Homocysteine Increases the Expression of Vascular Endothelial Growth Factor by a Mechanism Involving Endoplasmic Reticulum Stress and Transcription Factor ATF4*

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Vascular endothelial growth factor (VEGF) plays a key role in the development and progression of diabetic retinopathy. We previously demonstrated that amino acid deprivation and other inducers of endoplasmic reticulum-stress (ER stress) up-regulate the expression of VEGF in the retinal-pigmented epithelial cell line ARPE-19. Because homocysteine causes ER stress, we hypothesized that VEGF expression is increased by ambient homocysteine. DL-Homocysteine-induced VEGF expression was investigated in confluent ARPE-19 cultures. Northern analysis showed that homocysteine increased steady state VEGF mRNA levels 4.4-fold. Other thiol-containing compounds, including l-homocysteine thiolactone and DTT, induced VEGF expression 7.9- and 8.8-fold. Transcriptional run-on assays and mRNA decay studies demonstrated that the increase in VEGF mRNA levels was caused by increased transcription rather than mRNA stabilization. VEGF mRNA induction paralleled that of the ER-stress gene GRP78. Homocysteine treatment caused transient phosphorylation of eIF2α and an increase in ATF4 protein level. Overexpression of a dominant-negative ATF4 abolished the VEGF response to homocysteine treatment and to amino acid deprivation. VEGF mRNA expression by ATF4−/− MEF did not respond to homocysteine treatment and the response was restored with expression of wild-type ATF4. These studies indicate that expression of the pro-angiogenic factor VEGF is increased by homocysteine and other thiol-containing reductive compounds via ATF4-dependent activation of VEGF transcription.

Vascular endothelial growth factor (VEGF) is a secreted glycoprotein with endothelial cell-specific mitogenic properties and causes permeability changes in endothelial cell layers (1). VEGF has a distinct role in physiologic angiogenesis and is thus essential for normal embryogenesis (2). Regulation of VEGF expression is involved in many angiogenesis-driven pathologies including diabetic retinopathy (DR), a leading cause of morbidity among diabetic patients and the leading cause of new blindness for persons of working age (3). DR is characterized by loss of retinal capillaries leading to progressive retinal ischemia, increased retinal vascular permeability, and new retinal vessel growth. During proliferative stages of DR plasma and vitreous levels of VEGF are elevated in diabetic patients (4). Many different cell types in the eye produce VEGF, including retinal pigment epithelium, endothelial cells, pericytes, glial cells, and ganglion cells (5). An understanding of the stimuli that up-regulate VEGF expression in the diabetic retina is essential to development of effective treatments for DR. Several theories have been proposed to explain the VEGF-driven newvascularization of the retina. Oxygen tension markedly affects VEGF expression, increasing VEGF transcription and stabilizing VEGF mRNA (6). In addition, VEGF expression is increased by advanced glycation end products (AGE) (7), high glucose (8, 9), glucose deprivation (10), and by exposure to chemical inducers of ER stress (11). Thus, factors other than hypoxia may influence retinal VEGF expression and play a role in triggering the vascular complications associated with diabetes.

Although controversial (12, 13), several studies have shown an association between homocysteinemia and vascular diseases, including the development of DR (14, 15), ocular venous occlusion (16, 17), and neovascular age-related macular degeneration (18). However, a mechanism by which elevated homocysteine could contribute to development of retinal neovascularization is obscure. Moore et al. (19) demonstrated that intraocular injection of homocysteine in mice stimulated N-methyl-D-aspartate (NMDA) receptors and apoptotic cell death in the retinal ganglion cell layer in a manner that resembled early diabetic degenerative processes. However, these authors did not examine the effect of homocysteine on retinal VEGF expression, permeability or neovascularization. Another effect of homocysteine is perturbation of protein disulfide formation and protein folding within the endoplasmic reticulum (ER) (20, 21).

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¶ The abbreviations used are: VEGF, vascular endothelial growth factor; AARE, amino acid response elements; ATF4, activating transcription factor 4; DN, dominant negative; DR, diabetic retinopathy; DTT, dithiothreitol; eIF2α, eukaryotic initiation factor 2α; GADD153, growth arrest and DNA damage inducible gene 153; GFP, green fluorescent protein; GRP78, glucose-regulated protein 78; IRE1, human homologue of the yeast Ire1p inositol prototrophy gene; MEF, mouse embryonic fibroblasts; NSRE, nutrient stress response elements; PERK, PKR-like ER kinase; UPR, unfolded protein response; WT, wild-type; XBP-1, X-box binding protein-1; ER, endoplasmic reticulum; DMEM, Dulbecco’s modified Eagle’s medium; AS, asparagine synthetase; Hcys, homocysteine.
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21). Homocysteine elicits ER stress-responsive genes, including GRP78, GADD153, and ATF4 (22, 23). Werstuck et al. (24) showed that the altered gene expression in homocysteine-treated cells was due to activation of the ER stress response pathway known as the unfolded protein response (UPR).

The UPR represents a set of signaling cascades by which conditions within the ER are communicated to the protein translation machinery and to the nucleus in order to balance the folding capacity of the ER with the protein processing demand (25). These cascades are activated when the function of the ER is perturbed, or when the substrate protein burden on the ER outstrips the processing capacity. One branch of the UPR is initiated by activation of PERK, an eIF2α kinase. Phosphorylation of eIF2α inhibits GDP/GTP exchange and thus the re-formation of ternary translation initiation complexes, thereby slowing global protein translation. Decreased initiation paradoxically leads to increased expression of ATF4.

The structure of ATF4 mRNA includes a number of short upstream open reading frames (uORF) that precede the functional coding sequence. Phosphorylation of eIF2α creates conditions that favor downstream re-initiation and synthesis of ATF4 in ER-stressed and nutrient-deprived cells (26). Ultimately, ATF4 induces the expression of numerous genes, including genes involved in amino acid import, metabolism, and assimilation (27).

In this study, we demonstrate that homocysteine is a novel inducer of VEGF expression in a human retinal pigmented epithelial cell line (ARPE-19) and that homocysteine increases VEGF expression due to ATF4-dependent activation of VEGF transcription.

EXPERIMENTAL PROCEDURES

Cell Culture—ARPE-19 cells (American Type Culture Collection) were maintained in Dulbecco’s modified Eagle’s medium (DMEM, low glucose formulation) supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B. Mouse embryo fibroblasts (MEF) obtained from homozygous and heterozygous ATF4 knockout mice (28) were maintained in DMEM (high glucose formulation) supplemented with 15% (v/v) fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 4 mM glutamine, and 10 µM β-mercaptoethanol. For Northern blotting experiments, cells were plated in 10-cm tissue culture dishes and grown to confluency. One day prior to treatment, cells were rinsed twice with Dulbecco’s phosphate-buffered saline and fed with fresh medium. rt-Homocysteine, t-Homocysteine, thiolactone, l-homocysteine thiolactone, or dithiothreitol (DTT) (Sigma) were prepared fresh in DMEM and sterilized by filtration before added to the cell cultures. Reduced t- and t-homocysteine were generated as previously described (29). Briefly, o- and l-homocysteine thiolactone were incubated in 5 µL NaOH for 5 min at 20 °C, the solutions were then neutralized with HCl, diluted in DMEM, and administered to the cell cultures. For ELISA experiments, 105 cells per well were plated in 6-well plates, grown to confluence, fed with fresh medium 1 day prior to rinse with DPBS (2×), and fed with fresh medium (3 ml per well) containing rt-homocysteine or normal medium. For mRNA decay experiments, cells were treated with homocysteine for 3 h, followed by a dose of 5 µg/ml actinomycin D (actD) (Sigma) and then incubated for various times as described in a figure legend. For experiments utilizing adenoviral vectors, subconfluent cultures of ARPE-19 cells were treated with either ATF4 wild-type (WT), ATF4 mutant (DN), or the empty AdEasy vector (empty). Twenty hours postinfection, the percentage of cells infected was determined from the expression of green fluorescent protein (GFP). Anoxic conditions were created by sealing cell cultures within plastic bags along with a filter paper bag containing finely divided iron (Anaerocult™, Merck, Darmstadt, Germany). Upon addition of water, an oxidative reaction produces an oxygen-free environment confirmed by a colorimetric indicator strip (Anaerotest™, Merck).

Northern Blot Analysis—Northern blotting was performed as previously described (30) using cDNAs corresponding to human VEGF (dEST189750), GADD153 (dEST 298470), GRP78 (HAEAC89, ATCC), and asparagine synthetase (B752279 IMAGE Clone), and normalized to 18 S rRNA. 18 S rRNA template was generated using mouse RNA and the one-step PCR reaction kit GeneAmp® Gold RNA PCR Reagent kit (Applied Biosystems, Foster City, CA) with the following primers (5'-GCTACACATCTCAAGGAGGAGGCGC-3') and (5'-CCGTTGGTACCTAAATTAGGC-3'). Total RNA was isolated by the one-step acid-phenol guanidinium procedure (31) utilizing RNA-Stat60™ Reagent (TelTest, Friendswood, TX) according to the manufacturer’s protocol followed by an additional acid-phenol, phenol/ chloroform/isooamyl alcohol, chloroform extraction, and ethanol precipitation in the presence of 0.1 volumes of 3 M sodium acetate. Total RNA (12 µg/lane) was fractionated on 0.2 M formaldehyde/1% (w/v) agarose gels and transferred overnight onto a 0.45 micron Magna nylon membrane (Osmonics, Westborough, MA) in 10× SSC. The RNA was cross-linked using a CL-1000 UV cross-linker (UVP, Upland, CA) before hybridization. Specific probes were generated by labeling the cDNA with [32P]dCTP using a random primer DNA labeling kit (Amersham Biosciences). Membranes were hybridized with radiolabeled DNA probe for 6–8 h at 60 °C in a solution containing 7% (v/v) SDS, 0.25 M NaHPO4, pH 7.2, as described elsewhere (32). Blotting results were quantified by overnight exposure to a phosphor screen followed by analysis using a STORM phosphorimager and ImageQuant™ software (Molecular Dynamics, Sunnyvale, CA). For each sample, hybridization to 18 S rRNA was used to normalize results for mRNAs. Fold-inductions were determined by dividing normalized mRNA band intensity volumes for experimental samples to that of control (untreated or time 0) samples. RNA decay analysis was performed using the single compartmental model (see Ref. 33 for review of this model to mRNA decay data). Half-life was calculated as ln2/k, where k is the decay constant estimated by analysis of kinetic data. Decay constants used to calculate half-lives of VEGF mRNAs were estimated by fitting semi-log plots of (corrected, normalized mRNA concentration) versus time (30).

RT-PCR Analysis—Complementary DNA (cDNA) was synthesized from 100 ng of total RNA using a RNA PCR reagent kit GeneAmp® Gold RNA PCR Reagent kit. Polymerase chain reaction (PCR) amplification was performed under the following conditions: 95 °C for 1 min, 54 °C for 1 min, and 72 °C for 1 min; 40 cycles; followed by a 4-min 72 °C incubation performed in a DNA thermal cycler (PerkinElmer Life Sciences). The PCR products were size-fractionated by agarose gel electrophoresis using 3.3% (w/v) acrylamide (Diversified Biotech, MA) according to manufacturer’s recommendations and stained with 0.5 µg/ml ethidium bromide. Products were photographed under UV light using the GENEGENOME® imaging system (Synergene, Cambridge, UK). Forward and reverse primers used for the amplification of the edited segment of XBP-1 mRNA were 5'-GAAGCAGGAGGAGTGAAGTGAGG-3' and 5'-CATGGAAGATGTTCCGAGGAGG-3', respectively.

Nuclear Run-on Assay—Trilipic 15 cm plates were treated as described in the figure legend. 3 h post-treatment cells were mechanically removed from flask by scraping in cold phosphate-buffered saline. Cells were pelleted at 500 × g for 5 min at 4 °C. Cell pellets were resuspended in 4 ml of lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 10% (v/v) glycerol, 1 mg/ml MgCl2, 0.5% (v/v) Nonidet P-40), gently homogenized on ice for 10 min. Nuclei were pelleted and washed once with cold lysis buffer. Nuclear pellets were resuspended in 130 µL of RNase free water along with 1 µL of Rnasin and 150 µL of 2× reaction buffer (10 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 0.3% KC1, 20 µM ATP, 1 µM CTP, 1 µM GTP, and 1 µM UTP). Transcription was initiated by addition of 10 µL of 10 mM/cell of [32P]dATP. Nuclei were then incubated in a 30 °C water bath for 30 min. The reaction was then terminated by addition of 50 µL of DNase I buffer (60 mM Tris-HCI, 3.0 mM NaCl, 30 mM MgCl2, 12 mM CaCl2) and ~600 units of DNase I followed by incubation for 10 min at 30 °C. Protein digestion was then carried out at 42 °C for 30 min with the addition of 20 µL (5% (w/v) SDS, 0.5 µM Tris-HCl, pH 7.4, 0.125 µM EDTA) to the transcription reaction described above. After lysis digestion, the nuclear RNA was extracted with the RNeasy® protocol (Qiagen, Valencia, CA) according to the manufacturer’s protocol. Labelled nuclear RNA was captured by immobilized, in vitro transcribed chloramphenicol acetyl transferase, GAPDH, and VEGF antisense RNA probes. The DNA templates were derived by PCR using the following primers: (5'-AAACGACCAAACTAAACCACCACTACAATCG-3') and (5'-TATAAGCAGCACTATAGGCGTTCTTCTGGTTCGCTTACCTGGGGC-3'), GAPDH (5'-TGCACTGGTCCTTCTGAGGTGGGAGGG-3') and (5'-TATAAAGCAGCACTATAGGCGTTCTTCTGGTTCGCTTACCTGGGGC-3'), chloramphenicol acetyltransferase (5'-TAATTACGACGTAATTTTACTATATGAGGTGTATGAGGTGTATGAGGAGGG-3') and (5'-TTAAATCAGCAGCACTATAGGCGTTCTTCTGGTTCGCTTACCTGGGGC-3'). 1 µg of each template DNA was then used to synthesize unlabeled antisense RNA probes using MAXIScript™ in vitro transcription kit (Ambion, Austin, TX). RNA was then blotted onto nylon membrane as described previously (35). Mem
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branes were blocked for 30 min in 5 ml of ULTRAhyb® (Ambion) at 68 °C. Hybridization with the labeled nuclear RNA (1–3 × 106 cpm/ml) was then carried out for 24 h at 68 °C in ULTRAhyb®. Washing of membranes and detection of captured RNA transcripts was performed as described for Northern blotting analysis (above).

ELISA—ELISA assays were performed using commercial VEGF ELISA kits from R&D Systems (Minneapolis, MN). Conditioned media was collected from cells, aliquoted, and stored frozen until assayed. Samples were diluted 10-fold in dilution buffer provided with the kit prior to assaying. Assays were performed in triplicate, and the readings were compared with standard curves obtained with human recombinant VEGF165 provided with the kit.

ATF4 Adenoviral Vector Construction—Wild-type and DN mutant ATF4 cDNAs were expressed using the AdEasy adenoviral vector system (36) provided by Bert Vogelstein (Howard Hughes Institute, Johns Hopkins University). Plasmids containing murine wild-type ATF4 (WT) cDNA and DN mutant ATF4 (DN) cDNA were kindly provided by Jawed Alam (Department of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center) who generated the ATF4 WT plasmid (pEPMATF4muc) by inserting the murine ATF4 cDNA into the PmlI site of the pEF/myc/mito vector (Invitrogen), thus removing the mitochondrial targeting sequence (37). These authors constructed the ATF4∆RR (DN) plasmid (pEFMATF4m) by PCR-generated overlap extension to create seven amino acid substitutions within the DNA-binding domain (292RYRQKKR298 to 292GYLEAAA298). The mutant cDNA was cloned into the PmlI-NotI sites of the pEF/myc/mito vector, again removing the mitochondrial targeting sequence. To clone into the AdEasy system, WT and DN coding sequences from these vectors were amplified by PCR using a 5′-NotI-containing (underlined) forward primer (5′-AACAAACACGCGGGCGCTGTGTAACACCAT-GACCGAG-3′) and a 5′-HindIII-containing (underlined) reverse primer (5′-GTGTTAACGCTTTAGACTTGGCCGCCCATTCGAC-3′). The PCR reaction was carried out with Taq polymerase (Applied Biosystems) under the following conditions: 95 °C 1 min, 55 °C 2 min, 72 °C 1 min, for 35 cycles followed by a 7-min 72 °C incubation. The PCR products were cloned into the pAdTrack-CMV adenoviral shuttle vector encoding kanamycin resistance and containing a second expression cassette encoding GFP. The shuttle vectors were then linearized with the restriction enzyme PmeI and electroporated into DH5α competent cells along with the adenoviral backbone plasmid pAdEasy-1. Clones containing recombinant plasmids pAE-ATF4-Wt and pAE-ATF4-DN, formed by homologous recombination, were subsequently selected for kanamycin resistance and identified by plasmid size in conjunction with endonuclease analysis. At this stage the coding sequences and insertion points of pAE-ATF4-Wt and pAE-ATF4-DN cDNAs were confirmed by the University of New Mexico School of Medicine DNA Sequencing Facility. Recombinant adenoviral vectors were generated by transfecting the 293 packaging cell line with PacI-linearized pAE-ATF4-Wt and pAE-ATF4-DN, formed by homologous recombination, and then selecting for the presence of kanamycin resistance. The adenoviral stocks were then titered in 293 cells to yield 3 × 1010 plaque-forming units (PFU)/ml. The adenoviral stock was prepared by concentration of the viral supernatant using a centrifugation at 80,000 × g at 4 °C for 1 h followed by dialysis against phosphate-buffered saline. The virus was then titered in 293 cells to yield 104 PFU/ml. The pH of the virus stock was adjusted to 5.0 by addition of Tris-HCl (pH 8.0), and the virus was concentrated by ultracentrifugation. The virus was then diluted to 108 PFU/ml and stored at –80 °C until used.

RESULTS
Response of VEGF, GADD153, and GRP78 mRNA Expression in ARPE-19 Cells to Homocysteine and other Thiol Containing Compounds—To test the hypothesis that expression of VEGF mRNA is responsive to homocysteine and reductive stress, confluent ARPE-19 cells were cultured for 4 h in normal medium (Control), medium containing 10 mM dl-homocysteine, medium containing 10 mM l-cysteine, medium containing 10 mM 1-homocysteine thiolactone (l-Hcy-Thio), or medium containing 5 mM DTT (B). Total RNA was isolated and Northern blotting analysis with a VEGF probe was performed. In addition, the expression of the genes for GADD153 (28) and GRP78 (29) was analyzed by Northern blotting and RT-PCR analysis. Northern blotting analysis revealed that the expression of VEGF mRNA was induced by dl-homocysteine, l-cysteine, and 1-homocysteine thiolactone (Fig. 1A). RT-PCR analysis showed that the expression of GADD153 mRNA was induced by dl-homocysteine, l-cysteine, and 1-homocysteine thiolactone (Fig. 1B). The expression of GRP78 mRNA was induced by dl-homocysteine, l-cysteine, and 1-homocysteine thiolactone (Fig. 1C). The results suggest that the expression of VEGF, GADD153, and GRP78 mRNA is responsive to homocysteine and reductive stress.

FIG. 1. Expression of VEGF and ER stress-responsive genes GRP78 and GADD153 in response to homocysteine and other thiol containing compounds. A, Northern blotting analysis with coinciding numerical values of relative mRNA inductions. Confluent ARPE-19 cells were cultured for 4 h in normal medium (Control), medium containing 10 mM dl-homocysteine, medium containing 10 mM l-cysteine, medium containing 10 mM 1-homocysteine thiolactone (l-Hcy-Thio), or medium containing 5 mM DTT (B). Total RNA was isolated and Northern blotting analysis was performed. C, RT-PCR analysis of XBP-1 mRNA splicing by IRE1. RT-PCR analysis was performed with primers flanking the XBP-1 mRNA splice site using total RNA. Smaller bands demonstrate the removal of the 26-bp segment by IRE1 endonuclease.

ARPE-19 cells were cultured for 4 h in the following: 1) normal medium; 2) medium containing 10 mM dl-homocysteine; 3) medium containing 10 mM l-cysteine; 4) medium containing 10 mM 1-homocysteine thiolactone (the intramolecular ring counterpart to homocysteine); and 5) medium containing 5 mM DTT (a reducing agent known to cause ER stress). Total RNA was isolated, and normalized mRNA contents for each sample were compared with that of the untreated control (Fig. 1A). The ARPE-19 cells contained detectable amounts of a single mRNA species corresponding to VEGF. The amounts of VEGF mRNA relative to 18 S rRNA were increased 4.4-fold by dl-homocysteine, 7.9-fold by 1-homocysteine thiolactone, and 8.8-fold by DTT. Cysteine caused an apparent 1.8-fold increase in VEGF mRNA content that was not statistically significant. To differentiate between the d- and l- isomers of homocysteine, d- and l-homocysteine thiolactone were hydrolyzed to produce d- and l-homocysteine, and cells were treated with 10 mM of each compound. Both d- and l-homocysteine were equally capable of inducing VEGF expression (data not shown), suggesting that the effect did not depend upon the cellular metabolism of homocysteine.

The expression of ER stress-responsive genes was also examined to determine if these treatments caused activation of the UPR. The expression of GADD153 was also up-regulated 4.5-fold by dl-homocysteine, 1.9-fold by l-homocysteine thiolactone, and 3.8-fold by DTT. l-cysteine did not significantly up-regulate GADD153 expression compared with the control. In
addition, GRP78 was up-regulated 7.3-fold by DL-homocysteine, 3.3-fold by L-homocysteine thiolactone, and 4.8-fold by DTT. L-Cysteine did not significantly affect GRP78 mRNA expression. These results are in good agreement with past studies demonstrating the effect of homocysteine on these genes (22, 23).

The ability of thiol-containing compounds to activate an ER stress response was further evaluated by measuring the splicing of X-box binding protein-1 (XBP-1) mRNA. Upon activation of the UPR, the ER transmembrane protein IRE1 acquires an endonuclease activity that subsequently excises a 26-base pair segment from XBP-1 mRNA, thereby, upon ligation, creating the open reading frame that encodes the transcription factor (38). RT-PCR analysis with primers flanking the splicing site served as an extremely sensitive measure of UPR activation. Fig. 1B shows that a large fraction of XBP-1 mRNA is spliced in cells treated with DL-homocysteine, L-homocysteine thiolactone, and DTT. No XBP-1 splicing was detected in the control cells. Treatment with 10 mM cysteine caused only a very small amount of XBP-1 splicing that is illustrated by overloading of that PCR sample.

The Time and Dose Response of VEGF to Homocysteine—To better define the rapidity of this response, a time course study was carried out with hourly measurements from 1 to 5 h in the presence of medium containing 0.1, 1.0, and 10 mM DL-homocysteine (Fig. 2). The time course for 0.1 mM DL-Hcys showed a 2.0-fold increase in VEGF mRNA content within 1 h of treatment. The response to 0.1 mM DL-Hcys was maximal at 2.0 h with a 3.8-fold induction and decreased rapidly thereafter. The up-regulation of GRP78 mRNA expression correlated closely with the up-regulation of VEGF mRNA during the 0.1 mM time course. The time course seen with 1.0 mM showed a similar but more extensive induction of VEGF mRNA, with an apparent increase within 1 h that reached 5.2-fold maximum induction at 2 h, followed by a rapid decline of mRNA levels. The 10 mM homocysteine time course experiment demonstrated an initial VEGF mRNA up-regulation occurring within 1 h and increasing until 5 h, at which time the relative VEGF mRNA level reached 12-fold the initial content. The response to 10 mM homocysteine returned to basal levels at 16 h (data not shown). The time course of GRP78 mRNA response also mirrored the VEGF response in cells treated with 1.0 and 10 mM homocysteine. These data show that the induction of VEGF in response to homocysteine treatment was time-and-dose-dependent and that the up-regulation of an ER stress-responsive gene resembled the induction of VEGF.

Secretion of VEGF by Homocysteine-treated Cells—Global
protein synthesis is attenuated during the UPR through phosphorylation of the translation initiation factor eIF2α (39). Since VEGF is a glycosylated and secreted protein, its synthesis is dependent on ER function. We therefore tested whether VEGF was secreted during homocysteine-induced ER stress. The levels of VEGF secreted in the medium from cells treated with 10 mM homocysteine were increased 1.3-fold (p = 0.024) relative to untreated controls (data not shown). This level of secretion corresponds closely with the previously demonstrated 1.3-fold increased secretion of VEGF observed with glutamine-deprived ARPE-19 cells (11). Although VEGF protein accumulation was significantly increased, it was not proportional to the observed increase of VEGF mRNA levels caused by the same treatment. While this is probably due in part to the transient nature of homocysteine-treated cells harvested, and the nuclear run-on assay was performed. B, quantitative analysis of triplicate assays.

**Fig. 3. Effect of homocysteine on VEGF mRNA decay rate.** Cells were treated for 3 h in the absence or presence of 10 mM homocysteine, followed by addition of 5 µg/ml actinomycin D. Total RNA was collected at the times indicated and Northern blotting analysis performed. Semilog graph of normalized relative mRNA levels, corrected by subtraction of the values obtained after 6 h of decay, versus time along with lines obtained by linear regression are shown.

**Fig. 4. Effect of homocysteine on VEGF mRNA decay rate.** Cells were treated for 3 h in the absence or presence of 10 mM homocysteine, followed by addition of 5 µg/ml actinomycin D. Total RNA was collected at the times indicated and Northern blotting analysis performed. Semilog graph of normalized relative mRNA levels, corrected by subtraction of the values obtained after 6 h of decay, versus time along with lines obtained by linear regression are shown.

**The Effect of Homocysteine on VEGF mRNA Stability—**Hypoxia and glutamine deprivation increase VEGF expression by both transcriptional and post-transcriptional mechanisms (11, 40). We investigated the effect of homocysteine on mRNA degradation rate to differentiate between transcription and post-transcriptional effects on VEGF expression. Confluent ARPE-19 cells were treated with 10 mM Dl-homocysteine for 3 h, causing VEGF mRNA levels to increase, and then were treated with 5 µg/ml actinomycin D (actD). Control and homocysteine-treated cells were harvested at 0.24 h⁻¹ (r = 0.80) and 0.48 h⁻¹ (r = 0.99) were obtained for VEGF mRNA decay in control and treated cells, respectively, suggesting that homocysteine did not cause stabilization of VEGF mRNA. In fact, there was an apparent destabilization of VEGF mRNA upon homocysteine treatment. Although the basal VEGF decay rate obtained agrees with previous reports from other cell types (41–43), it should be noted that accurate measurement of VEGF mRNA decay in untreated cells was difficult due to the low basal level. Regardless, it appeared that mRNA stabilization could not account for the increase of VEGF mRNA levels produced by homocysteine. This suggests that homocysteine causes transcriptional activation of the VEGF gene.

**Transcriptional Up-regulation of VEGF—**To further investigate the role of transcription in the up-regulation of VEGF mRNA a nuclear run-on assay was conducted in ARPE-19 cells treated with either normal medium or 10 mM Dl-homocysteine. A slot blot capture of radiolabeled nuclear RNA. Triplicate 15-cm plates were treated with normal medium (Control) or 10 mM Dl-homocysteine for 3 h at which time the nuclei were harvested, and the nuclear run on assay was performed. B, quantitative analysis of triplicate assays.

**The Role of ATF4 in the Expression of VEGF in ARPE-19 Cells—**To examine the role of ATF4 in the up-regulation of VEGF expression, the AdEasy adenoviral vector system was used to express dominant-negative (ATF4 DN) and wild-type (ATF4 Wt) proteins (37). First, the effects of various amounts of Wt and DN mutant ATF4 adenovector stocks (1:20 to 1:160 dilutions) on the intracellular level of ATF4 antibody-reactive protein were examined at 20-h postinfection (Fig. 6A). Viral infection was assessed by determining the percentage of cells that exhibited GFP fluorescence. ATF4 expression was evaluated by Western blotting analysis. Both of the exogenously produced ATF4 proteins were antigenic. Although infection rates were comparable, the expression of the ATF4 DN mutant was approximately twice that of the ATF4 Wt protein at each viral stock dilution. Exogenously produced proteins were expressed at levels much higher than those of the endogenous ATF4. A titration curve for the empty virus was determined by

**Fig. 4. Effect of homocysteine on VEGF transcription rate.** A, slot blot capture of radiolabeled nuclear RNA. Triplicate 15-cm plates were treated with normal medium (Control) or 10 mM Dl-homocysteine for 3 h at which time the nuclei were harvested, and the nuclear run on assay was performed. B, quantitative analysis of triplicate assays.
evaluating the percentage of cells exhibiting GFP fluorescence 20-h postinfection. Viral infection of ARPE-19 cells with 1:40, 1:80, and 1:80 dilutions respectively of Wt, DN, and empty virus resulted in infection rates >90% for each (data not shown).

To evaluate the role of ATF4 function in the induction of VEGF expression by various stresses, ARPE-19 cells were infected with empty, ATF4 Wt, and ATF4 DN adenovectors. 20 h after contact with the viruses, cells were fed with fresh medium, and then cells from each virus treatment group were subjected to the following: no treatment (incubated with control medium for 5 h), incubated with medium containing 10 mM DL-homocysteine for 4 h, incubated with medium containing no glutamine (−Q) for 5 h, or incubated in normal medium but subjected to anoxia for 5 h. VEGF mRNA expression was then analyzed by Northern blotting (Fig. 6). VEGF mRNA was up-regulated by DL-homocysteine, nutrient deprivation (−Q), and anoxia. Compared with cells infected with empty vector, the expression of Wt ATF4 increased VEGF mRNA levels in the control cells and had little effect on VEGF levels in DL-homocysteine-treated and glutamine-starved cells. In contrast, the expression ATF4 DN greatly inhibited VEGF expression under all conditions, including anoxia. However, under anoxic conditions the response was somewhat different, in that both the Wt and DN proteins greatly reduced VEGF expression. These results indicate that ATF4 positively affects VEGF mRNA expression, as would be expected if this factor activates VEGF transcription. In addition, ATF4 function is necessary for induction of VEGF expression by homocysteine and glutamine deprivation. However, because both Wt and DN ATF4 protein diminish induction of VEGF mRNA expression during anoxia, it is most likely that these proteins are sequestering an ATF4 binding partner that plays a role in hypoxic induction of this gene.

The expression ATF4 Wt slightly increased the expression of GADD153 mRNA in the control cells. The induction of GADD153 expression in response to homocysteine, nutrient deprivation, or anoxia was inhibited by the expression of ATF4 DN. Surprisingly, both the Wt and DN viruses blocked induction of GADD153 by DL-homocysteine. GRP78 was recently shown to be an ATF4-responsive gene (44). These data confirm the recent findings of Luo et al. (44) using these same adenoviral vectors. That study demonstrated that ATF4 Wt protein expression increased GRP78 mRNA expression and ATF4 DN expression repressed GRP78 expression under all treatment conditions (44). Asparagine synthetase (AS), a gene known to be ATF4 responsive (45), was greatly induced by the Wt virus in all treatment groups. Unexpectedly, both Wt and DN ATF4 induced AS under anoxic conditions.

VEGF Expression in ATF4−/− MEF—To further confirm the ATF4 DN results, the effect of homocysteine on VEGF expression was examined in MEF cultures obtained from homozygous (−/−) and heterozygous (+/−) ATF4 knockout mice. The absence of ATF4 was demonstrated in ATF4−/− MEF via Western blotting (Fig. 7A). Again, this antibody detected two nonspecifically immunoreactive bands corresponding to the bands observed with ARPE-19 lysates (Fig. 5A). However, only in lysates from
ATF4+/− MEF was a band of the expected size of ATF4 protein detected. Both MEF cell types were treated with either 10 mM dl-homocysteine (4 h) or glutamine-starved for 5 h. The band corresponding to ATF4 greatly increased in intensity with homocysteine treatment, but was not increased with glutamine deprivation.

To determine the role of ATF4 in VEGF expression, the treatments described in Fig. 6 were repeated with the

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**Fig. 7.** Effects of homocysteine treatment and nutrient deprivation on VEGF expression in ATF4+/− MEF. A, Western blot analysis of ATF4 expression in wt-Hcys treated and glutamine (−Q)-starved cells in ATF4+/− and ATF4+/− MEF. MEF were treated with 10 mM dl-homocysteine (4 h) or glutamine starved for 5 h. B, Northern blot analysis of VEGF, GRP78, and GADD153 expression in MEF. MEF were treated with either 10 mM dl-homocysteine (4 h), glutamine-starved (5 h), or anoxia (5 h). Fold inductions of VEGF mRNA are shown. C, Northern blotting analysis of VEGF, GRP78, and GADD 153 mRNAs in ATF4+/− MEF. Cells received no treatment (control), 10 mM dl-homocysteine (4 h), homocysteine + ATF4 Wt, or homocysteine + ATF4 DN. Fold inductions of VEGF mRNA are shown.
ATF4+/- and ATF4−/− MEF cells. ATF4+/- MEF had a higher basal level of VEGF mRNA compared with the ATF4−/− MEF (Fig. 3B). The ATF4−/− MEF were not capable of increasing VEGF mRNA levels following homocysteine treatment. In contrast, both the ATF4−/− and ATF4+/− MEF were capable of inducing VEGF following glutamate starvation and anoxia. When ATF4 WT protein was introduced by adenoviral infection, ATF4−/− MEF did increase VEGF mRNA expression in response to homocysteine treatment (Fig. 7C). In contrast, ATF4 DN had very little effect on the VEGF mRNA induction. This analysis also confirmed that induction of GRP78 mRNA expression was dependent upon ATF4 protein. Although ATF4 was not essential for GRP78 mRNA induction by homocysteine (Fig. 7B), ATF4 WT protein did greatly increase the induction (Fig. 6C). This analysis also demonstrated that ATF4 protein, if not essential, is important to GADD153 mRNA induction in response to homocysteine. In conclusion, ATF4 plays a vital role in the induction of VEGF expression by MEF and ARPE-19 cultures in response to homocysteine treatment.

DISCUSSION

The present study includes the following novel findings. 1) Homocysteine induced the expression of VEGF mRNA in a time- and dose-dependent manner in a retinal-pigmented epithelial cell line, and D- and L-homocysteine had the same effect. 2) Homocysteine thiolactone and DTT also induced VEGF expression. 3) Homocysteine did not cause VEGF mRNA stabilization. 4) Homocysteine increased transcription from the VEGF gene. 5) Homocysteine, its thiolactone and DTT induced the expression not only of VEGF but also ER stress-responsive genes and the activation of IRE1 endonuclease activity (as measured by XBP-1 mRNA splicing). 6) Homocysteine treatment produced a transient phosphorylation of eIF2α followed by an increase in ATF4 protein level. 7) Inhibition of ATF4 function abrogated the VEGF response to homocysteine and to glutamine deprivation by ARPE-19 cells; and 8) ATF4−/− MEF were unable to induce VEGF in response to homocysteine treatment and the VEGF response was restored in the ATF4−/− MEF with expression of WT ATF4.

Since VEGF was elevated by both the D- and L-isomers of homocysteine, it is likely that the up-regulation of VEGF is a chemical effect that does not require metabolism of this amino acid. The up-regulation of VEGF by homocysteine thiolactone and DTT suggest that the thiol group may be key to the induction of VEGF. We suggest that the reductive potential of these compounds interferes with thiol oxidation and protein disulfide formation in the ER, thus inhibiting correct protein folding. Such is the case for DTT (46, 47). The amino acid l-cysteine had no significant effect on VEGF expression. In addition, homocysteine, thiolactone and DTT caused ER stress, whereas cysteine did not. A previous study also reported that cysteine did not cause ER stress (24). Whether this is the result of a lower reductive potential of the thiol group, cellular distribution, or cellular metabolism is not known.

Treatment of ARPE-19 cells with fresh medium containing homocysteine resulted in temporary increases in VEGF and GRP78 mRNA levels. The transient nature of these responses may be due to the fact that homocysteine is unstable in medium and thus causes a short-lived reductive insult. However, by measuring the amount of reactive free thiols in medium using 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), we estimated that reduced homocysteine decays with a half-life of 25 h in the medium used (data not shown). Thus, an initial concentration of 10 mM homocysteine would still be ~6 mM by 16 h, at which time the VEGF response had effectively run its course.

Regardless of the cause, VEGF mRNA levels responded to homocysteine in a temporal manner that correlated closely to that of GRP78 mRNA. Because the response of GRP78 expression is known to be transcriptional, the correlation is consistent with the idea that transcription of VEGF is ER stress-responsive. This relation supports our past finding linking nutrient deprivation and chemical inducers of ER stress to the transcription of VEGF (11). mRNA decay rates indicated that homocysteine did not cause VEGF mRNA stabilization. In fact, mRNA decay curves showed a slight destabilization of VEGF mRNA by homocysteine. This is an unexpected result that should be interpreted with caution since measurement of VEGF decay below basal levels was difficult to achieve. We have interpreted the results only as indicative of a transcriptional up-regulation of VEGF, rather than a post-transcriptional mechanism. Nuclear run-on assays confirmed that homocysteine increased the rate of transcriptional initiation of the VEGF gene.

Furthermore, our results demonstrate that homocysteine causes a transient phosphorylation of eIF2α followed by an increase in ATF4 protein levels. This complements previous studies that indicated that ATF4 mRNA expression is induced by homocysteine (23, 48). ATF4 is a transcription factor that is expressed in response to nutrient and ER stress. ATF4 mRNA levels are increased in response to activation of the UPR and translation of the ATF4 protein is responsive to phosphorylation of eIF2α (26).

Conditions that are known to activate eIF2α kinases include ER stress, amino acid deprivation, the presence of double-stranded RNA and heme deficiency. ATF4 may therefore be a common integrated response to signaling by stress-induced eIF2α kinases. The phosphorylation of eIF2α and the resultant increase in ATF4 precede temporally the up-regulation of VEGF mRNA, which is consistent with their involvement in VEGF gene regulation. A DN ATF4 adenosinoviral vector demonstrated that VEGF up-regulation in response to homocysteine and glutamine deprivation is dependent upon ATF4 function. Overexpression of the transcription factor ATF4 increased VEGF mRNA levels and expression of a DN mutant form of this transcription factor was capable of diminishing both homocysteine and glutamine starvation-induced VEGF mRNA levels in ARPE-19 cells. The present results also confirmed that AS and GRP78 expression are greatly affected by ATF4 activity. The role of ATF4 in control of GRP78 expression was just recently discovered (44).

The ATF4 DN mutant functions by competitively inhibiting the formation of functional complexes containing endogenous ATF4 proteins. Thus, the ability of the DN mutant to abrogate the VEGF response to homocysteine, nutrient stress, and anoxia supports the role of ATF4 in the up-regulation of VEGF. However, the sequestering of ATF4 binding partners in nonfunctional complexes with the DN mutant can also inhibit the normal function of those proteins. Thus, the negative effect of ATF4 DN overexpression must be interpreted with caution. Significantly, overexpression of wild-type ATF4 was sufficient to increase VEGF mRNA levels in unstressed cells, and did not inhibit the expression in nutrient and ER-stressed cells. This rules out a mechanism of action that relies on sequestration of other factors. Such a mechanism may, however, explain the negative effects of both wild-type and DN proteins on VEGF expression in anoxic cells. Anoxia has been shown to upregulate several members of the bZIP (basic/leucine zipper domain) transcription factor class, including ATF4 (49). Fig. 6 suggests that ATF4 and its binding partners play a role in the control of VEGF under a wide range of conditions.

The results of the ATF4 DN were confirmed in ATF4−/− MEF cultures. The absence of VEGF induction in the homocysteine treated ATF4−/− MEF suggest that ATF4 is an essential factor in homocysteine induced VEGF. The ability of WT ATF4 protein expression to restore this response shows that ATF4 has a clear role in the up-regulation of VEGF under reductive stress. The
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decrease in basal levels of VEGF in the ATF4/−/− MEF suggests a broader role for ATF4 in basal VEGF expression. The ability of anoxia and glutamine deprivation to induce VEGF in the ATF4/−/− MEF points toward complexity of VEGF regulation. Glutamine deprivation was expected to resemble homocysteine based on the effects of expressing the ATF4 DN in ARPE-19 cells. However, unlike homocysteine treatment, glutamine deprivation did not increase ATF4 protein levels in the ATF4/−/− MEF. Thus, glutamine deprivation was seemingly able to induce VEGF expression by a mechanism independent of ATF4. Similar results were obtained with breast carcinoma cells.2 These data indicate varying modes of transcriptional control of VEGF in different cell types and with different stresses.

Genes regulated by ATF4 include ones involved in amino acid import, metabolism and assimilation (27). We propose that ATF4 also up-regulates VEGF in order to satisfy increased nutritional import, metabolism and assimilation (27). We propose that ATF4 involved in this induction.

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Homocysteine Increases the Expression of Vascular Endothelial Growth Factor by a Mechanism Involving Endoplasmic Reticulum Stress and Transcription Factor ATF4

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