Inhibition of NGF deprivation–induced death by low oxygen involves suppression of BIM<sub>EL</sub> and activation of HIF-1

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Introduction

The availability of neurotrophic factors plays an important role in determining whether developing neurons live or die as they innervate their target tissues (Burek and Oppenheim, 1996). A well-characterized model for studying trophic factor dependency involves withdrawing NGF from dissociated sympathetic neurons maintained in cell culture (Martin et al., 1988; Deckwerth and Johnson, 1993; Edwards and Tolkovsky, 1994). NGF withdrawal activates the intrinsic apoptotic pathway characterized by release of cytochrome c from mitochondria and subsequent activation of caspases (Deckwerth and Johnson, 1998; Neame et al., 1998). Cytochrome c release is coordinated by members of the BCL-2 family, most notably BAX and BIM<sub>EL</sub> (Deckwerth et al., 1998; Putcha et al., 2001; Whitfield et al., 2001), which in turn are regulated by c-Jun NH<sub>2</sub>-terminal kinases (JNKs; Harris and Johnson, 2001; Whitfield et al., 2001; Lei and Davis, 2003; Putcha et al., 2003).

A number of reports have shown that reducing O<sub>2</sub> tension can significantly impact cell survival, yet the mechanisms underlying these effects are not well understood. Here, we report that maintaining sympathetic neurons under low O<sub>2</sub> inhibits apoptosis caused by NGF deprivation. Low O<sub>2</sub> exposure blocked cytochrome c release after NGF withdrawal, in part by suppressing the up-regulation of BIM<sub>EL</sub>. Forced BIM<sub>EL</sub> expression removed the block to cytochrome c release but did not prevent protection by low O<sub>2</sub>. Exposing neurons to low O<sub>2</sub> also activated hypoxia-inducible factor (HIF) and expression of a stabilized form of HIF-1α (HIF-1α<sub>PP</sub>-AG) inhibited cell death in normoxic, NGF-deprived cells. Targeted deletion of HIF-1α partially suppressed the protective effect of low O<sub>2</sub>, whereas deletion of HIF-1α combined with forced BIM<sub>EL</sub> expression completely reversed the ability of low O<sub>2</sub> to inhibit cell death. These data suggest a new model for how O<sub>2</sub> tension can influence apoptotic events that underlie trophic factor deprivation–induced cell death.

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Abbreviations used in this paper: BAF, boc-aspartyl(OMe)-fluoromethylketone; EGLN, egg-laying nine; ERK, extracellular signal-regulated kinase; HIF, hypoxia-inducible factor; HRE, hypoxia response element; JNK, c-Jun NH<sub>2</sub>-terminal kinase; MOI, multiplicity of infection.

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and transactivation of a large and diverse group of HIF-responsive genes (for review see Safran and Kaelin, 2003).

Here, we show that exposing sympathetic neurons to low O\textsubscript{2} during NGF deprivation significantly reduces their rate of cell death. Induction of BIM\textsubscript{EL} and loss of mitochondrial cytochrome c were both suppressed in NGF-deprived neurons exposed to low O\textsubscript{2}. Forced expression of BIM\textsubscript{EL} restored cytochrome c release but did not reverse the protective effect of low O\textsubscript{2}, suggesting that additional mechanisms were important for inhibiting cell death. Results from several experiments implicated HIF as a potential mediator of the neuroprotective effect of low O\textsubscript{2}. This was confirmed by microinjection experiments combining targeted deletion of HIF-1α with ectopic expression of BIM\textsubscript{EL} in the same neurons. These results provide a new model for how O\textsubscript{2} tension influences the apoptotic events that underlie trophic factor deprivation–induced death.

Results
Reducing O\textsubscript{2} inhibits death caused by NGF deprivation
To assess whether reducing O\textsubscript{2} tension affects the survival of sympathetic neurons, dissociated neurons that had been maintained in vitro for 5 d under standard culture conditions (i.e., 5% CO\textsubscript{2} and 95% air, equal to 20% O\textsubscript{2}) were refed with fresh NGF-containing media or deprived of NGF and immediately placed in incubators equilibrated to 20%, 2%, or 1% O\textsubscript{2}. Survival was assessed after staining cells with the DNA-binding dye Hoechst 33,342 and examining neuronal nuclei for evidence of chromatin condensation. Exposing NGF-maintained neurons to 1–2% O\textsubscript{2} for up to 4 d had no effect on their survival (unpublished data). On the other hand, low O\textsubscript{2} exposure markedly increased the survival of NGF-deprived neurons, with the greatest effect seen at 1% O\textsubscript{2} (Fig. 1, A and B). Under these conditions, 75% of neurons exhibited normal nuclear morphology as late as 48 h after NGF deprivation. Although slightly smaller, the cell soma also resembled those of NGF-maintained neurons, whereas the neuronal processes appeared less well protected. Based on these observations, we speculated that reducing O\textsubscript{2} might interfere with certain aspects of the cell death pathway known to be activated during trophic factor deprivation.

Low O\textsubscript{2} inhibits release of cytochrome c from mitochondria
Using immunofluorescence, we investigated the effect of low O\textsubscript{2} on the localization of cytochrome c after NGF withdrawal. Neurons maintained with NGF at 20% O\textsubscript{2} have a punctate, cytoplasmic distribution of cytochrome c immunofluorescence, indicative of its mitochondrial localization (Fig. 2, A and B). As seen by others (Deshmukh and Johnson, 1998; Neame et al., 1998), withdrawing NGF from these neurons results in an almost complete loss of punctate cytochrome c immunofluorescence. In contrast, the majority of neurons exposed to 1% O\textsubscript{2} during NGF deprivation retained punctate cytochrome c immunofluorescence similar to NGF-maintained neurons.

We also examined the effect of low O\textsubscript{2} and NGF withdrawal on cytochrome c protein levels detected by immunoblotting whole cell lysates. In a previous study, immunoblots of mitochondrial and cytosolic fractions prepared from NGF-deprived neurons revealed the expected decrease in mitochondrial cytochrome c, but not an accumulation of cytochrome c in the cytosolic fractions (Putcha et al., 2000). Consequently, immunoblotting cytochrome c from whole cell lysates yielded the same results as analyzing subcellular fractions, a procedure that requires obtaining neurons from many more animals. As shown in Fig. 2 C, total cytochrome c levels decreased in neurons deprived of NGF and exposed to 20% O\textsubscript{2}. However, there was no change in the level of cytochrome c in the NGF-deprived cells exposed to 1% O\textsubscript{2}. Thus, these data together with the immunofluorescence results described above indicate that the neuroprotective effect of reduced O\textsubscript{2} lies upstream to the loss of mitochondrial cytochrome c.

Induction of BIM\textsubscript{EL} after NGF withdrawal is suppressed by low O\textsubscript{2}
Because the BCL-2 family proteins BAX and BIM\textsubscript{EL} are critical for cytochrome c release after NGF withdrawal, we compared their protein levels before and after NGF withdrawal in neurons.
cultured under standard and reduced \(O_2\). We also analyzed expression of the pro-survival protein BCL-X\(_L\) and for changes in the phosphorylation of c-Jun. Phosphorylation of c-Jun increases in neurons deprived of NGF under standard culture conditions (Ham et al., 1995; Virdee et al., 1997) and we observed an essentially identical increase in neurons exposed to low \(O_2\) (Fig. 3 A). BAX and BCL-X\(_L\) protein levels remained unchanged after 20 h of NGF withdrawal, regardless of \(O_2\) tension. BIM\(_{EL}\) protein levels, on the other hand, increased an average of fourfold when neurons were deprived of NGF under standard \(O_2\) conditions; but when NGF deprivation was performed at 1% \(O_2\), a much smaller increase in BIM\(_{EL}\) was observed (Fig. 3, A and B; also see Fig. 4 B). This effect appeared to be specific because low \(O_2\) did not inhibit c-Jun phosphorylation or decrease the expression of any of the other proteins examined.

Recent studies have suggested that phosphorylation of BIM\(_{EL}\) by extracellular signal-regulated kinases (ERKs) is involved in targeting BIM\(_{EL}\) to the proteasome (Biswas and Greene, 2002; Ley et al., 2003). We considered the possibility that a similar mechanism might underlie the suppression of BIM\(_{EL}\) expression by low \(O_2\). Exposing control NGF-maintained neurons to 1% \(O_2\) for 20 h resulted in a small increase in phosphorylated ERK1/2 (Fig. 4 A, top). Nonetheless, phosphorylated ERK1/2 levels decreased similarly during NGF deprivation regardless of \(O_2\) tension (Fig. 4 A), suggesting that reducing \(O_2\) was not sufficient to stimulate ERK activation in sympathetic neurons in the absence of NGF signaling.

If low \(O_2\) exposure were to trigger proteasome-mediated destabilization of BIM\(_{EL}\), then inhibiting the proteasome might be expected to restore BIM\(_{EL}\) levels to those found in normoxic, NGF-deprived neurons. To test this possibility, neurons were deprived of NGF under 20% or 1% \(O_2\) in the presence or absence of the proteasome inhibitor MG132 and then analyzed by immunoblotting for changes in BIM\(_{EL}\) expression. As shown above, NGF deprivation resulted in increased BIM\(_{EL}\) levels only in normoxic neurons (Fig. 4 B, compare second lane with fifth lane). Treatment with MG132 did little to reverse the suppression of BIM\(_{EL}\) in neurons exposed to low \(O_2\) (Fig. 4 B, compare fifth lane with sixth lane). Together, these results indicate that the defect in BIM\(_{EL}\) induction during NGF deprivation performed at low \(O_2\) is not due to ERK-dependent targeting of BIM\(_{EL}\) to the proteasome.

Similar to BIM\(_{EL}\) protein, the increase in BIM\(_{EL}\) mRNA during NGF deprivation was also suppressed by low \(O_2\) (Fig. 4 C). Both c-Jun and the FOXO family member FKHRL1 have been implicated in up-regulating BIM\(_{EL}\) transcription in cells deprived of NGF (Sohn et al., 2003). To test whether c-Jun or FKHRL1 were involved in low \(O_2\)-induced BIM\(_{EL}\) induction, we examined the phosphorylation state of c-Jun during NGF deprivation under standard culture conditions (Fig. 3 A and B). Phosphorylation of c-Jun increased in neurons deprived of NGF under standard culture conditions (Ham et al., 1995; Virdee et al., 1997) and we observed an essentially identical increase in neurons exposed to low \(O_2\).
of NGF (Putcha et al., 2001; Harris and Johnson, 2001; Whitfield et al., 2001; Gilley et al., 2003). c-Jun is activated relatively soon after NGF withdrawal through JNK-dependent phosphorylation of Ser-63 (Virdee et al., 1997; Eilers et al., 1998). Activation of FKHR1 after NGF withdrawal involves the dephosphorylation of sites originally phosphorylated by Akt protein kinase (Zheng et al., 2002; Gilley et al., 2003). Thus, FKHR1 activation typically coincides with inactivation of Akt (Burgering and Kops, 2002).

To test whether the JNK/c-Jun and Akt/FKHR1 pathways might be altered in neurons kept under different O2 conditions, we immunoblotted c-Jun and Akt using antibodies specific for their phosphorylated and activated forms. In neurons maintained at 20% O2, the amount of phosphorylated c-Jun increased within 5 h of NGF removal and remained elevated for at least 20 h. Conversely, phosphorylated Akt levels decreased within 5 h of NGF deprivation and remained low (unpublished data). These same changes in c-Jun and Akt phosphorylation occurred when the experiment was performed on neurons exposed to 1% O2 (Fig. 4 D), suggesting that the reduced O2 tension used in these studies is unlikely to impact either c-Jun activation or Akt inactivation after NGF withdrawal.

We also examined whether low O2 might directly influence FKHR1 by altering its phosphorylation at Thr-32, a site targeted by Akt (Brunet et al., 1999). As shown previously (Gilley et al., 2003), neurons deprived of NGF under standard O2 conditions had reduced levels of Thr-32–phosphorylated FKHR1 compared with neurons maintained with NGF (Fig. 4 E). Unexpectedly, the amount of FKHR1 phosphorylated at this site did not decrease when NGF was withdrawn from neurons exposed to 1% O2, despite the concurrent decrease in Akt phosphorylation. These results suggest that activation of FKHR1 in response to NGF withdrawal might be inhibited in neurons exposed to reduced O2. Such a scenario, if corroborated, could help explain the suppression of BIMEL mRNA expression that occurs under these conditions (Gilley et al., 2003).

**Ectopic BIM**\(_{EL}\) reverses the ability of low O2 to block cytochrome c release but not cell death

The results described above suggest a model in which low O2 inhibits cell death by suppressing BIMEL induction, which in turn inhibits the release of cytochrome c from mitochondria and ultimately cell death. As a test of this idea, we infected neurons with a control adenovirus expressing EGFP or one expressing a BIMEL/EGFP fusion protein. The next day, the cells were deprived of NGF and switched to a 1% O2 environment for an additional 24 h, after which the cells were examined by immunofluorescence for cytochrome c localization. Parallel cultures were deprived of NGF for 48 h to assess the effects of BIMEL expression on cell survival. The majority of control EGFP-expressing neurons exposed to low O2 during NGF deprivation retained intense, punctate cytochrome c immunofluorescence (Fig. 5, A and B), similar to the results shown with uninfected neurons in Fig. 2. In contrast, very few of the NGF-deprived neurons exposed to low O2 and expressing BIMEL/EGFP retained punctate cytochrome c labeling. Despite their apparent lack of mitochondrial-localized cytochrome c, the majority of cells expressing BIMEL/EGFP remained healthy 48 h after withdrawing NGF (Fig. 5 C). Thus, whereas forced expression of BIMEL can overcome the block to cytochrome c release, there must be additional processes induced by low O2 (apparently downstream or independent of cytochrome c release) that can sustain cell survival in the absence of NGF.
Low O₂ exposure results in increased HIF activity. Because BIMEL expression failed to reverse the neuroprotective effect of low O₂, we wondered if activation of HIF might contribute to the enhanced survival seen under these conditions. In initial experiments, we infected neurons using an adenovirus that expresses a luciferase reporter gene under the control of four tandem hypoxia response elements (Ad-HRE-luciferase). Not surprisingly, NGF-maintained neurons exposed to 1% O₂ had increased HIF reporter gene activity compared with neurons kept under standard cell culture conditions (Fig. 6 A). NGF-deprived neurons exposed to 1% O₂ displayed a similar elevation in HRE-luciferase activity, demonstrating that low O₂-mediated activation of HIF in these neurons can occur in the absence of NGF signaling. HIF-1α protein levels also increased under the same conditions suggesting that at least part of the increase in HIF activity is due to HIF-1 (Fig. 6 B).

Stabilized HIF-1αPP→AG inhibits NGF deprivation-induced death

Having found that low O₂ activates endogenous HIF in the neurons, we wondered whether stabilization of HIF in the absence of low O₂ would be sufficient to inhibit trophic factor deprivation–induced cell death. A stabilized form of HIF-1α (HIF-1αPP→AG) was created by mutating the two proline residues that target HIF-1α for degradation to alanine and glycine. Transfection experiments in COS-7 cells done in the presence and absence of coexpressed EGLN prolyl hydroxylase demonstrated that HIF-1αPP→AG was active and relatively resistant to EGLN-mediated inactivation (Fig. SI A, available at http://www.jcb.org/cgi/content/full/jcb.200407079/DC1). In addition, sympathetic neurons coinfected with an adenovirus expressing HIF-1αPP→AG fused to EGFP (Ad-HIF-1αPP→AG/EGFP) and Ad-HRE-luciferase had substantially higher luciferase activities compared with cells coinfected with Ad-EGFP and Ad-HRE-luciferase (Fig. S1 B). To determine whether HIF-1αPP→AG can influence trophic factor deprivation–induced cell death, neurons were infected with Ad-HIF-1αPP→AG/EGFP or Ad-EGFP and the next day deprived of NGF. By 24 h of NGF deprivation, about half of the Ad-EGFP infected and uninfected neurons showed signs of chromatin condensation and nuclear fragmentation (Fig. 7, A and B). In contrast, the nuclei of nearly all HIF-1αPP→AG/EGFP

Figure 5. BIMEL expression promotes loss of mitochondrial cytochrome c but does not decrease survival in NGF-deprived neurons exposed to low O₂.

Neurons were infected with Ad-BIMEL/EGFP or Ad-EGFP adenoviruses (MOI = 100). The next day, NGF was withdrawn and the cells were immediately transferred to 1% O₂. (A and B) After 24 h of NGF deprivation, the cells were fixed and analyzed for immunofluorescence using anti-cytochrome c antibody. The three panels for each treatment are of the same field of view. Bar, 15 μm. The percentage of EGFP-expressing, BIMEL/EGFP-expressing, and control (uninfected) cells showing punctate cytochrome c immunofluorescence was determined (means ± SEM, n = 3). Arrows in A point to a neuron expressing BIMEL-EGFP (top), its Hoechst-stained nucleus (middle), and its lack of punctate cytochrome c immunofluorescence (bottom). (C) Neurons were treated as described above except that NGF deprivation was continued for 48 h. The cells were then stained with Hoechst dye and scored for viability (mean ± SEM, n = 3). Except in the case of uninfected cells, only neurons positive for BIMEL/EGFP or EGFP expression were scored in B and C.

Figure 6. Low O₂ exposure activates HIF in the presence or absence of NGF. (A) Neurons were infected with Ad-HRE-luciferase (MOI = 10) and 48 h later either continued in the presence of NGF or deprived of NGF for an additional 12 h at 20% or 1% O₂. Cells were then lysed and relative luciferase activities were determined (mean ± SEM of triplicate wells; similar results were obtained in a second independent experiment). (B) Neurons were either deprived of NGF and incubated at 1% O₂ or kept in the presence of NGF and cultured at 20% O₂. After 20 h, HIF-1α protein expression was analyzed by immunoprecipitation followed by immunoblotting with an anti-HIF-1α antibody.
Figure 7. HIF-1α<sub>PP→AG</sub> inhibits NGF deprivation-induced death by acting at a point downstream to cytochrome c release. Sympathetic neurons were infected with Ad-EGFP or Ad-HIF-1α<sub>PP→AG</sub>/EGFP virus (MOI = 100) or left uninfected. The next day, the cells were refed with fresh NGF-containing medium or with medium lacking NGF and cultured for an additional 24 or 48 h. (A) The images show Ad-EGFP and Ad-HIF-1α<sub>PP→AG</sub>/EGFP-infected neurons 24 h after NGF withdrawal. Top panels show EGFP or HIF-1α<sub>PP→AG</sub>/EGFP fluorescence, whereas the bottom panels show the corresponding Hoechst-stained nuclei in the same field of view (marked by arrowheads). Bar, 15 μm. (B) Cell survival (mean ± SEM, n = 3) was quantified in neurons expressing EGFP or HIF-1α<sub>PP→AG</sub>/EGFP using the criteria described in Materials and methods. (C) MTT assays were performed on infected cultures 24 and 48 h after NGF withdrawal. Data represent mean ± SEM (n = 3) and are reported as a percentage of the activity in uninfected NGF-maintained neurons. (D) Neurons were infected with Ad-HIF-1α<sub>PP→AG</sub>/EGFP as above. After an additional 20 h in the presence or absence of NGF, the cells were analyzed for cytochrome c immunofluorescence. The images show Ad-HIF-1α<sub>PP→AG</sub>/EGFP fluorescence (top), Hoechst-stained nuclei (middle), and cytochrome c immunofluorescence (bottom) for a single field of view for each treatment. Bar, 20 μm. (E) Neurons were infected with Ad-HIF-1α<sub>PP→AG</sub>/EGFP and then treated with or without NGF as outlined in D. A set of uninfected neurons was transferred to an incubator equilibrated to 1% O<sub>2</sub>, whereas a second set of uninfected cells and the cells infected with Ad-HIF-1α<sub>PP→AG</sub>/EGFP remained at 20% O<sub>2</sub>. After 20 h the cells were analyzed for cytochrome c immunofluorescence. Data represent the percentage of cells with punctate cytochrome c and are the mean ± SEM from three experiments. (F) Caspase assays were performed using the substrate DEVD-AFC and lysates prepared from uninfected neurons or neurons infected with Ad-HIF-1α<sub>PP→AG</sub>/EGFP or Ad-EGFP. NGF deprivation was for 24 h. Caspase activity is reported as relative fluorescence units/microgram protein (mean ± SEM, n = 3).

Expressing neurons appeared healthy, closely resembling those of NGF-maintained neurons. By 48 h, over 80% of neurons expressing HIF-1α<sub>PP→AG</sub>/EGFP remained healthy compared with just 25% of the control cells. Survival was also assessed using a MTT metabolic activity assay. NGF-deprived neurons infected with Ad-HIF-1α<sub>PP→AG</sub>/EGFP retained significantly more MTT metabolic activity than control neurons infected with Ad-EGFP. NGF deprivation was for 24 h. Caspase activity was reported as relative fluorescence units/microgram protein (mean ± SEM, n = 3).

**HIF-1α<sub>PP→AG</sub> does not block the loss of cytochrome c from mitochondria**

We next addressed whether the release of cytochrome c from mitochondria is inhibited in neurons expressing HIF-1α<sub>PP→AG</sub>. Cells infected with Ad-HIF-1α<sub>PP→AG</sub>/EGFP were maintained with or without NGF and analyzed by immunofluorescence for cytochrome c. In the presence or absence of NGF, cells expressing HIF-1α<sub>PP→AG</sub>/EGFP had the same pattern of cytochrome c immunofluorescence as control neurons—punctate and intense in the presence of NGF, diffuse and faint in its absence (Fig. 7, D and E). Despite the apparent loss of mitochondrial cytochrome c in neurons expressing HIF-1α<sub>PP→AG</sub> and deprived of NGF, caspase activity assays done using a DEVD-based fluorescent substrate showed that the activation of caspase-3–like enzymes during NGF deprivation was inhibited in cells infected with Ad-HIF-1α<sub>PP→AG</sub>/EGFP (Fig. 7 F). Together, these results suggest that HIF-1α<sub>PP→AG</sub>, in contrast to low O<sub>2</sub>, inhibits caspase activation and ultimately cell death without substantially blocking the loss of mitochondrial cytochrome c.

**Low O<sub>2</sub>-mediated protection is partially reversed by disrupting HIF-1α expression and completely abrogated when disruption of HIF-1α is combined with ectopic BIM<sub>EL</sub>**

The activation of HIF after exposure to low O<sub>2</sub> and the ability of mutant HIF-1α<sub>PP→AG</sub> to inhibit death after NGF withdrawal led us to test the significance of HIF-1α for the neuroprotective effect of low O<sub>2</sub>. For these experiments, we isolated sympathetic neurons from transgenic mice homozygous for a loxp flanked (floxed) HIF-1α gene (HIF-1α<sup>loxp</sup>; Ryan et al., 2000). Previous studies have shown that introducing Cre recombinase into HIF-1α<sup>loxp</sup> cells results in efficient deletion of the HIF-1α gene and loss of HIF-1-dependent gene induction (Ryan et al., 2000; Seagroves et al., 2001).

To determine whether expression of Cre recombinase in HIF-1α<sup>loxp</sup> sympathetic neurons results in a substantial loss of HIF-1α, we infected the neurons with an adenovirus that expresses Cre (Ad-Cre) or with the control Ad-EGFP virus. Immunofluorescence confirmed Cre expression in virtually all of
the cells by 24 h after infection (unpublished data). RT-PCR
analysis revealed greatly reduced HIF-1α expression specifically in Ad-Cre infected cultures (Fig. 8 A), suggesting that efficient deletion of the floxed HIF-1α gene had occurred. Loss of HIF-1α had no effect on the ability of NGF to promote survival for at least 4 d, and initial NGF deprivation experiments suggested that HIF-1α deletion did not significantly affect short-term protection afforded by low O2 as measured at 24 h after NGF withdrawal (unpublished data; see below).

Finally, we tested whether suppression of BIMel and activation of HIF-1 might both be important for the survival enhancing effect of low O2. Thus, we attempted to disrupt HIF-1α and overexpress BIMel in the same neurons. To ensure efficient coexpression of Cre and BIMel/EGFP, we microinjected HIF-1αfl/fl neurons with an equimolar mixture of Ad-Cre and Ad-BIMel/EGFP expression plasmids. Additional neurons were injected with each plasmid alone and with an EGFP expression plasmid. The next day the neurons were deprived of NGF and exposed to 1% O2. After 48 h, nearly 75% of EGFP-expressing neurons remained healthy, as judged by the appearance of their nuclei after staining with Hoechst dye (Fig. 8 B). In contrast, only ~30% of the Cre-injected neurons appeared healthy after 48 h, suggesting that HIF-1 is important for the longer-term (>24 h) neuroprotective effect of low O2. The most dramatic result was seen in neurons cojected with Cre and BIMel/EGFP. Virtually all of these cells had condensed or fragmented chromatin after 48 h of NGF deprivation. We conclude that both suppressing BIMel induction and activating HIF-1 are important for the survival enhancing effects of low O2 on trophic factor deprived neurons.

Discussion

Most cell culture studies on neuronal apoptosis are performed using neurons exposed to O2 concentrations well above those experienced in vivo. Because changes in O2 concentration can influence a wide range of biochemical processes, we decided to investigate the effects of reducing O2 on apoptotic mechanisms in a well-characterized model of neurotrophic factor deprivation. The results from this study support a model in which exposure to low O2 inhibits cell death caused by NGF withdrawal at two levels—one upstream of the loss of cytochrome c from mitochondria and a second that is downstream or independent of cytochrome c release. The block to cytochrome c release appears to be due at least partially to inadequate BIMel expression. Overexpressing BIMel reversed the block to cytochrome c release but still failed to override the protective effect of low O2, thus revealing a second place where low O2 acts to inhibit apoptosis.

Certain events triggered by NGF withdrawal were not affected by lowering O2. For example, dephosphorylation of ERKs and Akt and activation of JNKs (as judged by c-Jun phosphorylation) occurred similarly in neurons exposed to 20% or 1% O2. These findings differ from those obtained in models of ischemic and hypoxic preconditioning in which neuroprotection requires activation of Akt (Ruscher et al., 2002; Wick et al., 2002). In our paradigm, lowering the O2 concentration interrupted the apoptotic program subsequent to JNK activation, resulting in reduced BIMel expression. A similar situation has been reported in colon cancer cells, where O2 deprivation was found to down-regulate expression of the pro-apoptotic BCL-2 family protein BID (Erler et al., 2004).

Although the nature of the downstream block to cell death remains uncertain, several observations point to a role for HIF. First, HIF was activated in NGF-deprived neurons exposed to low O2. Second, introducing HIF-1αPP→AG mimicked the downstream block by inhibiting caspase-3 activation and cell death without preventing cytochrome c release. Third, whereas the neuroprotective effect of low O2 was partially reduced in neurons lacking HIF-1α, protection was completely lost in neurons expressing ectopic BIMel (to override the first block) and lacking HIF-1α.

How might low O2 inhibit caspase activation after cytochrome c release? One possibility is that neurons exposed to low oxygen may express or maintain higher levels of proteins capable of blocking caspase activation. For example, Potts et al. (2003) reported that down-regulation of the X-linked inhibitor of apoptosis protein (XIAP) was necessary for caspase activation and cell death after NGF withdrawal, even after cytochrome c was present in the cytoplasm. Thus, an increase in XIAP expression under reduced O2 conditions might be sufficient to inhibit trophic factor deprivation–induced death. A similar mechanism was recently suggested based on the induc-
tion of IAP-2 expression in oxygen-deprived kidney cells (Dong et al., 2003). Additional mechanisms for blocking cell death after cytochrome c release may involve down-regulation of Apaf-1 or downstream caspases (Jia et al., 2001; Devarajan et al., 2002). We are currently investigating whether the expression level of any of these proteins is influenced by lowering O2 tension. Although our efforts so far have focused on well-studied pro-survival and pro-apoptotic pathways, it is important to realize that reducing O2 tension will at some point influence numerous other biochemical processes. Cellular effects of low oxygen can include changes in ion channel function, re- 

active O2 species generation, and energy metabolism, as well as alterations in the activity of a large number of enzymes (Bickler and Donoho, 2002).

In cells maintained under standard culture conditions of 20% O2, HIF activity is kept at an extremely low level do to the highly efficient degradation of its α subunits. This occurs in large part because the O2-dependent enzymes responsible for targeting HIF-α subunits for ubiquitination (HIF prolyl hydroxylases) have a KM for O2 that is near its concentration in air (Hirsilä et al., 2003). Because HIF prolyl hydroxylase activity is reduced in cells at physiologic O2 tensions (Epstein et al., 2001), basal HIF activity in most cells in vivo should be substantially greater than in cells in culture. Given this scenario together with a neuroprotective role for HIF-1, down-regulating basal HIF activity may be an important part of the overall cell death program. How could inactivation of HIF-1 be ensured in neurons deprived of NGF? In a previous study, we showed that NGF deprivation results in an increase in the expression of the SM-20 gene (Lipscomb et al., 1999). Recently, SM-20 was found to be the rat orthologue of EGLN3, one of three HIF prolyl hydroxylases in mammals (Lipscomb et al., 2001). Thus, up-regulation of SM-20/EGLN3 could serve to quench an HIF-dependent neuroprotective pathway. At O2 tensions that are too low to support EGLN3 activity, this mechanism might be compromised resulting in sustained HIF activation and inhibition of cell death (Freeman et al., 2004).

In summary, basic cellular mechanisms controlling cell death and survival are generally characterized in cells maintained under supraphysiologic oxygen conditions. As shown here, some of these mechanisms can be influenced by reducing O2 tension. We believe these results highlight the importance of carefully defining the O2-sensitivity of mechanisms that determine cell viability.

Materials and methods

Materials

Cell culture reagents were purchased from Invitrogen Life Technologies. FBS and NGF were purchased from Harlan Bioproducts for Science. Boc-asparagine (Ome)-fluoromethylketone (BAF) was purchased from Enzyme Systems Products. MG132 was purchased from BIOMOL Research Laboratories, Inc. and Hoechst 33,342 was purchased from Molecular Probes. Additional reagents were purchased from Sigma-Aldrich unless otherwise indicated.

Cell culture

Primary cultures of sympathetic neurons were prepared from the superior cervical ganglia of embryonic day-21 Sprague-Dawley rats (Harlan) or newborn HIF-1α−/− mice (Ryan et al., 2000) as described elsewhere (Lipscomb et al., 1999). After preplating to enrich for neurons, cells were plated on collagen- or polyornithine/laminin-coated dishes in NGF-containing media (90% MEM, 10% FBS, 2 mM l-glutamine, 20 μM uridine, 20 μM fluoro deoxyuridine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 ng/ml NGF). For NGF deprivation, neurons were rinsed twice with PBS before addition of media lacking NGF and containing neutralizing anti-NGF antiserum. All treatments were initiated 5–6 d after plating. The caspase inhibitor BAF (50 μM) was included in the media for immunoblotting and cytochrome c immunofluorescence experiments. BAF treatment prevents terminal phases of cell death without blocking upstream events including JNK activation, BIMu up-regulation, and cytochrome c release (Freeman et al., 2003). Thus, up-regulation of SM-20/EGLN3 could serve to quench an HIF-dependent neuroprotective pathway. At O2 tensions that are too low to support EGLN3 activity, this mechanism might be compromised resulting in sustained HIF activation and inhibition of cell death (Freeman et al., 2004).

Assessing viability and caspase activity

After removal from the incubator, cells were quickly rinsed with PBS and then fixed in 4% PFA. The cultures were then stained with Hoechst 33,342 (1 μg/ml in PBS) and visualized under phase-contrast and epifluorescence using a 40× objective and a microscope (model Diaphot 300; Nikon). Viability was assessed based on the appearance of the neurons and the Hoechst-stained nuclei (Lipscomb et al., 2001). Healthy neurons had round, smooth, and refractile cell bodies with clearly discernible nuclear membranes and nucleoli, and diffuse Hoechst-stained chromatin. In contrast, dying or dead cells were characterized by condensed or unde-

tectable chromatin and fragmented and atrophied cell bodies. For ex-

periments with recombinant adenosviruses, 150–200 neurons expressing EGFP or HIF-1αPP/EGFP were scored for each treatment per experiment. MIT assays (Magyarir et al., 1998) and caspase-3 activity assays (Straub et al., 2003) were described previously.

Immunofluorescence

Immediately after removal from the incubator, cells were fixed in 4% PFA for 30 min at 4°C. Immunofluorescence was done as described previously (Lipscomb et al., 2001) using a 1:500 dilution of mouse anti–cytochrome c antibody (BD Biosciences) or a 1:1000 dilution of anti-Cre antibody followed by a TRITC-conjugated goat anti–mouse secondary antibody (Jack-son ImmunoResearch Laboratories) diluted 1:300. Cells were rinsed with PBS before staining with Hoechst 33,342. In all NGF-maintained neurons, the cytochrome c immunofluorescence pattern is punctate throughout the cytoplasm and in neuronal processes but excludes the nucleus. After 20 h of NGF deprivation done in the presence of BAF, all of the neurons exhib-

ited a faint fluorescence signal that was evenly distributed throughout the cytoplasm and nucleus. None of the cells showed an obvious partial loss of punctate staining. For each experiment, the pattern of cytochrome c imm-

unofluorescence was scored by an observer blinded to the experimental treatments. Digital images were captured using a microscope (model Dia-

phot 300; Nikon) equipped with a DAGE-MTI CCD camera and Scion Image software (Scion Corp.).

Immunoblotting

Cells were rinsed once with ice-cold PBS and solubilized in SDS-PAGE sample buffer immediately after removal from the incubator. Lysates were boiled for 10 min and centrifuged for 5 min at 12,000 g before separation by SDS-PAGE. Separated proteins were transferred to nitro-

cellulose membranes, which were blocked in TBS containing 5% nonfat dry milk and 0.1% Tween-20 (TBST) for 1 h at RT. Membranes were incubated overnight at 4°C in the same buffer containing the following pri-
mary antibodies at the indicated dilutions: anti–cytochrome c, 1:500 (BD Biosciences); anti-BIM, 1:1,000 (StressGen Biotechnologies); anti-

BAX, 1:1,000 (Upstate Biotechnology); anti–BCL-XL, 1:1,000 (Santa Cruz Biotechnology); anti–phospho-c-Jun (Ser-63), 1:1,000 (Cell Sig-

naling); anti–phospho-Akt (Ser-473), 1:1,000 (Cell Signaling); anti-Akt, 1:1,000 (Cell Signaling); anti–phospho-ERK1/2, 1:1,000 (Cell Signaling); anti-ERK1/2 (1:1,000); anti–phospho-JNK (Thr-183, Tyr-185), 1:1,000 (Cell Signaling); anti–phospho-p90RSK (Thr-358), 1:1,000 (Cell Signaling); anti-HIF-1α, 1:1,000 (Novus Biologicals). The blots were then incubated for 1 h with appropriate HRP-conjugated secondary antibodies diluted

In the expression of the SM-20 gene (Lipscomb et al., 1999). After preplating to enrich for neurons, cells were plated on collagen- or polyornithine/laminin-coated dishes in NGF-containing media (90% MEM, 10% FBS, 2 mM l-glutamine, 20 μM uridine, 20 μM fluoro deoxyuridine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 ng/ml NGF). For NGF deprivation, neurons were rinsed twice with PBS before addition of media lacking NGF and containing neutralizing anti-NGF antiserum. All treatments were initiated 5–6 d after plating. The caspase inhibitor BAF (50 μM) was included in the media for immunoblotting and cytochrome c immunofluorescence experiments. BAF treatment prevents terminal phases of cell death without blocking upstream events including JNK activation, BIMu up-regulation, and cytochrome c release (Freeman et al., 2003). Thus, up-regulation of SM-20/EGLN3 could serve to quench an HIF-dependent neuroprotective pathway. At O2 tensions that are too low to support EGLN3 activity, this mechanism might be compromised resulting in sustained HIF activation and inhibition of cell death (Freeman et al., 2004).

In summary, basic cellular mechanisms controlling cell death and survival are generally characterized in cells maintained under supraphysiologic oxygen conditions. As shown here, some of these mechanisms can be influenced by reducing O2 tension. We believe these results highlight the importance of carefully defining the O2-sensitivity of mechanisms that determine cell viability.
The HIF-1α protein, the ORF of human HIF-1α, was expressed in E. coli (Qiagen). Quantitative RT-PCR to analyze BIMEL and cyclophilin expression was performed as described previously (Lipscomb et al., 2001) using 2 μg of anti-HIF-1α antibody. Immunoprecipitated proteins were then immunoblotted using the same anti-HIF-1α antibody. For all immunoblotting experiments, the data shown are representative of the results from three or more independent experiments.

RT-PCR

Total RNA was extracted from equal numbers of neurons plated on collagen-coated 6-well dishes using an RNaseasy Min kit (Qiagen). Semi-quantitative RT-PCR to analyze BIM, and cyclophilin expression was performed as described previously (Estus et al., 1994). PCR products shown in Fig. 4 C were labeled by incorporation of α[32P]-dCTP over 21 and 17 cycles of amplification, respectively. HIF-1α and cyclophilin PCR products in Fig. 8 A were amplified over 30 and 25 cycles, respectively, and analyzed on agarose gels stained with ethidium bromide.

Plasmids and site-directed mutagenesis

The plasmid containing HRE-luciferase was a gift from G. Semenza (Johns Hopkins University, Baltimore, MD) and R. Ratan (Harvard Medical School, Boston, MA). For expression of HIF-1α and HIF-1α/EGFP fusion protein, the ORF of human HIF-1α was generated by PCR using Pfu Turbo DNA polymerase (Stratagene) and confirmed by DNA sequencing. The HIF-1α cDNA was subcloned into pcDNA3 and pEFGP-C1 (CLONTECH Laboratories, Inc.). Site-directed mutagenesis of HIF-1α and HIF-1α/EGFP was done using QuickChange (Stratagene) to change Pro-402 and Pro-364 to Ala and Gly. The resulting plasmids, HIF-1α[Pro-402→Ala] and HIF-1α[Pro-364→Gly]/EGFP, were verified by DNA sequencing.

Ectopic gene expression

Replication-deficient adenovirus vectors were produced using the AdEasy system (Qbiogene) following the manufacturer’s protocol. EGFP and HIF-1α[Pro-402→Ala]/EGFP were subcloned into pShuttle-CMV; the HRE-luciferase cassette was subcloned into pShuttle. Recombination between the pShuttle-derived plasmids and pAdEasy-1 was performed in BJ5183 adenoviral packaging cells. Recombinant replication-defective adenovirus was purified as described above. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200407079/DC1.

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