Endoplasmic Reticulum (ER) Stress and Hypoxia Response Pathways Interact to Potentiate Hypoxia-inducible Factor 1 (HIF-1) Transcriptional Activity on Targets Like Vascular Endothelial Growth Factor (VEGF)*

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Background: The UPR and HIF signaling pathways are cytoprotective responses activated by inadequate cellular environments.

Results: These pathways can cooperate to increase the expression of shared targets like VEGF.

Conclusion: The UPR enhances HIF-1 transcriptional activity.

Significance: The simultaneous activation of both pathways in tumors may result in greater vascularization.

Cells respond to suboptimal microenvironments by activating stress signaling pathways, like the unfolded protein response and hypoxia-induced transcription factors HIF-1/2, to restore homeostasis. Both cytoprotective pathways have been well studied in isolation at the biochemical and molecular levels. Mounting evidence reveals that they can be activated simultaneously in tumor cells and, likely, in other tissues experiencing inadequate microenvironments and that they share some transcriptional targets, like the proangiogenic factor VEGFA. However, the potential interaction between these pathways is poorly understood. Cell culture experiments revealed that as a consequence of unfolded protein response activation, ATF4 bound to the human VEGFA promoter and activated its transcription, whereas HIF-1 did so in response to hypoxia. When both pathways were activated together, VEGFA transcripts were induced to a higher level than when either stress was applied alone. Surprisingly, this was not due to the combined actions of the stress pathway-specific transcription factors. Instead, we found that endoplasmic reticulum stress potentiated HIF-1 activity to transactivate VEGF expression as well as another well characterized target, BNIP3. These data reveal an unexpected interaction between two important cytoprotective responses that are likely to have significant consequences in environmentally compromised tissues and tumor cells.

When cells experience inadequate oxygen or nutrients because of limitations in their blood supply, signal transduction pathways are activated, leading to the secretion of factors that induce the production of new blood vessels from surrounding endothelial cells in a process known as angiogenesis (1, 2). Although angiogenesis is a normal physiological process, it is particularly critical for the survival of tumor cells because of their rapid proliferation and high metabolic demands. The dynamic nature of the tumor vasculature and the proximity of cells to a blood vessel give rise to variations in the supply of oxygen, resulting in regions in a solid tumor ranging from extremely low oxygen levels (anoxia-hypoxia) to normal levels (normoxia) (2). The poorly oxygenated tumor cells adapt to this limiting environment by activating the hypoxia-inducible factor (HIF)2 response. The HIF-1 and 2 transcription factors are comprised of a unique oxygen-labile α subunit and a shared, constitutively expressed β subunit (3). During normoxic conditions, the α subunit is rapidly degraded by the von Hippel-Lindau E3 ubiquitin ligase (3, 4), but when oxygen is limiting, the α subunit is stabilized, allowing it to dimerize with its counterpart, HIF-1β, to form an active transcription factor. Its targets regulate systemic responses like angiogenesis and erythropoiesis as well as cellular responses, like glucose and energy metabolism (5). VEGFA is a well known HIF-1α target that is up-regulated under hypoxic conditions in solid tumors and is one of the most potent proangiogenic factors required for neo-vascularization of solid tumors.

There is mounting evidence that a second stress signaling pathway, the unfolded protein response (UPR), is activated in multiple types of tumors because of their high metabolic rates, as well as limiting supplies of glucose and extreme decreases in oxygen availability (6). These conditions adversely affect normal protein folding in the endoplasmic reticulum (ER), leading to the accumulation of unfolded proteins that activate the response. The UPR protects cells via a complex transcriptional and posttranscriptional response that is mediated by three sig-

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2 The abbreviations used are: HIF, hypoxia-inducible factor; UPR, unfolded protein response; ER, endoplasmic reticulum; hnRNA, heteronuclear RNA; PERK, PKR-like ER kinase; CHOP, C/EBP homologous protein.
nal transducers; Ire1, PERK, and ATF6 (7). Most recently, the UPR has been shown to control the expression of both positive and negative regulators of angiogenesis in cell culture models (8, 9) as well as in animal studies (10), with a particular focus on VEGFA. Data obtained with mouse embryonic fibroblasts, which lacked various components of the UPR signaling pathway, argued that multiple branches of the response appeared to play a role in the transcriptional up-regulation of this proangiogenic factor (8, 9). However, because the wild-type counterparts of the various deficient mouse lines had very different basal and stress-inducible levels of VEGF expression, it was not possible to determine the relative contribution of each component to the up-regulation of VEGF.

Despite the fact that cells with an inadequate blood supply might be expected to activate both pathways simultaneously, the molecular interaction between these pathways on a shared target like VEGF has not been determined. Thus, our study of the UPR in regulating VEGF transcription in human tumor cells; second, to assess the effects on VEGF expression when both stresses were applied simultaneously to cells; and third, to determine the relative contribution of each pathway under these conditions. We report that although XBP-1(S) and ATF4 both bind to the human VEGFA promoter in a stress-inducible manner, ATF4 was found to be the primary regulator of UPR-induced VEGF mRNA expression in two human neuroblastoma cell lines. Furthermore, we demonstrated that the HIF and UPR pathways cooperated to induce greater VEGF expression than was observed when either pathway was activated alone. However, unexpectedly, this induction occurred primarily via UPR-induced potentiation of HIF-1 transcriptional activity, an observation that was extended to an additional HIF target, BNIP3. We report that the UPR enhances the phosphorylation of HIF-1α, which has been shown previously to enhance its activity (11).

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The human neuroblastoma cell lines NB1691 and SK-N-AS were cultured as described previously (12). Low-glucose conditions were achieved by culturing cells in RPMI medium containing 1 mM glucose. For hypoxia experiments, cells were grown in 10% serum, and when 60–70% confluent, the cells were transferred to a hypoxia chamber (In Vivo2 300, Ruskin) with conditions of 1% oxygen and 10% carbon dioxide at 37 °C.

**Western Blot Analysis**—Cell lysates were prepared as described previously (8). Proteins were separated by reducing SDS-PAGE, transferred to a PVDF membrane, and probed with the indicated primary antibodies. Rabbit anti-ATF4, goat anti-Hsc70, rabbit anti-XBP-1(S), and goat anti-Lamin B1 were purchased from Santa Cruz Biotechnology, and mouse anti-HIF-1α was from BD Biosciences. The blots were incubated with the appropriate HRP-conjugated secondary antibody and developed using the Pierce enhanced chemiluminescent substrate (Thermo Scientific).

**Total mRNA and hnRNA Quantification by Quantitative RT-PCR**—Total RNA was extracted using the RNeasy Qiagen mini-prep kit and 2 μg was subjected to reverse transcription using a high-capacity cDNA synthesis kit (Invitrogen). Real-time PCR reactions were performed in duplicate using a SYBR Green PCR Master Mix kit (ABI). Amplification of the corresponding genes was achieved using specific primers (listed below) and measured continuously using an ABI 7900HT detection system. The transcription rate of VEGF was determined by measuring VEGF hnRNA using quantitative RT-PCR primers across exon 1 and intron 1 of the human gene. For all other genes examined, total mRNA was quantified. The signals obtained for both mRNA and hnRNA were compared with GAPDH, which served as an internal control. The value for untreated cells was set to 1, and the value for the various treatments was presented as a fold increase over the value for untreated cells. The primers used were as follows: GAPDH, 5’GTCGGAGTCAACCGATTTGGTCG3’ (forward) and 5’ATGGAATTTGCATGGTTGGAATCA3’ (reverse); VEGF hnRNA, 5’GCTGTCTTGGGTGCAATTTGGAG3’ (forward) and 5’CCAATGGCCCGGTGG3’ (reverse); GADD34, 5’GCCAGAAAAAGTGCTCTTCTC3’ (forward) and 5’TCAGCTCTCCTCTGGGCC3’ (reverse); BNIP3, 5’TTAATCAACCCGGAAAGCGCAC3’ (forward) and 5’GACTCCAGTTCTCTATCAA3’ (reverse); and HIF1α, 5’GAATGCTCAGAGGGAAGCGAA3’ (forward) and 5’ACAGTCACCTGTTGCTGCA3’ (reverse).

**Chromatin Immunoprecipitation**—ChIP analyses were performed using a ChIP-IT® Express chromatin immunoprecipitation kit (Active Motif). Extracts from 10⁷ cells were incubated overnight with antibodies against either ATF4, provided by Dr. David Ron (University of Cambridge, UK), rabbit anti-XBP-1(S) polyclonal antisera (Santa Cruz Biotechnology, catalog no. sc-7160X), anti-HIF-1α (Abcam), or rabbit anti-BiP polyclonal antisera (13), which served as a negative control. Two percent of the extract volume was removed before immunoprecipitation and served as input control. Purified, immunoprecipitated DNA and input DNA were then analyzed by PCR. The primers used for the PCR reaction were as follows: “HIF” binding site, 5’GGCTTTGGGGAGATTTGCTCTA3’ (forward) and 5’GCAGAAGACCGCAGAAGTTGGACGA3’ (reverse); “ATF4” binding site, 5’GGTCGGGCTCCAGAAACCATGAACAT3’ (forward) and 5’GCAGCGGCAACGCAAAGCC3’ (reverse); and “XBP1” binding site, 5’GGTGGGAGCTCTGGGAGCTGG3’ (forward) and 5’CCAGGGAAGAGATTTCGGACAA3’ (reverse).

**Quantitation of VEGF Secretion**—The Quantikine human VEGF ELISA kit (R&D Systems) was used to measure the amount of VEGF secreted in culture supernatants over a 24-h period. Quantification of VEGF was determined on the basis of a standard curve and was expressed as picograms/milliliters/1 × 10⁶ cells.

**Transient and Stable RNA Interference**—Cells were cotransfected with either one of two different ATF4 siRNAs (Integrated DNA Technologies), one of two XBP-1 siRNAs (Integrated DNA Technologies), or NCI (negative control) together with FITC-labeled non-targeting RNA (siGlo, Thermo Scientific). FITC-positive cells in Fig. 3, E and F, were isolated 48 h after transfection using a BD FACS Aria II cell sorter. The cells were replated and treated with UPR inducers as indicated in the figure. The effects on both VEGFA transcription and secretion were determined. NB1691 cells stably expressing HIF-1α...
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shRNA or negative control shRNA were provided by Dr. Andrew Davidoff (St. Jude Children’s Research Hospital) (14). Generation of Doxycycline-inducible ATF4 shRNA in the SK-N-AS Cell Line—shRNA sequences targeting human ATF4 were cloned into the pSuperior-puro vector (Oligoengine), which expresses shRNAs from a doxycycline-inducible H1 promoter. The shRNA sequences used to target ATF4 were cloned into the pSuperior vector with BglII and Xho1 overhangs as follows: 5’GATCCCCCCCTTCTGACCAAGTTGGATTTCCAGAGAATCCAGGTCAGAAGGTTTTTA3’ (forward) and 5’TCGATAAAAAACCTCTGACCAAGTTGGATTTCTTGAAAATCCAGGTCAGAAGGTTTTTA3’ (reverse). Two separate vectors, one containing the tetracycline repressor and the other encoding the ATF4 shRNAs, were stably transfected in the SK-N-AS cell line. Blasticidin (3 g/mL of medium) and puromycin (1 g/mL of medium) were used to select for clones stably expressing the tet-repressor and pSuperior vectors, respectively. After obtaining antibiotic-resistant double transfectants, a number of single cell clones were isolated and screened for regulated knockdown of ATF4 expression. Cells were treated with or without doxycycline for 24 or 48 h before incubating in low-glucose or thapsigargin-containing medium for the indicated times. The level of ATF4 expression was determined using Western blot analyses.

Immunoprecipitation and Phosphatase Assay—For immunoprecipitation, cell lysates were prepared as described previously (8). Total protein concentration was measured using a Bradford assay, and equal amounts of protein were immunoprecipitated with 2 μg HIF-1α antibody (BD Biosciences) and protein A-agarose. Immunoprecipitated material was washed with lysis buffer and split. Half of the lysate remained untreated, and the other half was treated with A protein phosphatase (New England Biolabs) for 30 min at 30 °C. Post-treatment, lysates were separated by SDS-PAGE and transferred to a PVDF for Western blotting.

Ultrasound-guided Para-adrenal Xenografts—All animal studies were approved by the institutional Animal Use and Care Committee prior to initiation. Ultrasound procedures were performed using the VEVO-770 high-frequency ultrasound system fitted with an RMV-706 probe. SK-N-AS cells were suspended in Matrigel (BD Worldwide) at a concentration of 2 × 10⁶ cells/mliter. Para-adrenal injections were performed in CB17-SCID mice as described previously (12).

In Situ Hybridization—When xenograft tumors reached 600–800 mm³ in size, they were removed, fixed in 4% paraformaldehyde, frozen, and sectioned into 12-μm slices using a HM500M cryostat. Slides were processed for in situ hybridization as described previously (15). Plasmids containing cDNAs encoding CHOP (a target of the PERK branch of the UPR (16)), ERdj4 (a target of the Ire1 branch (19)), BiP (a target of ATF6 activation (6)), BNIP3 (a HIF-1 target (5)), and VEGFA (a target of both pathways) were linearized prior to in vitro transcription to generate probes of ~500–900 bases. The resulting cRNA probes were labeled with digoxigenin and visualized with antidigoxigenin antibodies conjugated to alkaline phosphatase (anti-digoxigenin AP Fab fragments, catalog no. 11093274910, Roche). Serial sections were used for hybridization with each probe, and H&E staining was performed on flanking sections. High-magnification images were obtained using a Nikon E800 microscope equipped with a DXM1200 camera using a ×10/0.30 numerical aperture Plan Fluor objective.

RESULTS

Activation of the UPR Increases VEGF Secretion and Transcription Rate in Human Tumor Cell Lines—Two recent studies reported that ER stress regulates VEGFA expression in murine cells (8, 9), so we first determined whether it represented a UPR target that was conserved in human cell lines. We chose a large number of neuroblastoma cell lines and one medulloblastoma line (Daoy) were left untreated (NT, black bars) or treated with thapsigargin (Tg, striped bars) for 24 h. Cell culture media from the indicated samples were collected, and VEGF secretion was measured by ELISA. Caspase 8 (Casp8) protein (Y, present; n, absent) and N-Myc protein status (L, low; H, high; M, moderate) for the neuroblastoma lines are indicated. Arrows indicate the two cell lines that were used more extensively in this study.

![VEGF secretion is induced by the UPR in a number of human tumor lines. 23 human neuroblastoma cell lines and one human medulloblastoma line (Daoy) were left untreated (NT, black bars) or treated with thapsigargin (Tg, striped bars) for 24 h. Cell culture media from the indicated samples were collected, and VEGF secretion was measured by ELISA. Caspase 8 (Casp8) protein (Y, present; n, absent) and N-Myc protein status (L, low; H, high; M, moderate) for the neuroblastoma lines are indicated. Arrows indicate the two cell lines that were used more extensively in this study.](attachment:image.png)
the stress conditions used were specific for their pathways alone. ATF4 and HIF1α protein levels were examined by Western blot analysis, which further confirmed the pathway-specific activation of these transcription factors with the treatment conditions used in the experiments (data not shown).

We next determined whether the increased secretion of VEGF observed with ER stress was due to transactivation of the human VEGF gene by measuring hnRNA (heteronuclear RNA), which is processed rapidly and provides a good estimate of the transcription rate (18). Indeed, VEGF transcription was activated by ER stress in both cell lines, although the increase was more robust to these stressors than to hypoxia in the NB1691 cells. In addition, thapsigargin was a more potent inducer of VEGF transcription than either low glucose or hypoxia in both cell lines.
cell lines (Fig. 2C). The effect of these cell stress activators on VEGF secretion was determined. Although detectable amounts of VEGF were secreted from untreated SK-N-AS and NB1691 cells (Fig. 2D), we observed an increase of VEGFA in the media from both cell lines with all of the stress inducers, which was again more dramatic in the NB1691 cell line.

**ATF4 and XBP-1 Bind to the Human VEGF Promoter in Response to UPR Activation, but ATF4 Plays the Major Role in Regulating VEGF**—Because ER stress was found to transcriptionally up-regulate VEGF expression, we next wished to determine which UPR transcription factor was responsible. Analysis of the human VEGF promoter revealed potential binding sites for the UPR-inducible transcription factors ATF4 and XBP-1(S) (Fig. 3A). Occupation of the ATF4 and XBP-1(S) sites in response to ER stress was determined by ChIP assays. Although we were unable to detect binding of ATF4 to any of the six potential sites upstream of the transcription start site (data not shown), we did detect stress-inducible binding of ATF4 to a site at position +1.2 kb relative to the transcription start site (Fig. 3B), which is analogous to where ATF4 binds in the mouse promoter (8). XBP-1(S) binding was detected at a region 0.5 kb upstream of the transcription start site after treatment with the UPR inducer thapsigargin (Fig. 3C). We did not detect binding of XBP-1(S) to any of the other potential sites on the promoter (data not shown).

Both ATF4 and XBP-1(S) have been shown recently to bind to the mouse VEGF promoter, but it was not possible to assess the relative role each factor played in the transcriptional up-regulation of this gene because the various mouse null cell lines used had very different basal and stress-induced VEGF mRNA levels (8). Thus, to determine the relative contribution of ATF4 and XBP-1 in regulating VEGF expression in response to ER stress, we individually transfected the two cell lines with siRNA pools specific for each of these UPR-induced transcription factors to reduce their expression. The siRNAs targeting human ATF4 significantly decreased UPR-induced expression of this factor without reducing XBP-1(S) protein levels, which were actually slightly increased in the SK-N-AS cells (Fig. 3D). Similarly, cells transfected with XBP-1(S) siRNA had a significantly reduced expression of its target but did not affect ATF4 expres-
Importantly, hypoxia (1% O2) did not induce either ATF4 or XBP-1(S) (Fig. 3D, lanes 6 and 9). We found that decreasing the expression of ATF4 significantly reduced VEGF transcription after ER stress (Fig. 3E) and diminished secretion to near basal levels (Fig. 3F). Conversely, XBP-1(S) did not appear to contribute to the transcription rate of VEGF (Fig. 3E), but inhibiting its expression did reduce VEGF secretion by ~25% (Fig. 3F). This might imply a role for XBP-1(S) in the posttranslational processing of VEGF, which is in keeping with the fact that XBP-1(S) regulates expression of ER chaperones and cofactors that are known to play a role in the maturation of other secretory pathway proteins (19). Thus, although both transcription factors bound in a stress-inducible manner to the VEGF promoter, XBP-1(S) did not appear to play a significant role in regulating VEGF transcription in the SK-N-AS cell line. Similar data were obtained in the NB1691 cell line (data not shown).

The UPR and HIF Pathways Cooperate to Induce VEGF Secretion and Transcription Rate—We next examined the effects on VEGF expression when both pathways were activated simultaneously in cultured cells. In both the NB1691 and SK-N-AS cell lines, we observed a greater increase in VEGF secretion when the cells were treated with a combination of hypoxia and either thapsigargin or low-glucose medium than when either pathway was activated alone (Fig. 4). To confirm this finding, we examined two additional lines, MCF-7 breast carcinoma cells and Rh30 rhabdomyosarcoma cells, and, similarly, observed that more VEGF was secreted when both pathways were activated together (data not shown). When the VEGFA transcription rate was examined, we found that it was also up-regulated to a greater extent with the combination of stresses (Fig. 4B), and that, for the most part, increases in transcription rates were very similar to those observed when VEGFA secretion was monitored. This is in keeping with a previous study that reported enhanced VEGFA mRNA expression with the combination of hypoxia and low glucose in a human lung carcinoma line (20). These data argue that the two stress pathways can cooperate to induce VEGFA transcription when they are applied together.

The Increased Transcription of VEGF in Response to the Activation of Both Pathways Is Not Dependent on ATF4—We took two approaches to determine the relative contribution of each of these pathway-specific transcription factors to the up-regulation of VEGF expression.
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The first was to verify the binding of ATF4 and HIF-1α to their composite sites under these conditions, and the second was to assess the effects of inhibiting the expression of each transcription factor independently on the transcription of VEGFA when the combination of these stresses was applied. Unexpectedly, we observed a readily detectable decrease in ATF4 binding to the site at +1.5 kb on the VEGFA promoter in NB1691 cells (Fig. 5A) and SK-N-AS (data not shown) when the UPR was activated in combination with hypoxia as compared with UPR activation alone (Fig. 5A). Western blot analysis showed that ATF4 protein levels were not quite as high with the combination of stresses as compared with UPR activation alone (Fig. 5B), which was also observed in the SK-N-AS line (data not shown). We next examined the effects of reducing ATF4 expression on VEGFA transcription when both stress pathways were coactivated. Two separate siRNA sequences that specifically target ATF4 mRNA were used in the NB1691 cell line, along with a negative control siRNA that has no sequence homology to the human genome. We obtained a significant...
decrease in ATF4 protein expression upon UPR activation alone and in combination with hypoxia when either of the siRNA sequences was used as compared to the negative control siRNA in the NB1691 line (Fig. 5C). For the SK-N-AS cell line, stable clonal cell lines expressing doxycycline-inducible ATF4 shRNA were established. UPR-induced ATF4 protein expression was inhibited after doxycycline treatment (Fig. 5D). ER stress induction of GADD34, a target of ATF4, was also decreased significantly in the NB1691 cell line expressing the ATF4 siRNA sequences (Fig. 5F) and in the SK-N-AS cells with doxycycline-inducible ATF4 shRNA sequences (Fig. 5H). Similar to the results obtained using transient expression of siRNA in SK-N-AS cells (Fig. 3E), ATF4 played a major role in regulating VEGF transcription (Fig. 5, E and G) and secretion (data not shown) in response to UPR activation alone. However, in keeping with our ChIP data (Fig. 5A), when both the UPR and HIF signaling pathways were activated together, ATF4 did not appear to play a significant role in regulating VEGF transcription and had no effect on secretion. Importantly, ATF4 was still a functional transcription factor when both stress responses were coactivated because ATF4 siRNA significantly reduced GADD34 expression under these conditions in both cell lines (Fig. 5, F and H).

**HIF-1α Is the Major Regulator of VEGF Expression When Both the UPR and HIF Pathways Are Coactivated**—We next examined the binding of HIF-1α to the human VEGFA promoter, which has several potential HIF-1α binding sites (21) (Fig. 6A). Our results demonstrate that, indeed, HIF-1α binds to the VEGF promoter at a site ∼0.9 kb upstream of the transcription start site when the cells are treated with hypoxia alone

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**FIGURE 6. HIF-1α regulates VEGF expression induced by the combination of both stresses.** A, potential HIF-1α sites in the human VEGF promoter. TSS, transcription start site. B, cross-linked chromatim from NB1691 cells that were left untreated or treated as indicated for 8 h were immunoprecipitated with anti-HIF-1α. Precipitated chromatin and input controls were used for PCR amplification. The regions amplified are indicated by paired horizontal arrows in A, and the functional binding site is indicated by a bold vertical arrow in A. These data are representative of two independent experiments. C, Western blot analysis of HIF-1α that were either left untreated or treated as indicated in the figure for 8 h. Hsc70 protein levels were used as loading control. NT, not treated; Lglu, low glucose; Hyp, hypoxia. D and E, NB1691 (D) and SK-N-AS (E) cells stably expressing either HIF-1α shRNA or a negative control were either left untreated or treated as indicated. HIF-1α protein levels were determined for each treatment group by Western blot analysis to measure knockdown efficiency. F and G, total RNA was extracted from the indicated treatment groups, and VEGF mRNA levels were measured from NB1691 cells (F) and SK-N-AS cells (G). Experiments were done in duplicate or triplicate. Error bars indicate mean ± S.D. for each determination. Data were analyzed using unpaired Student’s t test. *, p < 0.05; **, p < 0.001; NS, not significant.
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and also with the combination of the two stresses (Fig. 6B). Although this was not a quantitative RT-PCR, the binding of HIF-1α to this site reproducibly appeared somewhat greater with the combination of stresses (Fig. 6B). Unlike ATF4, the HIF-1α protein levels were induced to similar levels with hypoxia alone and when hypoxia was combined with low glucose (Fig. 6C). To determine the contribution of HIF-1α in regulating VEGF expression when both pathways were activated together, we examined NB1691 and SK-N-AS cell lines that stably expressed HIF-1α shRNA or a negative control shRNA (14). As reported previously, HIF-1α protein was readily induced in the negative control lines when they were incubated under low-oxygen conditions (1% O₂) alone, but in the cells expressing shRNA to HIF-1α, there was a significant decrease in its expression (14). We found similar results when these lines were treated with a combination of hypoxia and UPR inducers (Fig. 6, D and E). As a consequence, BNIP3 transcripts, a downstream target of HIF-1α, were reduced significantly in cells treated with hypoxia alone and in combination with UPR inducers (data not shown). The contribution of HIF-1α to VEGFA expression under conditions where both stress pathways were activated simultaneously was determined next. We found that VEGF transcription (Fig. 6, F and G) and secretion (data not shown) were reduced to near basal levels with hypoxia alone in the lines expressing shRNA to HIF-1α as expected, but also when hypoxia was combined with either low glucose or thapsigargin. These data demonstrate that HIF-1α plays the major role in regulating VEGF expression when both stress pathways are induced together and further reveal that UPR activation appears to enhance HIF-1α activity.

Assessing How UPR Activation Enhances HIF-1α Activity—To begin to understand how ER stress might potentiate the ability of HIF-1α to transactivate target genes like VEGFA and BNIP3, we examined several mechanisms reported previously. First, we investigated whether ER stress might increase the transcription of the HIF-1α gene, which has been shown previously to be up-regulated via direct binding of NFκB to its promoter in response to reactive oxygen species (22). Importantly, NFκB is also activated downstream of the PERK branch of the UPR (23). However, we observed no increase in HIF-1α mRNA levels under any of our experimental conditions (data not shown). We next examined the expression of HIF-1β protein, which encodes the constitutively expressed subunit in HIF-1 heterodimers that is needed to produce an active transcription factor (3). Although a previous report demonstrated that glucose depletion in MIN6 β-cells increased HIF-1β protein levels (24), the conditions used in our experiments did not affect HIF-1β levels under any of the stress conditions we used (data not shown). Nor did we observe any evidence for increased nuclear localization of HIF-1α with the combination of stresses as compared with hypoxia alone (data not shown). Lastly, we performed time course experiments and examined the accumulation of HIF-1α protein. Although the kinetics of stabilization appeared similar, we observed the appearance of a slower migrating form of HIF-1α at 1 h that became more predominant over time (Fig. 7A, lanes 2–7). In the cells where both pathways were activated simultaneously, the slower migrating band became more prominent at earlier times, and the shift to this form was more complete (Fig. 7A, lanes 9–13). Because HIF-1α is the target of multiple kinases, which, in some cases, can increase its activity (25–27), the immunoprecipitated samples were treated with protein phosphatase to determine whether the slower migrating species represented phosphorylated HIF-1α (Fig. 7B, lanes 7, 9, and 11). After treatment, the higher molecular weight band collapsed to a single faster band (Fig. 7B, lanes 8, 10, 12), demonstrating that HIF-1α is phosphorylated to a greater extent by UPR activation in this cell line.

Spatial Organization of UPR and HIF Signaling in Neuroblastoma Tumor Sections—Our study revealed stress-specific regulation of VEGF transcription as well as an unexpected interaction between the UPR and HIF pathways. To begin to assess the potential significance of these results in a tissue setting, we determined the relative expression of the UPR and HIF pathways in xenograft tumors. We chose the SK-N-AS cell line because it had very low basal levels of VEGF secretion that were increased significantly by ER stress. Cells were implanted into the para-adrenal space, and when the tumors reached 600–800 mm³ in size, as determined by ultrasound, they were removed, fixed, and sectioned. Gross analysis of the neuroblastoma xenografts revealed extensive vascularization around the tumor mass. To analyze UPR and HIF pathway activation in these xenografts, downstream targets specific to each pathway, as well as VEGF, a target of both pathways, were examined by in situ hybridization on consecutive tumor sections. In addition, tumor sections were stained with H&E to identify regions with high metabolic activity (Fig. 8, A, Ai, Aii, Ei, Eii, and Eiii), as indicated by dark purple staining. Inspection of H&E-stained sections at higher magnification (Fig. 8, Ai, Aii, Ei, Eii, and Eiii) also allowed us to detect blood vessels, which were confirmed with antibodies to CD34 (data not shown). When the expression of BNIP3 mRNA, a HIF-1 target, was examined on low magnification, we observed heterogeneous signals (Fig. 8, B and F) that, on higher magnification, revealed robust expression further from the blood vessels, with little or no expression in the area surrounding the blood vessel (Fig. 8, Bi, Bii, Fi, and Fii).
distance from the center of a blood vessel to the closest boundary of BNIP3 expression was between 70–100 μm, in keeping with published data (28). When representative targets of each of the three branches of the UPR (i.e. CHOP, ERdj4, and BiP) were similarly examined at low magnification, we observed a fairly uniform expression pattern (Fig. 8, D, H, and I, respectively), which coincided with more metabolically active areas of the tumor, as indicated by H&E staining (A and E). Higher magnification in each of these sections revealed that UPR expression was distributed uniformly around blood vessels and throughout the metabolically active areas of the tumor (Fig. 8, Di, Dii, Hi, Hii, Ii, and Iii). VEGFA expression, a target of both pathways, more closely mirrored that of the UPR targets CHOP, ERdj4, and BiP and was observed throughout the metabolically active areas of the tumor (Fig. 8, C and G). In summary, certain regions of the tumor appear to have activated the UPR alone in which ATF4 is likely to contribute to VEGF expression, whereas others show evidence of activating both pathways simultaneously, which are likely to be dependent on HIF-1α made more potent by UPR activation. We did not observe areas of the tumor where only the hypoxic pathway was activated.

**DISCUSSION**

The UPR and hypoxia-activated pathways represent two well studied cytoprotective responses that can be induced by insufficient microenvironments to restore or enhance tissue vascularization by increasing expression of the proangiogenic factor...
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VEGF. This can occur in normal developmental and physiological contexts, like establishing the vasculature of a placenta (29) and promoting wound healing (30, 31), as well as pathological conditions, like increasing tumor vascularization (10, 32). Although a large number of studies exist to demonstrate activation of both pathways in these various tissues and conditions, nearly all of them study the pathways independently, and, thus, very little is known about possible interactions between these pathways on shared transcriptional targets like VEGF. As a result, there is no understanding of the relative contribution of each pathway on its transcription. Our first step was to determine which UPR-induced transcription factor regulated VEGF transcription in our lines, because this has been somewhat controversial (8, 9, 33). Previous studies have shown that the transcription factor ATF4, which can be activated by a number of different cellular stress responses, binds to the VEGFA promoter in response to arsenite (34), oxidized phospholipids (33), and more conventional UPR inducers like thapsigargin (8, 9).

In this study, we show that, although XBP-1(S) also binds to this promoter in response to ER stress, depleting XBP-1(S) levels had very little effect on VEGF transcription, whereas reducing ATF4 expression significantly reduced VEGF mRNA to pre-stress levels in cells experiencing ER stress. A role for ATF4 in regulating several proangiogenic factors in response to low glucose was observed recently in a human head and neck squamous cell carcinoma cell line by introducing shRNA to ATF4 with lentiviruses (32). It is noteworthy that studies on vascularization of the mouse placenta demonstrated an essential role for the inositol-requiring protein 1 (IRE1) branch of the UPR in VEGF production and blood vessel formation (35). However, this was independent of its only known downstream transcriptional target, XBP-1(S), raising the possibility of differences in either species- or tissue-specific regulation. Although XBP-1(S) did not noticeably contribute to the transcription of VEGF in our study, we did observe a decrease in the secretion of VEGF when XBP-1 expression was inhibited. As XBP-1(S) controls the up-regulation of some ER chaperones and cofactors, it is possible that it contributes to the maturation of VEGF protein in the ER during UPR activation. Along these lines, a number of studies have shown that the ER stress-inducible chaperone GRP170, which is also known as oxygen-regulated protein ORP150, plays a very important role in VEGF processing and secretion (36).

Our cell culture data, obtained from multiple cell lines, demonstrated that, when both pathways are activated simultaneously, there is greater transcription of the VEGFA gene than when either pathway is induced alone, which also results in greater secretion of this proangiogenic factor. This was not totally unanticipated because both pathways produce transcription factors that bind directly to distinct regions on the VEGF promoter (8, 21). However, surprisingly, this increased induction was almost entirely due to HIF-1α, with little or no contribution from the UPR-induced transcription factor ATF4. Even though ATF4 was still transcriptionally active on other targets, we were unable to detect its binding to the VEGF promoter, and reducing its expression had no effect on VEGF transcription or secretion. It is unclear whether HIF1 binds earlier or better to the VEGF promoter, thereby obstructing ATF4 binding, or whether hypoxic conditions affect the chromatin structure at the ATF4 binding site.

Our data suggest that the increased transcription of VEGF observed when both pathways are coactivated is due to a UPR-dependent enhancement of the transcriptional activity of HIF-1 because BNIP3 mRNA, another target of HIF-1, was also increased when the two pathways were activated together. We examined several possible mechanisms for UPR-induced enhanced HIF-1 activity on the basis of previous reports (22, 24, 37). However, we found no differences in either HIF-1α mRNA levels or HIF-1β protein levels with the combination of the two pathways compared with hypoxia alone, nor did we detect an increased nuclear localization of HIF-1α. Phosphorylation of HIF-1α has been shown to stabilize this transcription factor (11) and can either positively (25, 26) or negatively (38, 39) regulate its activity. Our experiments showed evidence for an increased magnitude of HIF-1α phosphorylation with the combination of stresses in two human neuroblastoma lines. A number of kinases have been identified that target HIF1α and increase its activity, including p38MAPK (11), CK2 (25), and ERK1/2 (27). Of these, p38MAPK (40) is activated by the UPR, making it an appealing candidate for enhancing HIF-1α activity when both stresses are applied.

To begin to understand the possible significance of ATF-4-induced VEGF expression versus UPR-enhanced HIF-1-dependent VEGF regulation, we examined tumor sections to assess areas in which each pathway was activated. Notably, very little has been done to quantify the extent of overlap of these two pathways within a tumor sample. We examined the expression of targets of each pathway by in situ hybridization to identify regions of the tumor where the pathways were activated. We found that the expression of BNIP3 (HIF1) transcripts was restricted to regions of the tumor that were further from the vasculature, in keeping with published data (28). These areas also expressed CHOP, Bip, and ERdj4 mRNA (UPR), arguing that both pathways were activated in these areas. Unlike BNIP3, the UPR-induced transcripts were expressed throughout the tumor, revealing that UPR activation was more widespread and correlated with the most metabolically active regions of the tumor. No areas were detected in which only HIF-1 was activated. On the basis of our cell culture data, it is likely that, in regions of the tumor where only the UPR was activated, ATF4 would play a major role in the transcriptional up-regulation of VEGF. Conversely, in regions where both pathways were activated, ATF4 would be unlikely to contribute. Instead, the activation of the UPR would increase HIF-1 transcriptional activity and increase the processing of VEGF protein via its up-regulation of ER chaperones. The detection of VEGF transcripts throughout the tumor argues that UPR-induced ATF4 is likely to control its expression in the less hypoxia areas. Although the contribution of ATF4 to tumor vascularization will require much more in-depth animal studies, it is noteworthy that reduction of PERK expression (17, 32, 41), which regulates ATF4 during ER stress, results in tumors that grow slower or are less well vascularized.

In summary, we discovered an interaction between two well studied cytoprotective (UPR and HIF) pathways and shed light on the molecular mechanism underlying this interaction. We
found that pathway-specific transcription factors can control shared targets like VEGF when each pathway is activated alone. However, when the pathways are activated simultaneously, the hypoxia-induced HIF-1 transcription factor plays the major role in regulating VEGF expression, and the UPR contributes by potentiating its activity. The detection of regions of a tumor in which both pathways are activated as well as areas where only the UPR is activated suggests that there are likely to be distinct pathways for regulating VEGF expression within a tumor mass.

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