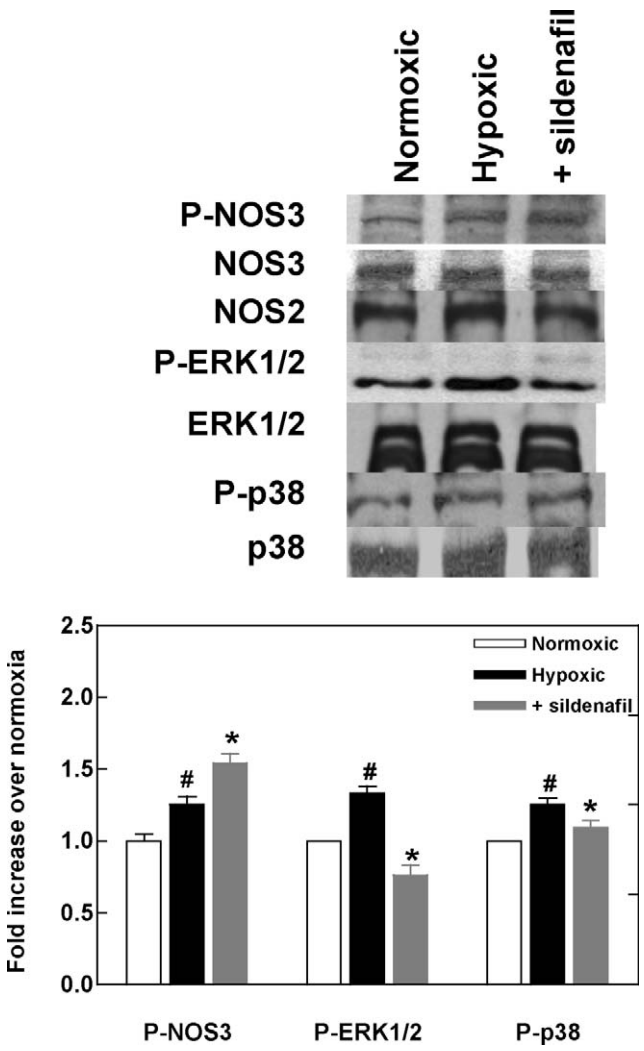


Figure 5. Effect of hypoxia and sildenafil administration on the signaling pathways. Upper panel. Representative Western blots showing the expression of cytosolic P-NOS III (Ser-1177), total NOS III, total NOS II, P-ERK1/2, total ERK1/2, P-p38, and total p38. Lower panel. Densitometric analysis of the phosphorylated proteins referred to the respective total, expressed as fold increase over normoxia ($n=$ 6/group). The P values for the one-way ANOVA test are 0.002, 0.0002, and 0.004, respectively. #, $P < 0.05$ hypoxic versus normoxic; *, $P < 0.05$ hypoxic versus hypoxic+sildenafil (Bonferroni posttest).

neuron apoptosis without concomitant HIF-1α reduction. This observation helps to address the mechanism underlying hypoxia-induced neuron apoptosis. Indeed, the signaling pathway stemming from sildenafil appears to match that stemming from hypoxia as far as the increases in P-NOS III, NOx, cGMP, and VEGF are concerned (Fig. 6). By contrast, these pathways appear to diverge for the stimulation of bcl-2, ERK1/2, and p38, and hence, the development of apoptosis.

The fact that immunohistochemical studies showed PDE-5 localization in neurons (27) raises the possibility that neuronal PDE-5 inhibition increases cGMP, activates cGMP-dependent protein kinase G, upregulates NOS III, and increases the level of bioactive NO (24), thereby establishing a synergism among PDE-5 inhibition, cGMP, and NO (28). Our findings support this scenario, in part. A messenger with a dichotomous role in the brain, NO released by NOS III is considered beneficial because it promotes vasodilation, whereas NO produced by neuronal and inducible NOS contributes to damage (29). There is evidence that NOS III phosphorylation at Ser-1177 activates the protein, whereas Thr-495 phosphorylation inhibits its activity (30). Although NO production does not necessarily correlate with NOS III activation, we observed good correspondence between NOx and NOS III phosphorylated at Ser-1177 (compare Figs. 4 and 5), whereas the level of inducible NOS (or NOS II) remained unchanged under all experimental conditions tested in this study. Remarkably, phosphorylation of NOS III at Ser-1177 was found to play an important role in augmenting regional cerebral blood flow after ischemia (30). However, the mechanisms underlying NO increase during hypoxia, with or without PDE-5 inhibition, are different. Hypoxia increases the conversion of plasma nitrites into bioactive NO through a variety of mechanisms; the most important of these involves the increased abundance of hemoglobin in the deoxygenated state (31, 32). PDE-5 inhibition during hypoxia increases bioactive NO and the NO-derived path by upregulating the NO/cGMP path, which activates NOS III either directly or through VEGF overexpression (Fig. 3), in agreement with data reported in a forebrain cerebral ischemia/reperfusion model (30). Furthermore, upregulation of the NO/cGMP path is fundamentally antiapoptotic because it upregulates bcl-2 (33). The described paths override those originating from HIF-1α activation, thereby explaining the additional effect of sildenafil over that of hypoxia alone.

The slight, nonsignificant hypoxia-induced bcl-2 upregulation at unaltered Bax expression observed in this study is consistent with data obtained in a study using rhabdomyosarcoma and Ewing sarcoma cells (34). That study reported that bcl-2 upregulation is independent of HIF-1α, as it appears here. The concomitant increase in TdT positivity in hypoxia alone indicates that apoptosis increases in a bcl-2–independent fashion. By contrast, sildenafil increased bcl-2 expression with only minor changes in Bax. The substantial reduction in TdT positivity by sildenafil indicates a critical role for bcl-2. As we are unaware of studies showing a direct effect of cGMP on bcl-2 or Bax, we examined the role of P-ERK1/2 and P-p38.

Hypoxia-induced ERK1/2 phosphorylation is consistent with high P-ERK1/2 levels in microvessels and astrocytes of neonatal rat brain exposed to 8% O_2 for 3 hrs (35), as well as in cultured neuroblastoma cells exposed to 0.1% O_2 for 24 hrs (36). Hypoxia-induced p38 phosphorylation is consistent with observations gathered in an *in vivo* model of focal cerebral ischemia, where P-p38 concentration increased in the ischemic striatum 24 hrs after cerebral artery occlusion (37). Phosphorylation of p38 is also a hallmark of hypoxia in murine cerebral microvascular

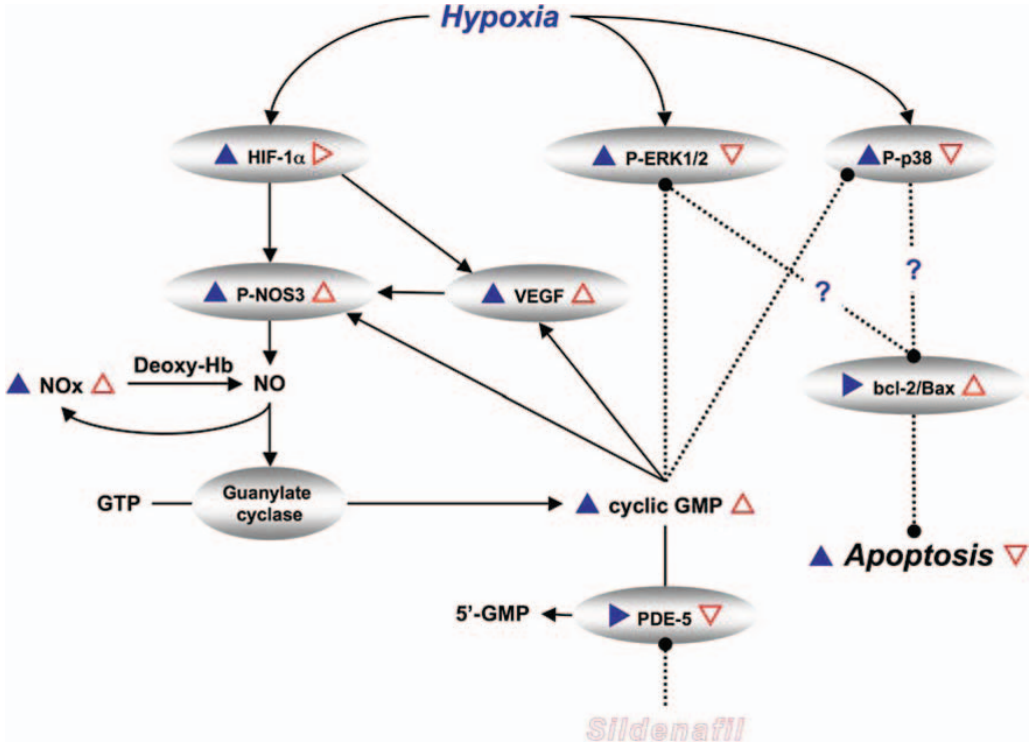


Figure 6. Proposed mechanism by which hypoxia and sildenafil differentially modulate apoptosis in neurons. The effects of hypoxia are blue on the left of the protein name by filled symbols (▲, ▼, and ▶ denote increase, decrease, and no effect, respectively). The effects of sildenafil are red on the right of the target protein by empty symbols (△, ▽, and ▷ denote increase, decrease, and no effect, respectively). Stimulatory and inhibitory effects are indicated with solid arrows (→) and dotted lines (···●), respectively. A color version of thsi figure is available in the the online journal.

endothelial cells (38) and in cultured cortical neurons (39). The level of P-p38 increases acutely (24 hrs at 10% O_2) in rats exposed to hypoxia (7), whereas chronic hypoxia maintains p38 in an activated state for up to 2 weeks (40).

Reduced ERK1/2 phosphorylation following PDE-5 inhibition is consistent with observations in pulmonary artery smooth muscle, whereby sildenafil administration inactivates ERK1/2 by a mechanism linked to increased activity of cGMP-dependent protein kinase G (41). Increased activity of cGMP-dependent protein kinase G was also shown to prevent p38 phosphorylation (42).

The effect of ERK1/2 and p38 phosphorylation on apoptosis is controversial. In one study, ERK1/2 activation was associated with bcl-2 upregulation and resistance to pharmacologically induced apoptosis (36). However, ERK1/2 phosphorylation contributes to cell death (43) through a mechanism involving caspase-3 activation, decreased inhibition of Akt, and reduced bcl-2/Bax ratio (44). Reduced Bax expression in sildenafil-treated mice may also be a consequence of Bax phosphorylation by P-p38, which causes mitochondrial translocation prior to apoptosis (45). As for p38, it can have both pro- and antiapoptotic effects, depending on the stressing stimulus and the upstream events leading to its activation. However, in cardiac myocytes, the axis leading to p38 activation through increased activity of cGMP-dependent protein kinase G appears to be a defense mechanism (46). A mass-spectrometry study identified p38 phosphorylation as a key event for bcl-2 phosphorylation and induction of apoptosis (47). Data reported in the present study seem to support the view that PDE-5 inhibition acts as an antiapoptotic agent through reduced p38 and ERK1/2 phosphorylation and raised antiapoptotic bcl-2 potential. It is likely that such a mechanism depends strictly on hypoxia severity and duration, as well as on the dose of sildenafil. In any case, the present study reveals that the signaling pathways stemming from chronic *in vivo* hypoxia and PDE-5 inhibition are synergistic in enhancement of the NO/cGMP mechanism, but diverge for their effect on apoptosis, with a potentially critical role played by ERK1/2 and p38.

Enhancing the NO/cGMP pathway during *in vivo* chronic hypoxia by administering sildenafil reduces hypoxia-induced neuron apoptosis. This effect was unrelated to the hypoxia-signaling pathway mediated by HIF-1α. Raising the level of cGMP upregulated the bcl-2/Bax ratio, probably through downregulation of the ERK1/2 and p38 paths. This mechanism may override that elicited by HIF-1α.

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