Response of VEGF Expression to Amino Acid Deprivation and Inducers of Endoplasmic Reticulum Stress

Steve F. Abcouwer, Philip L. Marjon, Robyn K. Loper, and David L. Vander Jagt

PURPOSE. Vascular endothelial growth factor (VEGF) plays an important role in initiation of the angiogenesis that leads to proliferative retinopathy. Several environmental conditions and chemical agents that influence the expression of VEGF can also cause endoplasmic reticulum (ER) stress. The hypothesis for the current study was that expression of VEGF is responsive to conditions that cause ER stress, including amino acid deprivation.

METHODS. Confluent cultures of a human retinal pigmented epithelial cell line (ARPE-19) were deprived of amino acids or treated with chemical inducers of ER stress. Treatment with cobalt was used to mimic hypoxia-induced expression of VEGF. Northern blot analysis was used to measure intracellular VEGF mRNA, and ELISA was used to measure secreted VEGF protein. Glucose-regulated protein 78 (GRP78) mRNA levels were compared with those of VEGF. Glyceraldehyde-phosphate dehydrogenase (GAPDH) mRNA was used as a control.

RESULTS. Conditions and chemical agents known to activate ER stress response (ERSR) pathways also induced the expression of VEGF. Deprivation of amino acids in the culture medium increased VEGF mRNA expression by 1.5- to 6-fold. Glucose deprivation or treatment of ARPE-19 cells with tunicamycin, brefeldin A, the calcium ionophore A23187, or thapsigargin increased the expression of VEGF mRNA in these cells by 8- to 10-fold. Expression of GRP78 mRNA was well correlated with that of VEGF mRNA under all conditions. These treatments also increased the secretion of VEGF protein by up to twofold. The increase in VEGF mRNA level in response to glucose deprivation was rapid (greater than 10-fold) and was observed in a physiologically relevant range of glutamine concentrations. The half-life of VEGF mRNA was increased 2.5-fold by glutamine starvation.

CONCLUSIONS. These results indicate that VEGF is an ER stress-responsive gene and suggest that cells can respond to nutrient deprivation by increasing VEGF expression through both transcriptional and posttranscriptional mechanisms.

E xpression of vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF), contributes to angiogenesis that occurs during normal development and in many pathologic states, such as tumor growth and proliferative retinopathy. It has been known for some time that the expression of VEGF is controlled by oxygen tension.1,12 Hypoxia upregulates the expression of VEGF in both cancer and ischemic retinal disease.5 Under hypoxic conditions, expression of VEGF is also enhanced by stabilization of the mRNA4,5 and by increased translation initiation at an internal ribosome entry site (IRE).6 Environmental factors other than oxygen tension also control VEGF expression. For example, glucose deprivation increases expression of VEGF in HepG2 human hepatoma cells,7 U-937 human monocytic cells,8 and C6 rat glial tumor cells.9 Three recent studies have implicated Ca2+ in the control of VEGF expression. Höper et al.10 demonstrated that A23187, a Ca2+-ionophore, and thapsigargin, a sarcoendoplasmic Ca2+-adenosine triphosphatase (ATPase) inhibitor, increased VEGF mRNA levels in a human monocytic cell line. In studies of the effects of basic fibroblast growth factor on VEGF secretion in osteoblast-like cells, Tokuda et al.11 observed that A23187 and thapsigargin stimulated release of VEGF. Similarly, Metzen et al.12 showed that thapsigargin stimulates VEGF secretion in human hepatoma cells.

Besides inducing expression of VEGF, glucose deprivation, A23187 and thapsigargin have in common another effect on mammalian cells. All three treatments perturb the function of the endoplasmic reticulum (ER), resulting in an ER stress response (ERSR). The ERSR describes a complex set of signal-transduction pathways and genetic responses induced by various conditions that cause ER stress.13,14 ERSR signaling pathways are activated by amino acid deprivation; glucose starvation; a number of chemical inducers that perturb ER homeostasis, inhibit protein glycosylation, or inhibit vesicle trafficking from the ER to the Golgi; reducing agents that interfere with protein disulfide formation in the ER; expression of ER-targeted proteins that are not folded correctly in the ER; overexpression of ER-targeted proteins leading to ER overloading; and cholesterol starvation that inhibits the formation of ER membranes. Four mammalian ERSR signaling pathways have been described: the unfolded protein response (UPR) pathway,13,14 the ER overload (ERO) response,13,14 the general control response (also called the integrated stress response),15,16 and the sterol regulatory element binding protein (SREBP) pathway.13 The first three of these response pathways are triggered by various and overlapping types of ER stress, including ER-perturbing drugs, glucose deprivation, amino acid deprivation, expression of misfolding proteins and overexpression of normal proteins. These pathways also cause specific and overlapping genetic responses, evidenced by the induction of different as well as common stress-responsive genes.
In the present study, a human retinal pigmented epithelial cell line (ARPE-19 cells) was used as a model system to test the hypothesis that VEGF expression is increased by conditions that cause ER stress. Retinal pigmented epithelial (RPE) cells are one of the cell types that increase expression of VEGF in experimental models of proliferative retinopathy. Proliferative retinopathy is a neovascular disease of the retina occurring in diabetics and premature infants and is one of the major causes of legal blindness. The molecular events that lead to inappropriate retinal neovascularization are poorly understood. However, in both diabetic retinopathy (DR) and retinopathy of prematurity (ROP), increased expression of VEGF by RPE cells and retinal glial cells (Müller cells) is thought to initiate and promote aberrant angiogenesis. We used Northern blot analysis to measure the effects of amino acid deprivation, glucose deprivation, and chemical inducers of ER stress on the expression of VEGF mRNA in confluent cultures of ARPE-19 cells. Exposure to CoCl2, which is known to increase expression of VEGF by mimicking the effects of hypoxia, was examined by splice-form amplification. In addition, under all treatment conditions, the expression of VEGF and mRNA for the 78-kDa glucose-regulated protein (GRP78) was compared. GRP78, which encodes the endoplasmic reticulum luminal protein BiP, is the prototypical ER stress-responsive gene and is known to be upregulated by the UPR pathway. Secretion of VEGF was also determined by measuring accumulated protein in the culture medium by ELISA. Our data suggest that expression of VEGF is responsive to amino acid availability and is increased by all treatments that cause ER stress, as indicated by induction of GRP78. A portion of this study has been published in abstract form.

**METHODS**

ARPE-19 cells were purchased from the American Type Culture Collection (Manassas, VA). The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, low glucose formulation) supplemented with 4 mM L-glutamine, 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B. For Northern blot analysis experiments, cells were plated in 10-cm tissue culture dishes, grown to confluence, fed with fresh medium 1 day before being rinsed twice with Dulbecco’s phosphate-buffered saline (DPBS), and fed with fresh media containing drugs or other treatments as described in the figure legends. For glutamine-deprivation experiments, glutamine-free medium (DMEM supplemented with 10% dialyzed FBS [dFBS] and no glutamine) containing added glutamine or drugs was used as described. Northern blot analysis for VEGF by mimicking the effects of hypoxia. Secretion of VEGF was also determined by measuring accumulated protein in the culture medium by ELISA. Our data suggest that expression of VEGF is responsive to amino acid availability and is increased by all treatments that cause ER stress, as indicated by induction of GRP78. A portion of this study has been published in abstract form.

**RESULTS**

Response of VEGF and GRP78 mRNA Expression to Inducers of ER Stress

To test the hypothesis that expression of VEGF mRNA is responsive to ER stress, confluent ARPE-19 cells were cultured for 18 hours in normal medium, in medium without glucose, and in medium containing (1) 0.1% (vol/vol) dimethyl sulfoxide (DMSO, as a carrier control); (2) 2 or 5 μg/mL tunicamycin (a glycosyltransferase inhibitor); (3) 10 μM of the Ca2+ ionophore A23187; (4) 10, 1, or 0.1 μg/mL brefeldin A (an inhibitor of ER-to-Golgi vesicle transport); or (5) 2 or 5 mM azetidine-2-carboxylate (a proline analogue shown to cause ER stress in some cells). Total RNA was isolated and Northern blot analysis for VEGF, GRP78, and GAPDH mRNA was performed. Samples were compared with the untreated control or with DMSO-treated cells (Fig. 1). The ARPE-19 cells contained detectable amounts of a single VEGF mRNA species of a size corresponding to the VEGF121 mRNA splice form. The presence of a covalent VEGF121 isoform was confirmed by splice-form-specific RT-PCR (data not shown). The amounts of VEGF mRNA relative to GAPDH were increased by glucose deprivation (10-fold), tunicamycin (8- and 6-fold at 2 and 5 μg/mL, respectively), A23187 (9-fold) and brefeldin A (9-fold at all three concentrations). Similarly, the expression of GRP78 mRNA was markedly increased by glucose deprivation (39-fold), tunicamycin (20- and 21-fold at 2 and 5 μg/mL, respectively), A23187 (45-fold), and brefeldin A (79-, 88-, and 82-fold at 10, 1, or 0.1 μg/mL, respectively). However, azetidine-2-carboxylate had a minimal effect on both VEGF and GRP78 mRNA expression.

As a positive control for VEGF induction, cells were treated with CoCl2, which induces VEGF expression by a mechanism that mimics hypoxia. CoCl2 increased the level of VEGF mRNA approximately twofold and increased GAPDH expression by a similar amount (Fig. 1). Several published studies have documented hypoxic induction of GAPDH expression (see Ref. 26 and references therein). CoCl2 had very little effect on the GRP78 mRNA level.

As an additional test of the hypothesis that ER stress induces expression of VEGF, ARPE-19 cells were also treated with the sarcoplastic reticulum Ca2+-ATPase inhibitor thapsigargin (data not shown). Hopper et al. demonstrated that both A23187 and thapsigargin increase expression of VEGF mRNA by five- to sixfold in a human monocytic cell line. We found that thapsigargin caused an approximately 3-fold increase in VEGF mRNA levels and a 68-fold induction of GRP78 mRNA content in ARPE-19 cells (data not shown). We conclude that glucose deprivation and all treatments that caused an appreciable ERSR

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in ARPE-19 cells increased expression of VEGF mRNA. However, the multiples of change in induction were less than those observed for GRP78 mRNA.

**Response of VEGF and GRP78 mRNA Expression to Amino Acid Deprivation**

ER stress can also be caused by amino acid deprivation. To test the hypothesis that VEGF expression is affected by amino acid starvation, cultures of ARPE-19 cells were incubated in medium completely devoid of amino acids or without a single amino acid normally found in medium. ARPE-19 cells were first cultured for several passages in complete MEM supplemented with 2 mM glutamine and 10% (vol/vol) FBS. Once adapted to these conditions, confluent ARPE-19 cells were cultured for 24 hours in normal medium (Ctrl), in medium without glucose (Glc Free), or in media containing the indicated concentrations of CoCl$_2$, azetidine-2-carboxylate (Aze), tunicamycin (Tuni), DMSO carrier (DMSO), the Ca$^{2+}$ ionophore A23187, or breveldin A (Bref A). Total RNA was isolated and Northern blot analysis was performed. Changes in mRNA levels are reported in the Results section.

**Response of VEGF and GRP78 mRNA Expression to Glutamine Deprivation**

Because the absence of glutamine caused the greatest induction of VEGF mRNA expression, the effect of depriving ARPE-19 cells of this amino acid was tested in more detail. To determine the rapidity and sustainability of this response, confluent ARPE-19 cells were cultured in glutamine-free DMEM-based medium for various times from 3 to 72 hours. Total RNA was isolated and Northern blot analysis was performed. Glutamine starvation caused a rapid increase (10-fold) in relative expression of VEGF mRNA by 3 hours that was sustained for 48 hours (Fig. 3A). Although the extent of GRP78 induction was twofold at 3 hours, the kinetics of this induction was slower than that of VEGF. However, the maximum relative inductions of both VEGF mRNA (16-fold) and GRP78 mRNA (9-fold) were observed 24 hours after glutamine starvation was initiated. Again, these relative inductions were increased by a decline in GAPDH mRNA levels, which were reduced to 59% of initial content at 24 hours.

To better define the rapidity of this response, a similar glutamine-starvation time-course study was performed with incubation times of 15 minutes to 4.5 hours. Relative VEGF mRNA levels were increased nearly twofold after 1 hour of glutamine starvation, and ninefold by 4.5 hours (Fig. 3B). The GRP78 mRNA level was not appreciably affected until 4.3 hours, at which time it was increased by 2.4-fold. GAPDH mRNA levels did not exhibit any appreciable or consistent decline. Thus, VEGF expression is rapidly induced by glutamine deprivation of other amino acids singly caused inductions that ranged from no effect to a threefold increase. Comparison of VEGF and GRP78 levels under all these media conditions showed a strong correlation ($r = 0.90$) between the two, suggesting that the responses of these two mRNAs to amino acid deprivation are related.

It should be noted that the apparent inductions in glutamine-deprived cells were boosted by a 41% depletion of GAPDH content. Similarly, we previously observed that GAPDH mRNA levels are slightly diminished by glutamine deprivation in human breast cancer cells. Even allowing for the effect of GAPDH normalization, glutamine deprivation caused the greatest increase in VEGF and GRP78 mRNA levels. This effect was unexpectedly even greater than that of total amino acid deprivation.

**Response of VEGF and GRP78 mRNA Expression to Glutamine Deprivation**

![Figure 1](image1.png)

**Figure 1.** Expression of VEGF and GRP78 mRNA in response to inducers of ER stress. Confluent ARPE-19 cells were cultured for 18 hours in normal medium (Ctrl), in medium without glucose (Glc Free), or in media containing the indicated concentrations of CoCl$_2$, azetidine-2-carboxylate (Aze), tunicamycin (Tuni), DMSO carrier (DMSO), the Ca$^{2+}$ ionophore A23187, or breveldin A (Bref A). Total RNA was isolated and Northern blot analysis was performed. Changes in mRNA levels are reported in the Results section.

![Figure 2](image2.png)

**Figure 2.** Expression of VEGF and GRP78 mRNA in response to amino acid deprivation. Confluent ARPE-19 cells were cultured for 24 hours in MEM containing the normal complement of amino acids (+All), without all amino acids (−All), or without each of the amino acids indicated. Total RNA was isolated and Northern blot analysis was performed. Changes in mRNA levels are reported in the Results section.
tamine starvation, and the induction occurs more rapidly than that of GRP78.

We next examined the response of VEGF and GRP78 mRNA expression to various times of culture in glutamine-free medium and in media with various glutamine concentrations. Confluent ARPE-19 cells were cultured in glutamine-free medium for the times indicated (A, B) or in media containing the indicated concentrations of glutamine (Gln) for 24 hours (C). Total RNA was isolated, and Northern blot analysis was performed. Changes in mRNA levels are reported in the Results section.

FIGURE 3. Response of VEGF and GRP78 mRNA expression to various times of culture in glutamine-free medium and in media with various glutamine concentrations. Confluent ARPE-19 cells were cultured in glutamine-free medium for the times indicated (A, B) or in media containing the indicated concentrations of glutamine (Gln) for 24 hours (C). Total RNA was isolated, and Northern blot analysis was performed. Changes in mRNA levels are reported in the Results section.

FIGURE 4. Effect of serum content of the medium on expression of VEGF and GRP78 mRNA in glutamine-free and containing media. Confluent ARPE-19 cells were cultured for 24 hours in medium containing 0 or 4 mM glutamine and various amounts of dFBS from 0% to 10% of volume, and total RNA was isolated and Northern blot analysis was performed. Whereas basal VEGF expression in glutamine-fed cells was not serum dependent, expression of VEGF mRNA by glutamine-starved cells was markedly decreased as serum concentration was lowered (Fig. 4). A similar effect was seen with GRP78 mRNA—that is, serum had no appreciable effect on expression of GRP78 mRNA in glutamine-fed cells, but markedly affected expression in glutamine-starved cells. Thus, glutamine deprivation caused large increases only when serum was present. In this experiment, VEGF mRNA and GRP78 mRNA levels again exhibited an excellent correlation ($r = 0.94$).

The effects of glutamine on VEGF and GRP78 mRNA half-lives were examined. Confluent ARPE-19 cells were starved of glutamine for 18 hours, causing an increase in VEGF mRNA levels. Cultures were then treated with 5 μg/mL actinomycin D (actD) to inhibit transcription. After 30 minutes of incubation with actD, a culture was harvested for RNA isolation and half of the remaining cultures were fed with 4 mM glutamine. The remaining half of the cultures were not fed glutamine and thus remained glutamine starved. Glutamine-replete and glutamine-deprived cultures were then harvested at several times (0.5, 1, 2, 4, 8, and 24 hours) after feeding. By Northern blot analysis, we examined the decays of VEGF and GRP78 mRNAs after inhibition of transcription by actD. Half-lives of mRNA species were estimated by least-squares analysis of ln(GAPDH-normalized mRNA levels) versus time, obtaining a decay constant from the slope of the linear portion of each curve (0.5–8 hours). In these analyses, decay constants of 0.37 hour ($r = 0.97$) and 0.14 hour ($r = 0.96$) were obtained for VEGF mRNA decays in the presence and absence of glutamine, respectively (curves not shown). The calculated half-lives of VEGF mRNA were thus 1.9 and 4.7 hours in the presence and absence of glutamine deprivation, and the induction occurs more rapidly than that of GRP78.

The effect of serum concentration in the medium on expression of VEGF and GRP78 mRNA was examined for cells in both glutamine-free and glutamine-containing media. Confluent ARPE-19 cells were cultured for 24 hours in medium containing 0 or 4 mM glutamine and various amounts of dFBS from 0% to 10% of volume, and total RNA was isolated and Northern blot analysis was performed. Whereas basal VEGF expression in glutamine-fed cells was not serum dependent, expression of VEGF mRNA by glutamine-starved cells was markedly decreased as serum concentration was lowered (Fig. 4). A similar effect was seen with GRP78 mRNA—that is, serum had no appreciable effect on expression of GRP78 mRNA in glutamine-fed cells, but markedly affected expression in glutamine-starved cells. Thus, glutamine deprivation caused large increases only when serum was present. In this experiment, VEGF mRNA and GRP78 mRNA levels again exhibited an excellent correlation ($r = 0.94$).

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We next examined the response of VEGF and GRP78 mRNA expression to various glutamine concentrations in the medium. Confluent ARPE-19 cells were cultured in medium containing reduced concentrations of glutamine for 24 hours, total RNA was isolated, and Northern blot analysis was performed. This dose-response experiment demonstrated that both VEGF and GRP78 mRNA levels were increased to a similar extent with decreasing ambient glutamine availability (Fig. 3C). In glutamine-free conditions, the relative inductions of VEGF and GRP78 mRNA were at a maximum at nine and sevenfold, respectively. Both VEGF and GRP78 mRNA levels exhibited an appreciable increase at 0.25 mM glutamine that were five- and threefold those at 4 mM glutamine, respectively. Again, these relative inductions were increased by the effect of glutamine deprivation on GAPDH mRNA levels, which decreased with decreasing glutamine concentrations (by 50% at 0 mM glutamine). Regardless, there was an excellent correlation between VEGF mRNA and GRP78 mRNA levels ($r = 0.96$).

The effect of serum concentration in the medium on expression of VEGF and GRP78 mRNA was examined for cells in both glutamine-free and glutamine-containing media. Confluent ARPE-19 cells were cultured for 24 hours in medium containing 0 or 4 mM glutamine and various amounts of dFBS, or in media containing 4 mM glutamine and the indicated concentrations of FBS. Total RNA was isolated and Northern blot analysis was performed. Changes in mRNA levels are reported in the Results section.
glutamine, respectively. This estimate of basal VEGF decay rate is very close to estimates of 1.1, 1.6, and 2.0 hours obtained previously in other cell types. The present results suggest that, in the absence of glutamine, VEGF mRNA stability was increased by 2.5-fold. This increase is much less than the increases in steady state VEGF mRNA levels observed in glutamine-starved cells. Thus, mRNA stabilization cannot account for a large portion of the effect of glutamine availability on VEGF expression, suggesting that a transcriptional mechanism is also at work. In these same cells, decay constants of 0.14 hour ($r = 0.91$) and 0.093 hour ($r = 0.99$) were obtained for GRP78 mRNA decays in the presence and absence of glutamine, respectively (curves not shown). GRP78 mRNA half-lives were therefore 4.9 and 7.5 hours in the presence and absence of glutamine, respectively. These results are very similar to those obtained in an earlier study in our laboratory, which determined half-lives of 4.8 and 9.2 hours for GRP78 mRNA in glutamine-starved or -starved breast carcinoma cells, respectively.

**Secretion of VEGF by ER-Stressed Cells**

Global protein synthesis is diminished during conditions of nutrient starvation and ER stress. Furthermore, because VEGF is itself glycosylated and secreted, there is reason to question whether its expression can be maintained under conditions of ER dysfunction. To determine whether cells under conditions of ER stress are able to translate and secrete VEGF protein, the accumulation of VEGF in the medium was compared in control and ARPE-19 cells cultured under normal, glutamine-deprived, tunicamycin-treated, and CoCl$_2$-treated conditions. Triplicate cultures of confluent ARPE-19 cells in six-well plates were incubated with 3 mL per well of complete medium containing 4 mM glutamine (control), 0.25 mM glutamine (Gln), 5 µg/mL tunicamycin (+Tuni) or 100 µM CoCl$_2$ for 24 hours. An ELISA was used to determine accumulation of VEGF in the media. Data represent the mean concentration ± SD.

**FIGURE 5.** Secretion of VEGF protein by glutamine-deprived, tunicamycin-treated, and CoCl$_2$-treated cells. Triplicate cultures of confluent ARPE-19 cells in six-well plates were incubated with 3 mL per well of complete medium containing 4 mM glutamine (control), 0.25 mM glutamine (Gln), 5 µg/mL tunicamycin (+Tuni) or 100 µM CoCl$_2$ for 24 hours. An ELISA was used to determine accumulation of VEGF in the media.

**DISCUSSION**

The response of VEGF expression to hypoxia is thought to be the major factor contributing to its induction in a number of diseases. In vitro, hypoxia increases expression of VEGF in many cell types, including RPE, Müller, and tumor cells. Induction of expression in response to hypoxia has been studied extensively compared with induction by other agents. Induction by hypoxia is due to an increase in steady state VEGF mRNA. Increased transcription of VEGF is mediated by hypoxia inducible factor (HIF)-1. Although these mechanisms clearly play a role in control of VEGF expression, two questions remain unanswered: How general is the hypoxic mechanism of VEGF induction, and what other mechanisms contribute to the increase in expression of VEGF? For example, nutrient insufficiencies such as hypoglycemia and amino acid deprivation cause alterations in gene expression by mechanisms involving ERSR pathways. Might VEGF also be responsive to these mechanisms?

In the present study we examined the effects of the hypoxic induction of VEGF and GRP78 of amino acid availability, hypoglycemia, and chemical agents that initiate the ERSR, including brefeldin A, A13278, thapsigargin, and tunicamycin. The positive results obtained suggest that VEGF expression is increased in response to ER stress. The increases of steady state VEGF mRNA caused by ER stress were at least as large as those previously reported to be caused by hypoxia. Initial indications suggest that the response to glutamine deprivation is due to both increased transcription of the gene and the stabilization of mRNA. Additional experiments are needed to determine whether this is also true of other causes of ER stress. If so, the effects of ER stress would be similar to the effects of hypoxia, which causes both increased transcription of the VEGF gene and stabilization of the mRNA. In fact, previous studies have documented increases in the half-life of VEGF mRNA caused by hypoxia that are nearly identical with the effects of glutamine deprivation that we observed.

Additional experiments are needed to confirm a transcriptional response to ER stress and to determine which ERSR pathway(s) are responsible for transcriptional upregulation of expression of VEGF. For example, the UPR pathway induces expression of GRP78, GRP94, and calreticulin by activation of the activating transcription factor (ATF)-6 and its binding to ERSR enhancer (ERSE) sequences. A preliminary examination of the VEGF promoter region did not reveal the presence of any canonical ERSE sequences, neither the ERSE described by Yoshida et al. (CCAATN$_2$CCAG), nor the ERSE-II described by Kakame et al. (ATTTGNCACCAG). However, several isolated CCAAT sequences (that could function as binding
sites for NF-Y) and several isolated CCACG sequences (that could function as binding sites for ATF6) are present (data not shown). None of these sites were adjacent, as found in the ERSR, and therefore their function is unknown. In addition, the consensus ATF-6 binding site described by Wang et al.42 (TGACGT) was also not found in the VEGF promoter region.

Induction of VEGF in RPE cells by amino acid deprivation was observed to varying degrees with all the amino acids tested, although expression of VEGF was most sensitive to glutamine deprivation. Glutamine is the most abundant amino acid in the circulation and is used by many cells and tissues at much higher rates than any other amino acid.35 Glutamine is the primary interorgan nitrogen transport vehicle and serves as an appreciable oxidative fuel source.34,35 Thus, glutaminolysis provides cells with metabolic precursors, cellular energy, and reductive equivalents. Because glutamine is such an important nutrient, it is possible that mechanisms such as adenosine monophosphate (ATP) depletion or depletion of other metabolites contribute to the cellular response to its absence. The ERSR, and presumably the UPR, pathway was activated by deprivation of amino acids, judged by the induction of GRP78. However, induction of GRP78 in response to amino acid deprivation was not as strong as induction of VEGF. Conversely, expression of GRP78 was induced more than that of VEGF by glucose deprivation and the drugs tunicamycin, thapsigargin, A23187, and brefeldin A. These data suggest that VEGF may be more responsive to the general amino acid response pathway than to the UPR pathway. The general amino acid response (or integrated stress response) pathway is believed to act through translation upregulation of expression of the transcription factor ATF4 (also known as CREB2).40 ATF4 was shown to bind to a CAAT/enhancer-binding protein (C/EBP)-ATF composite site (CATTGCACTCAG) of GADD153/CHOP and activate its transcription in response to ER-stress.41 However, no sequences corresponding to the C/EBP-ATF composite site were found in the VEGF promoter. In fact, no sequences corresponding to the range of C/EBP binding sites62 were found.

Another possibility is that transcription of VEGF is responsive to the EOR pathway. This signal pathway is induced primarily by accumulation of protein in the ER membrane and results in activation of the nuclear transcription factor NF-κB.45 Several studies have demonstrated that NF-κB DNA-binding activity is increased in response to ER-perturbing agents including tunicamycin, brefeldin A, and thapsigargin.44-47 Several recent studies have implicated NFκB in the control of VEGF expression. Sunwoo et al.48 showed that the proteosome inhibitor PS-341 inhibited expression of VEGF by squamous cell carcinoma cells and associated this effect with inhibition of NFκB activity. Huang et al.49,50 demonstrated that inhibition of NFκB activity by transfection of human prostate cancer and ovarian cancer cells with IκBα inhibited the expression of VEGF, both in vitro and in vivo. However, Bancroft et al.51 found that transfection of squamous cell carcinomas with IκBα did not inhibit VEGF expression. Others have noted an absence of NFκB sites in the VEGF genes. However, we have found that human VEGF contains three NFκB consensus sites (GGGAAT-TCCT, GGGGCTTCTC, and GGGACTCTCC) and an NFκB-like site (GGGTATTTGCCC) in the promoter region (data not shown).

The present study did not specifically address the translation of VEGF mRNA under conditions of ER stress. There is reason to believe that VEGF mRNA would not be translated under these conditions, for concomitant with increased transcription and translation of GRP genes, there is global inhibition of protein synthesis. In part, this complex response is mediated by phosphorylation of the translation-initiation factor eIF2α,15,52 and/or by translational repression through cleavage of 28S ribosomal RNA.53 However, hypoxia also creates a condition in which global protein synthesis is inhibited. To escape this effect of hypoxia, translation of VEGF is initiated in a 5’ cap-independent manner at an internal ribosome entry site (IRES), allowing its preferential expression.66 Perhaps translation of VEGF during amino acid deprivation and ER stress may rely on this mechanism as well. Recently, Fernandez et al.54 demonstrated that enhanced expression of the cationic amino acid transporter, Cat-1, during amino acid starvation is due in part to initiation of translation at an IRES found in that mRNA.

Because VEGF is a glycoprotein and therefore is dependent on the function of the ER for expression, it is also important to consider how VEGF may be processed and secreted during conditions of ER stress. Recent studies by Ikeda et al.55 suggest a possible mechanism. One of the ER-resident chaperones is the human oxygen response protein, ORP150. Although only the response of ORP150 expression to hypoxia has been studied, the human ORP150 mRNA is homologous to that of GRP170 in rodent cells, which is known to be responsive to several ER stresses.56 It seems highly significant that ORP150 binds to VEGF, and that overexpression of ORP150 facilitates secretion of VEGF, whereas inhibition of ORP150 expression causes decreased secretion and increased intracellular retention of VEGF.57,58 Thus, induction of GRP170/ORP150 represents a mechanism whereby cells experiencing ER stress may be able to support the secretion of VEGF. It is conceivable that VEGF secreted under stressed conditions is not glycosylated normally because of dysfunction of the ER and Golgi. However, we did not detect any differences in electrophoretic mobilities of VEGF proteins secreted from control, glutamine-starved, and tunicamycin-treated cells (data not shown). Pertinent to this issue are the results of Peretz et al.,59 who found that expression of recombinant VEGF by BHK cells exhibits a 10% decrease when these cells are treated with tunicamycin. Furthermore, this study found that deglycosylation of VEGF does not affect the factor’s mitogenic activity. Thus, there is reason to believe that active VEGF can be produced and secreted by ER-stressed cells.

In summary, the data herein show that the expression of VEGF mRNA by ARPE-19 cells is induced by amino acid starvation, hypoglycemia, and several agents that cause ER stress. The mechanism of induction due to glutamine starvation appears to be both transcriptional and posttranscriptional. In this way, the response of VEGF expression to glutamine starvation is similar to the response to hypoxia. The mechanism of induction under other stress conditions remains to be determined. The data suggest that expression of VEGF is responsive to an ERSR pathway, although the particular pathway(s) responsible is not known. More important, the findings suggest the possibility that an ERSR pathway may play an important role in control of VEGF expression in nutrient deprived tissues. Although hypoxia is a strong inducer of VEGF expression, it is possible that deprivation of amino acids or other nutrients may also upregulate VEGF. Finally, although this study used only ARPE-19 cells, we have also found that amino acid deprivation and ER stress upregulate VEGF expression in other cell types, including Müller cells and tumor cells.60 Thus, the results of this study may be applicable not only to retinopathy, but also to other disease characteristics, such as tumor angiogenesis.

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References


