Prosurvival and Prodeath Effects of Hypoxia-inducible Factor-1α Stabilization in a Murine Hippocampal Cell Line*

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Hypoxia-inducible factor-1 (HIF-1) is a transcriptional activator involved in adaptation to hypoxic stress. Previous studies from our laboratory demonstrated that pharmacological activators of HIF-1 (e.g. deferoxamine, cobalt chloride) could also protect cultured primary neurons or an immortalized hippocampal neuroblast line (HT22) from oxidative stress-induced death. However, whether HIF-1 activation is sufficient to abrogate neuronal death resulting from oxidative stress or other hypoxia-independent death inducers remains unclear. To address this question we utilized a HIF-1α fusion protein that partially lacks the domain required for oxygen-dependent degradation of HIF-1α and that has a VP16 transcriptional activation domain from herpes simplex virus. HT22 cells were infected with a retrovirus encoding either the HIF-1α-VP16 fusion protein or the activation domain of the VP16 protein alone as a control. Expression of HIF-1α-VP16, but not VP16 alone, increased luciferase activity driven by a canonical hypoxia response element, increased mRNA of established HIF-1 target genes, and increased activity of one of these HIF-1 target genes. Unexpectedly, enhanced HIF-1 activity in HT22 cells enhanced sensitivity to oxidative death induced by glutathione depletion. Accordingly, suppression of HIF-1α expression using RNA interference rendered HT22 cells more resistant to DNA damage (induced by camptothecin) or endoplasmic reticulum stress (induced by thapsigargin and tunicamycin) than were VP16-expressing cells, and suppression of HIF-1α expression using RNA interference rendered HT22 cells more sensitive to death induced by DNA damage or endoplasmic reticulum stress. Together, these data demonstrate that HIF-1 can mediate prodeath or prosurvival responses in the same cell type depending on the injury stimulus.

Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric transcriptional activator that activates gene programs involved in hypoxic adaptation. It is composed of two subunits called HIF-1α and HIF-1β (1). Both subunits are expressed constitutively; however, HIF-1α is subject to ubiquitination and proteasomal degradation under normoxic conditions. The ubiquitination of HIF-1α is primarily regulated by a prolyl hydroxylase-catalyzed reaction (2). This reaction initially leads to hydroxylation of HIF-1α at proline 564 and proline 402 (3, 4). Hydroxylated HIF-1α recruits the ubiquitin-protein ligase, Von Hippel Landau (VHL) protein. VHL tags HIF-1α with ubiquitin and targets it for degradation (2, 5–7). Hydroxylation of critical residues on HIF-1α can be inhibited by hypoxia, iron chelators, and cobalt chloride (8, 9). With these treatments, HIF-1α is stabilized, it dimerizes with HIF-1β, and adaptive genetic responses involved in oxygen delivery, angiogenesis, and anaerobic glycolysis are activated (1, 2, 6, 10–13).

Several converging lines of inquiry argue that HIF-1 can mediate survival responses in the nervous system. First, activators of HIF-1 such as cobalt chloride and deferoxamine can prevent neuronal death due to oxidative stress and ischemia in vitro and in vivo (8, 14, 15). Second, HIF-1 target genes such as erythropoietin (EPO) and vascular endothelial growth factor (VEGF) have established neuroprotective properties. EPO has been shown to prevent neuronal death due to oxidative or nitrosative stress in vitro or to enhance recovery following stroke or spinal cord injury in vivo (16–20). Similarly, VEGF has been shown to protect motor neurons from growth factor deprivation and hydrogen peroxide treatment in vitro and ischemia in vivo (21–23). Moreover, conditional deletion of the hypoxia response element (HRE) in the VEGF gene leads to delayed motor neuron degeneration (24). Third, forced expression of HIF-1α can block death due to β-amyloid (25).

By contrast, HIF-1 also has been shown to be associated with prodeath responses in neurons. A dominant negative form of HIF-1α inhibits delayed neuronal death following hypoxia-ischemia in vitro (26). Additionally, expression of HIF-1-regulated “prodeath” BH3-only family members, such as BNIP3, has been shown to increase following cerebral ischemia (27–30). These apparently paradoxical results have stimulated efforts to define the factors that determine whether HIF-1 activation will mediate prodeath or prosurvival responses. Some have suggested that the cell type examined, the duration of the stimulus, and/or the nature of the stimulus can explain the

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disparate outcomes. Here we have shown that a cell line that constitutively expresses a form of HIF-1α resistant to oxygen degradation leads to increased or decreased resistance to cell death depending on the injury stimulus. These findings suggested that the nature of the injury stimulus is an important determinant of whether HIF-1 will be prodeath or prosurvival in a given cell type.

EXPERIMENTAL PROCEDURES

Plasmid and Retroviruses—A plasmid encoding a fusion protein of amino acids 1–529 of HIF-1α and the herpes simplex virus VP16 transactivation domain (pBABE-puro-HIF-1α-VP16) and a control plasmid encoding only VP16 (pBABE-puro-VP16) were described previously (31). Short interfering RNAs (siRNA) were cloned into the pSuperRetro vector (OligoEngine) under the control of polymerase-III H1-RNA gene promoter. SiRNAs that correspond to the HIF-1α promoter. SiRNAs that correspond to the HIF-1α were described previously (32). The following oligonucleotides were used: the siHIF-1α sense 5'-GATGCCCGTCTGATAGTCGAC-TCTGGATCTTCAAGAGGAAGATGTGAGCTCACATTTTTGGAAA-3' and the siHIF-1α antisense 5'-ACCTTGTTTCCCCAAAATCTGTGCTCACATCTTGATTCTTGGATCAAGTCGAGCGG-3'. The siVPH1 sequences. As a positive control, cells expressing the HIF-1α reporter were treated with 100 μM deferoxamine (DFO) overnight. All cell extracts were prepared and analyzed using the luciferase assay system (Promega, Madison, WI) according to the manufacturer's protocol. Luminescence was measured as a time point of total protein.

Nuclear Extracts and DNA Electrophoretic Mobility Shift Assay—Following infection or drug treatment, cells were scraped into cold phosphate-buffered saline, lysed, and assayed for lactate dehydrogenase (LDH) activity using a CytoTox96 nonradioactive cytotoxicity assay kit (Promega). Results were expressed as a relative light unit/mg of total protein.

Viability—Cell viability was assayed using a CellTitre 96 aqueous assay (Promega). Cells were plated in a 96-well plate at a density of 2.5×10^4 cells/ml in complete Dulbecco's modified Eagle's medium with 10% fetal bovine serum and incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO₂ for 24 h. Cells were washed with phosphate-buffered saline, lysed, and assayed for lactate dehydrogenase (LDH) activity using a CytoTox96 nonradioactive cytotoxicity assay kit (Promega). Results were expressed as a change in absorbance/mg of total protein.

Expression of Recombinant HIF-1α in Mouse Hippocampal Cell Line HT22—To evaluate the effect of HIF-1α stabilization on survival in response to oxidative stress in an HT22 cell line, we injected these cells with a retrovirus construct encoding the fusion protein containing amino acids 1–529 of HIF-1α and a VP16 transactivation domain or a retrovirus encoding the VP16 transactivation domain alone as a control. The HIF-1α-VP16 fusion, unlike native HIF-1α, should be stable under normoxic conditions because it lacks the prolyl residue at position 564, which is a target for oxygen-dependent hydroxylation and degradation. The fusion protein or the VP16 control was detected by Western blot in lysates from normoxic HT22 cells by using antibodies against HIF-1α or against VP16. Both antibodies under normoxic conditions recognized a band corresponding to the HIF-1α-VP16 protein, and it migrated to the predicted molecular mass of about 90 kDa in HT22 cells infected with the HIF-1α-VP16 retrovirus (not shown). These findings demonstrate that the HIF-1α fusion protein, unlike endogenous HIF-1α, is expressed under normoxic conditions.

To verify that the functional properties of the oxygen-stabilized, HIF-1α fusion protein are similar to endogenous HIF-1α, several experiments were performed. Four days after the growth of puromycin-selected HT22 transfomants, total RNA was isolated from control and infected cells 1 week following infection and with puromycin using the RNAasy mini kit (Qiagen) followed by in-column treatment with DNase (Qiagen). Reverse transcription reaction (30 min at 50 °C) and PCR amplification were carried out with a Reverse-IT™ one-step kit (Agdia, NY) in a thermal cycler PTC-100 (MJ Research, Inc.) with specific primers (Table I). Acidic ribosomal phosphoprotein and β-actin were monitored as housekeeping genes. Primer sequences utilized for VEGF A were described by Elson et al. (36). Primers for VP16, HIF-VP16, HIF-1α, enolase 1, and housekeeping genes were designed using the GeneWorks 2.5.1 program (Oxford Molecular Group Inc.). Total RNA at concentrations of 1, 10, and 50 ng was used for cDNA amplification to confirm the linear range of amplification. cDNA synthesis was carried out at 50 °C for 30 min, followed by a 4-min initial denaturation step and by 25 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min for VP16, HIF-1α-VP16, HIF-1α, β-actin, and PUMA primers. VEGF A, enolase 1, β-actin, and phosphoprotein were amplified in a two-step PCR reaction, at 94 °C for 30 s and 60 °C for 1 min.

Luciferase Assay—Stable cell lines expressing an HRE-luciferase reporter were obtained by transfection of HT22 cells with an enolase 1 promoter-reporter construct (33). Cells were transfected with a mix of luciferase reporter plasmid and pHyEGFP vector (BD Biosciences) in a 1:100 ratio using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After selection with hygromycin, cells were infected with retrovirus constructs containing either VP16 or HIF-1α-VP16 sequences. As a positive control, cells expressing the HIF-1α reporter were treated with 100 μM deferoxamine (DFO) overnight. All cell samples were prepared and analyzed using the luciferase assay system (Promega, Madison, WI) according to the manufacturer's protocol. Luminescence was measured as a time point of total protein.

Results

HIF-1α Stabilization Mediates Prodeath, Prosurvival Effects

RESULTS
RNA was isolated and expression of mRNA, which encodes the recombinant HIF-1α-VP16 protein in infected cells, was confirmed by semiquantitative RT-PCR. Two sets of primers were used. One set of primers (sense and antisense) was complementary to the VP16 transactivation domain; the second set of primers included a sense primer targeted to the HIF-1-α portion and an antisense primer to the VP16 portion (Table I and Fig. 1). RT-PCR assays verified that mRNA expression of each heterologous protein, VP16 or HIF-1α-VP16, was expressed at similar levels in each cell line generated. By contrast, the level of endogenous HIF-1α mRNA was not altered by expression of either recombinant protein (Fig. 1). The heterologous, oxygen-stabilized HIF-1α thus does not alter expression of endogenous HIF-1α.

The DNA binding properties of the recombinant HIF-1α-VP16 fusion protein were verified by electrophoretic mobility shift assays using an oligonucleotide with a canonical HIF-1 binding site (Fig. 2). Under basal, normoxic conditions, electrophoretic mobility shift assays of nuclear extracts from wild type HT22 cells or VP16 HT22 cells show two DNA binding complexes (Fig. 2, c and d). Supershift analysis demonstrated that these complexes contain ATF-1 and CREB (not shown). In response to DFO, an agent known to enhance binding of HIF-1-α and ATF-1/CREB under normoxic conditions, HT22 cells show the expected increase in DNA binding of the ATF-1/CREB binding complexes (c and d, lanes 2, 3, 9–11) (8) as well as another, slower migrating complex designated “b”. Note that in lanes 3 and 10, complex b is supershifted by an antibody to HIF-1α (supershifted complex designated a) indicating that complex b contains HIF-1α. A distinct, slower migrating complex, h, was detected in nuclear extracts from normoxic HT22 cells that stably express the HIF-1α-VP16 fusion protein (Fig. 2, lanes 5 and 9) but not from cells expressing the VP16 protein alone (Fig. 2, lane 4). This complex could be supershifted by a HIF-1α antibody (Fig. 2, lane 6) or a VP16 antibody (Fig. 2, lane 7), confirming that it contains HIF-1α-VP16. Of note, the level of oxygen-stabilized HIF-1α-VP16 protein was higher in DFO-treated HIF-1α-VP16 HT22 cells (compare lanes 5 and 9). The level of endogenous HIF-1α after the DFO treatment was comparable with that in controls, both in non-infected HT22 cells (lane 2) and VP16 HT22 cells (not shown). These data demonstrate that the HIF-1α-VP16 fusion can bind appropriately to the hypoxia response element under normoxic conditions. Furthermore, we have shown that the expression of this protein does not alter the basal or induced DNA binding of endogenous HIF-1α.

Expression of the HIF-1α-VP16 Fusion Protein in HT22 Cells Can Induce HIF-dependent Target Gene Expression—The ability of the HIF-1α-VP16 fusion protein to activate expression of established HIF-1-dependent target genes was confirmed using a HIF-1 luciferase-reporter assay as well as RT-PCR and immunoblotting of established HIF-1 target genes. For the reporter assay, we used a luciferase reporter construct that contained the HRE (68 bp) from the enolase 1 promoter (HRE-luc). HT22 cells stably transfected with HRE-luc-encoding plasmid were infected either with VP16 (control) or with HIF-1α-VP16. After selection with puromycin, cells were plated onto a 96-well plate and used for the luciferase activity assay. As a positive control, VP16-infected cells were treated with 100 μM DFO. Cells treated with DFO showed an expected 2–3-fold increase in HRE-luciferase activity (data not shown), whereas the cells expressing HIF-1α-VP16 had an approximately 60-fold increase in luciferase activity (*, p < 0.05, Fig. 3A). To determine whether changes in reporter expression induced by HIF-1α-VP16 are associated with changes in message of established HIF-1 target genes, we examined by RT-PCR enolase 1, vascular endothelial growth factor A (VEGF A), BNIP 3, Puma, and Noxa. Indeed, the message for all of these genes, but not β-actin (which does not have an HRE in its promoter), increased in HIF-1α-VP16-infected HT22 cells compared with VP16 alone (Fig. 3B). The increases were similar to those seen in the VP16-infected cells treated with DFO. In all cases examined (VEGF, enolase 1, and BNIP3, NIX, and PUMA), changes in message were correlated with changes in protein (not shown). Moreover, changes in activity of one of the established HIF-1-regulated genes, LDH, also was validated. Cells expressing the HIF-1α-VP16 viral construct had a 2-fold increase in LDH activity (*, p < 0.05, Fig. 3C) compared with cells expressing VP16 alone. Taken together, these data demonstrate that HIF-1α-VP16 expression can induce the transcriptional up-regulation of HIF-1-dependent genes and the activity of one of these genes under normoxic conditions in immortalized murine HT22 hippocampal neuroblasts.

Table I

| Primer name | Forward, 5′- | Reverse, 5′- |
|-------------|-------------|-------------|
| VP16        | TAGACGATTTTCGATCTGG | ACCGTACTCGTCAATTCC |
| HIF-1α      | TCACCTGACCAATATGCCC | ACCGTACTCGTCAATTCC |
| VEGF A      | GACATAGCTTCGACGAGAAGTC | TCGTAACTGGTGCTGTTGG |
| Enolase     | CTGTCGACCGTCGCTGAAAG | GCTTACCTCTCTATGGCTGGC |
| BNIP3       | ATGGAAATCTGTGCGAGAAGGCGGGA | CAGGCAACATCGATGGTGATGGG |
| NIX         | AGGACATAGCTGCAATGGTGAGT | GCCGAAATTCTCAGAAGGGTCTAGGAAAGT |
| PUMA        | CTGTAGAGATATCGAGCCG | AATTTCCAGGGGCTTTTCGCTC |
| β-Actin     | CTTCACTGAGATATCGAGCCG | CCTCAACCGCTCCCTGTCACCAG |
| PO          | TGGTGTCCCAGAAGAGGACC | TGCAAATAGGATGACCTGG |

FIG. 1. Expression of mRNAs encoding recombinant proteins in cells infected with retroviral constructs. Cells were infected with retroviruses encoding VP16 or the recombinant fusion protein HIF1α-VP16. Total RNA was collected following selection with puromycin. RT-PCR using specific primers determined the level of mRNA for VP16, for HIF1α-VP16, or for the C-terminal part of murine HIF-1α (only expressed in the endogenous HIF-1α). Phosphoriboprotein (PO) was monitored as a housekeeping gene.
activate HIF-1-dependent transcription in primary neurons and hippocampal cell lines (8). The HIF-1α-VP16 line provides an experimental paradigm in which to determine whether HIF-1 activation is sufficient to prevent oxidative stress-induced death in HT22 cells. Oxidative stress was induced in VP16 or HIF-1α-VP16 lines by continuous exposure of these cells to the excitatory amino acid, glutamate (2–20 mM). Glutamate induces concentration-dependent, oxidative stress-induced death with features of apoptosis (37, 38). Oxidative stress results from the ability of glutamate to competitively inhibit the transport of cystine into the cell via its plasma membrane transporter. Inhibition of cystine transport leads to deprivation of intracellular cysteine, the rate-limiting precursor in the synthesis of glutathione. Depletion of glutathione resulting from cellular cysteine deprivation leads to an imbalance between oxidants and antioxidants in favor of oxidants, and cell death ensues. Indeed, glutamate-induced death of HT22 cells can be completely abrogated by classical antioxidants such as vitamin E and N-acetylcysteine (49). Overnight treatment with 5 mM glutamate results in morphological changes and decreases in density consistent with apoptotic cell death in HT22 cells expressing VP16. Population assays revealed that 60 ± 3% of cells were dead. By contrast, overnight treatment of HT22 cells expressing HIF-1α-VP16 led to the death of 88 ± 1% of the cells (p < 0.05). Consistent with these population measurements, decreases in cell viability could be observed much sooner in HIF-1α-VP16 cells than the cells expressing VP16 alone (Fig. 4A). The potentiation of glutamate-induced death by HIF-1α-VP16 could be observed at a range of glutamate concentrations (Fig. 4B).

In culturing and plating the VP16- and HIF-1α-VP16-expressing HT22 cells, we noted that the HIF-1α-VP16-expressing cells divide more slowly and require passage nearly half as often as their sister cell lines that express VP16 alone. These changes in cell number were attributable to differences in cell growth rates resulting from cellular cysteine deprivation leads to an imbalance between oxidants and antioxidants in favor of oxidants, and cell death ensues. Indeed, glutamate-induced death of HT22 cells can be completely abrogated by classical antioxidants such as vitamin E and N-acetylcysteine (49). Overnight treatment with 5 mM glutamate results in morphological changes and decreases in density consistent with apoptotic cell death in HT22 cells expressing VP16. Population assays revealed that 60 ± 3% of cells were dead. By contrast, overnight treatment of HT22 cells expressing HIF-1α-VP16 led to the death of 88 ± 1% of the cells (p < 0.05). Consistent with these population measurements, decreases in cell viability could be observed much sooner in HIF-1α-VP16 cells than the cells expressing VP16 alone (Fig. 4A). The potentiation of glutamate-induced death by HIF-1α-VP16 could be observed at a range of glutamate concentrations (Fig. 4B).

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Despite the similar densities, HIF-1

initial plating numbers allowed us to achieve a similar density

compared with VP16-expressing HT22 cells at a range of concentrations (Fig. 5A). However, expression of the

generated and selected with puromycin. Expression of the hpGFP resulted in no change in HIF-1 message or protein expression compared with controls (Fig. 5A). However, expression of the hpHIF-1α, as expected, reduced HIF-1α message (Fig. 5A) and protein expression (Fig. 5B) compared with HT22 cell pools expressing hpGFP. Consistent with increased sensitivity of HT22 cells overexpressing HIF-1α-VP16 to glutamate, we found that HT22 cells with reduced endogenous HIF-1α message are more resistant to glutamate-induced cytotoxicity than those expressing hpGFP at a range of concentrations (Fig. 5C). The resistance of these cells to glutamate toxicity is likely because of a reduction in HIF-1α levels and not reduction in the levels of another protein or activation of the double-stranded RNA kinase, PKR, as the resistance could be reversed by forcing expression of HIF-1α-VP16, but not VP16 alone (Fig. 5D). Taken together, these results suggest that HIF-1α is a prodeath protein in HT22 hippocampal neuroblasts exposed to glutamate.

HIF-1α Can Function as a Prodeath or Antideath Protein in the Same Cell Type, depending on the Nature of the Death Stimulus—To evaluate whether HIF-1α is a general prodeath protein in HT22 cells, we next examined the effect of HIF-1α stabilization on other established prodeath stimuli. Camptothecin is a topoisomerase inhibitor that is known to induce DNA damage and apoptosis via p53 and cyclin-dependent kinase-mediated pathways (41). In contrast to the increased sensitivity of HIF-1α-VP16-overexpressing HT22 cells to glutamate, these cells were more resistant to cell death induced by camptothecin at a range of concentrations (Fig. 6A). Similar prosurvival effects of HIF-1α-VP16 were observed in HT22 cells exposed to tunicamycin (2 μM) or thapsigargin (3 μM) (Fig. 6B). In accordance with a protective role for HIF-1α in each of these paradigms, reduction of HIF-1α expression by RNA interference potentiated death induced by camptothecin, thapsigargin, or tunicamycin (Fig. 6, B, D, and F). By inhibiting glycosylation or the endoplasmic reticulum (ER) calcium-dependent ATPase, respectively, both of these agents disrupt processing of protein in the ER (42, 43). The accumulation of proteins in the ER leads to a stress response that includes the up-regulation of the putative prodeath protein Chop (44, 45). Indeed, prolonged ER stress ultimately triggers cell death. The ability of forced expression of HIF-1α to abrogate ER stress-induced death is intriguing given the established role of ER stress in hypoxic-ischemic-induced death in the central nervous system.

To begin to understand how glutamate-induced oxidative death differs mechanistically from camptothecin-induced or ER stress-induced death in HT22 cells, we examined the effect of antioxidants on each of these paradigms. As expected, we found that the structurally distinct antioxidants butylated hydroxylated anisole (10 μM) or N-acetylcysteine (100 μM) completely abrogated glutamate-induced death (48). By contrast, neither of these antioxidants could inhibit death of HT22 cells induced by camptothecin, thapsigargin, or tunicamycin (not shown). These findings identify a feature of the injury stimulus that may determine whether HIF-1 mediates prodeath or prosurvival responses.
FIG. 5. Knock down of HIF-1α in HT22 cells prevents oxidative glutamate toxicity. A, HT22 cells were infected with a retrovirus containing hpGFP (siGFP) or hpHIF-1α (siHIF) and selected for 1 week using puromycin. RT-PCR for HIF-1α or GFP was performed using primers described in Table I. B, observed reductions in message were associated with corresponding reductions in HIF-1α protein. Nuclear extracts from HT22 cells: lane 1, uninfected HT22 cells; lane 2, uninfected HT22 cells treated with 100 μM DFO (a pharmacological activator of HIF-1α expression). 100 μM DFO treatment of HT22 cells infected with: lane 3, siHIF, m.o.i. = 4; lane 4, siHIF, m.o.i. = 8; lane 5, siHIF, m.o.i. = 10; lane 6, siGFP, m.o.i. = 6. Immunoblot of nuclear extracts with an HIF-1α antibody reveals a specific decrease in DFO-stimulated HIF-1α protein levels in cells infected with the HIF-1α siRNA (lanes 3–5), but not the GFP siRNA (lane 6). Note that the nuclear protein Sp1 is unaffected by the siHIF or siGFP. C, percent cell viability of HT22 cells expressing siGFP, siHIF, VP16, or HIF-1α-VP16 24 h following the addition of glutamate (in mM). Note that reduction of HIF-1α levels is associated with resistance to glutamate toxicity, whereas forced expression of HIF-1α-VP16 is associated with enhanced sensitivity to glutamate. p < 0.01 at 5, 7.5, 10, and 15 mM glutamate, analysis of variance followed by Student-Newman-Keuls test. D, forced expression of human HIF-1α-VP16 in cells expressing siRNA to mouse HIF-1α restores sensitivity to glutamate (results are mean ± S.E. for three experiments performed in triplicate for HT22 cells treated with 15 mM glutamate for 12–16 h; p < 0.05 by analysis of variance followed by Student-Newman-Keuls test).

DISCUSSION

Prior studies from our laboratory showed that pharmacological activators of HIF-1α abrogate glutamate-induced oxidative death in primary neurons and neuronal cell lines (8). The current study was undertaken to determine whether HIF-1α is sufficient and/or necessary for protection from cell death induced by oxidative stress. We addressed this question by forcing expression of an oxygen-stabilized form of HIF-1α (HIF-1α-VP16) in a murine HT22 hippocampal cell line (Fig. 1). We verified that this construct induces the expression of established HIF-1-dependent genes without altering the regulation of endogenous HIF-1α (Figs. 1–3). Contrary to our expectations, we found that enhanced HIF-1 activity potentiated oxidative neuronal death (Fig. 4). Three observations suggested that potentiation of death by HIF-1α-VP16 appears to reflect a HIF-1-dependent physiological response and is not an artifact of attaching a VP16 transcriptional activation domain to HIF-1α or expressing non-physiological levels of HIF-1α. First, expression of VP16 alone does not potentiate oxidative stress-induced death (Fig. 4). Second, suppression of endogenous HIF-1α expression by RNA interference completely abrogates oxidative glutamate toxicity (Fig. 5B). Third, expression of HIF-1α-VP16 can restore sensitivity of HT22 cells with diminished endogenous levels of HIF-1α to glutamate (Fig. 5C). As previous studies from our laboratory showed that oxidative stress in cortical neurons is not sufficient to activate HIF-1α (8), the findings suggested that basal HIF-1α levels regulate sensitivity to oxidative glutamate toxicity.

The established ability of HIF-1α to regulate expression of genes that enhance sensitivity to cell death may provide mechanistic insight into the findings presented herein. Bruick (46) showed that HIF-1α can regulate the expression of BNIP3, a prodeath member of the Bcl-2 family of proteins. Indeed, we found that HIF-1α-VP16 leads to a stable up-regulation of BNIP3 at a message and protein level (Fig. 3 and data not shown). Previous studies have shown that BNIP3 expression is not sufficient to induce death in the absence of a secondary stress such as acidosis (47). Our findings that basal viability of HIF-1α-VP16-expressing HT22 cells is indistinguishable from those expressing VP16 despite the expression of BNIP3 in the former supports the notion that BNIP3 expression is not sufficient to induce cell death. The findings also suggest that oxidative stress may be added to the list of secondary stresses that permit BNIP3 to manifest its prodeath effects. Future studies will clarify whether potentiation of oxidative glutamate toxicity by HIF-1α in HT22 cells requires BNIP3 expression. Of course, it is possible that other putative prodeath genes regulated by HIF-1α, including NIX, Puma, Noxa, or 12-lipoxygenase, may act alone or together to mediate potentiation of glutamate-induced death by HIF-1α.

Our findings that forced expression of HIF-1α can act to potentiate oxidative glutamate toxicity must be reconciled with our prior observations that pharmacological activators of HIF-1α target HIF-prolyl hydroxylases prevent oxidative neuronal death. Because prolyl hydroxylase inhibition results in the stabilization of proteins other than HIF-1α, including IRP2 and RNA polymerase II, or the increased DNA binding of other transcription factors (CREB) (8), it is possible that increased levels of these other proteins are required to manifest the prosurvival effects of HIF-1α. It is also possible that the cytoprotective effects of prolyl hydroxylase inhibitors occur independent of HIF-1α.

HIF-1α is an established regulator of prosurvival or prodeath responses in the nervous system. Indeed, although HIF-1α is best known as a transcriptional activator that positively regulates the expression of genes (erythropoietin, VEGF) that com-
pensate for hypoxia and/or aglycemia, recent studies have highlighted the ability of this transcription factor to activate putative prodeath genes such as BNIP3, Puma, and Noxa (17, 20, 23, 48). The factors that determine whether HIF-1 will enhance or suppress neuronal survival are unclear. Here we have demonstrated that in HT22 hippocampal neuroblasts, the injury stimulus is important in determining whether HIF-1 activation will mediate prosurvival or prodeath effects. The ability of antioxidants to abrogate oxidative glutamate toxicity in HT22 cells, but not death induced by DNA damage or ER stress, raises the possibility that the cellular redox state determines which of its Janus faces that HIF-1 will reveal. Future studies will define the precise signaling pathways that encourage survival of death in the context of increased HIF-1 activity.

**Fig. 6.** HIF-1α confers resistance to DNA damage or ER stress in HT22 cells. HT22 cell pools stably expressing VP16 alone or HIF-1α-VP16 were plated in a 96-well plate at a density of 5 × 10⁴ cells/ml and treated 24 h after plating. Cell viability was assessed 24 h after addition of camptothecin, thapsigargin, or tunicamycin. A, cell viability of VP16 or HIF-1α-VP16-expressing HT22 cells exposed to the DNA damaging agent camptothecin for 24 h. Results are the mean ± S.E. for three independent experiments performed in triplicate. B, cell viability of HT22 cell pools expressing siHIF or siGFP exposed to camptothecin (μM) for 24 h. Note that forced HIF-1 expression prevents camptothecin-induced death, whereas reductions in HIF-1α levels potentiate camptothecin-induced death. C and E, cell viability of VP16- or HIF-1α-VP16-expressing HT22 cells exposed to the ER stressors tunicamycin (0–10 μM) or thapsigargin (0–10 μM) for 24 h. *, p <0.05 by t test. The viability of untreated cells is taken as 100%. D and F, cell viability of HT22 cell pools expressing siHIF or siGFP exposed to tunicamycin (0–10 μM) or thapsigargin (0–10 μM). Forced expression of HIF-1α abrogates ER stress-induced death, whereas suppression of HIF-1α expression by RNA interference potentiates ER stress-induced death.
therapy where the prodeath functions of HIF-1 must be augmented.

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