Hypoxia-Inducible Factor-1-Dependent and -Independent Regulation of Insulin-Like Growth Factor-1-Stimulated Vascular Endothelial Growth Factor Secretion

Mark G. Slomiany and Steven A. Rosenzweig

Department of Cell and Molecular Pharmacology and Experimental Therapeutics (M.G.S., S.A.R.) and Hollings Cancer Center (S.A.R.), Medical University of South Carolina, Charleston, South Carolina

Received March 7, 2006; accepted May 4, 2006

ABSTRACT

Hypoxia-induced stress plays a central role in retinal vascular disease and cancer. Increased hypoxia-inducible factor-1α (Hif-1α) expression leads to HIF-1 formation and the production of vascular endothelial growth factor (VEGF). Cytokines, including insulin-like growth factor-1 (IGF-1), also stimulate VEGF secretion. In this study, we examined the relationship between IGF-1 signaling, HIF-1α protein turnover and VEGF secretion in the ARPE-19 retinal pigment epithelial cell line. Northern analysis revealed that IGF-1 stimulated Hif-1α message expression, whereas the hypoxia-mimetic CoCl2 did not. CoCl2 treatment increased Hif-1α protein accumulation to a greater extent than IGF-1 treatment. However, IGF-1 stimulated a more significant increase in VEGF secretion. IGF-1-stimulated VEGF promoter activity was phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR (mammalian target of rapamycin)-dependent, whereas VEGF secretion was only partially reduced by inhibition of PI3K/Akt/mTOR and HIF-1 activities. Analysis of VEGF promoter truncation mutants indicated that sensitivity to CoCl2 was hypoxia response element (HRE)-dependent with the region upstream of the HRE conferring IGF-1 sensitivity. In conclusion, IGF-1 regulates VEGF expression and secretion via HIF-1-dependent and -independent pathways.

Vascular endothelial growth factor (VEGF) is a potent angiogenic and permeability enhancing factor causally linked to neovascularization in cancer, wound healing, diabetic retinopathy, and age-related macular degeneration (Aiello et al., 1995; Adams et al., 1996). In choroidal neovascularization (CNV), which develops in 10% of all cases of age-related macular degeneration, newly formed blood vessels enter the subretinal space, where leakage and bleeding lead to retinal detachment and photoreceptor death (Amin et al., 1994). This disease represents the most common cause of severe vision loss in elderly patients in developed countries (Amin et al., 1994). Therefore, identification of the mediators of ocular angiogenesis is a current area of intense interest for targeting the development of inhibitors of CNV.

The retinal pigment epithelium (RPE), is a highly specialized monolayer of epithelial cells interposed between the retinal photoreceptors and choroid, central to photoreceptor survival and function (Strauss, 2005). The RPE is a major source of proangiogenic (VEGF) and antiangiogenic (e.g., pigment epithelium-derived factor) factors, capable of modulating the progression of CNV (Amin et al., 1994; Punglia et al., 1997; Dawson et al., 1999). Animal model studies support a role for VEGF in the progression of CNV (Cui et al., 2000; Spilsbury et al., 2000; Krzystolik et al., 2002) in which, in addition to elevated VEGF levels in the vitreous, the RPE and surrounding subretinal membranes express elevated levels of VEGF and its receptor KDR/flk-1 (Wada et al., 1999).

Accumulating evidence has revealed that the retina contains all the components for a subretinal autocrine-paracrine IGF system (Waldhöllig et al., 1991). The RPE secretes IGF-1,
IGF-2, IGFBP-3, and IGFBP-6 (Moriarty et al., 1994), and possesses IGF-1 receptors (IGF-1Rs) (Lambooj et al., 2003; Rosenthal et al., 2004). The IGFs exhibit higher affinities toward the IGFBPs relative to their affinities for the IGF-1R, enabling the IGFBPs to act as IGF antagonists, reducing IGF bioavailability through sequestration (Rosenzweig, 2004). In the RPE, IGF-1 stimulates VEGF expression (Punglia et al., 1997). On this basis, the RPE is capable of modulating retinal function as well as contributing to the pathogenesis of CNV (Rosenthal et al., 2004; Strauss, 2005).

Elevated VEGF levels observed in proliferative retinal disorders have been attributed to the hypoxic response (Aiello et al., 1995; Adams et al., 1996). Adaptation to hypoxia involves changes in the protein expression and DNA binding activity of the hypoxia-inducible factors (HIFs), the most prevalent isoform being Hif-1α. This family of transcription factors consists of an oxygen tension- and cytokine-regulated α subunit and a constitutively expressed β subunit (Brahimi-Horn et al., 2005). Under normoxic conditions, Hif-1α undergoes O2-dependent hydroxylation at prolines 402 and 564, marking it for ubiquitination and proteasomal degradation. Under hypoxic conditions or treatment with the hypoxia mimic CoCl2, ubiquitination of Hif-1α is inhibited and Hif-1α accumulates as a consequence of prolyl hydroxylase inhibition. Stabilized Hif-1α dimerizes with Hif-1β, activating transcription of target genes containing hypoxia response elements (HREs), including VEGF (Brahimi-Horn et al., 2005). In addition, various cytokines, including IGF-1, modulate Hif-1α protein expression or stability through PI3K/Akt and/or p44/42 MAPK pathways (Miele et al., 2000; Fukuda et al., 2002; Treins et al., 2002). IGF-1 signaling to Hif-1α expression and VEGF secretion is incompletely understood. Furthermore, there is growing evidence of HIF-independent mechanisms in the regulation of VEGF synthesis and secretion (Milanini-Mongiat et al., 2002; Abdelrahim and Safe, 2005; Xu et al., 2005; Slomiany et al., 2006). To this end, we compared the effects of IGF-1 and the hypoxia-mimetic, CoCl2, on Hif-1α expression, HIF-1 transcriptional activity, and VEGF transcription, synthesis, and secretion. Our findings support a role for HIF-dependent and -independent pathways mediating IGF-1-induced VEGF expression and secretion.

Materials and Methods

Materials and Reagents. The spontaneously transformed RPE cell line, ARPE-19, was obtained from the American Type Culture Collection (Manassas, VA). Fetal bovine serum was purchased from Atlas Biologicals (Fort Collins, CO). Dulbecco’s modified Eagle’s medium, wortmannin, LY294002, and CsA were purchased from Sigma Chemical (St. Louis, MO). Recombinant human IGF-1 and VEGF were generously provided by Genentech (South San Francisco, CA). Recombinant human IGFBP-3 (N109D) was obtained from Upstate (Tryon, NC) and/or transfected with human IGFBP-3 in Puc119 was obtained from Dr. Jian-Xing Ma (Oklahoma University Health Science Center, Oklahoma City, OK). Full-length IGFBP-3 in Puc119 was obtained from Irene Smith (Genentech) and cloned into pcDNA3 by Dr. Beatrix A. Slomiany, and pRL-SV40 control plasmid (Promega) was obtained from Dr. D. T. Kurtz, Medical University of South Carolina (Charleston, SC). All other chemicals were of reagent grade or higher.

Tissue Culture. ARPE-19 cells were maintained as described previously (Slomiany and Rosenzweig, 2004a,b). For whole cell lysate, secretion, and microscopy studies, ARPE-19 cells were seeded at a density per well of 8.6 x 10^4 in six-well (9.6 cm^2 area per chamber) plates, grown to confluence, and serum-starved (fetal bovine serum was eliminated in all experiments) for 24 h before the indicated treatment. After pretreatment for 2 h with fresh serum-free medium containing inhibitors solubilized in ethanol, cells were treated with ethanol vehicle, inhibitors, IGF-1, and CoCl2. For transfections, ARPE-19 cells were seeded at a density per well of 2 x 10^5 in 24-well (1.9 cm^2 area per chamber) plates. After 24 h, 50 to 80% confluent cells were transfected and subjected to the treatment indicated below.

Immunoblot and Ligand Blot Analysis. After a 15-min or 4-h incubation, whole-cell lysates for Akt and Hif-1α immunoblots, respectively, were prepared using a modified radioligand binding assay buffer containing 50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and leupeptin, 2 mM sodium orthovanadate, and 10 mM NaF. Protein content was determined by bicinchoninic acid assay, and 100-μg aliquots were solubilized in SDS sample buffer. VEGF and IGFBP-3 were quantified in conditioned medium after a 12-h incubation following precipitation in 10% trichloroacetic acid, washing the pellet with acetone, and solubilization in SDS sample buffer. Lysates and conditioned medium so collected were resolved on 10 and 15% nonreducing polyacrylamide gels, respectively, were prepared using a modified radioimmunoprecipitation assay buffer containing 50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and leupeptin, 2 mM sodium orthovanadate, and 10 mM NaF. Protein content was determined by bicinchoninic acid assay, and 100-μg aliquots were solubilized in SDS sample buffer. VEGF and IGFBP-3 were quantified in conditioned medium after a 12-h incubation following precipitation in 10% trichloroacetic acid, washing the pellet with acetone, and solubilization in SDS sample buffer. Lysates and conditioned medium so collected were resolved on 10 and 15% nonreducing polyacrylamide gels, respectively, transferred to nitrocellulose (Osmotics, Westbrook, MA) with a TE-70 SemiPhor apparatus (Hoefer Scientific Instruments, San Francisco, CA), and subjected to ligand or immunoblot analysis. IGFBP-3 was quantified by ligand blot analysis (Robinson and Rosenzweig, 2004).

Immunoblots were blocked, probed, and visualized as described previously (Slomiany and Rosenzweig, 2004b) with either 1 μg/ml VEGF polyclonal antibody, 1 μg/ml Hif-1α monoclonal antibody, 1 μg/ml Hif-1β monoclonal antibody, 1:10,000 β-actin monoclonal antibody, 1 μl/ml phospho-Akt (Ser-473), or Akt polyclonal antibodies. To reprobe Hif-1α immunoblots for Hif-1β and β-actin levels or phospho-Akt blots for Akt, phospho-44/42 MAPK, and β-actin levels, antibodies were removed from the nitrocellulose via the 3 x 10^-7 M acetic acid, and 100-μg aliquots were solubilized in SDS sample buffer. VEGF and IGFBP-3 were quantified in conditioned medium after a 12-h incubation following precipitation in 10% trichloroacetic acid, washing the pellet with acetone, and solubilization in SDS sample buffer. Lysates and conditioned medium so collected were resolved on 10 and 15% nonreducing polyacrylamide gels, respectively, were prepared using a modified radioimmunoprecipitation assay buffer containing 50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and leupeptin, 2 mM sodium orthovanadate, and 10 mM NaF. Protein content was determined by bicinchoninic acid assay, and 100-μg aliquots were solubilized in SDS sample buffer. VEGF and IGFBP-3 were quantified in conditioned medium after a 12-h incubation following precipitation in 10% trichloroacetic acid, washing the pellet with acetone, and solubilization in SDS sample buffer. Lysates and conditioned medium so collected were resolved on 10 and 15% nonreducing polyacrylamide gels, respectively, transferred to nitrocellulose (Osmotics, Westbrook, MA) with a TE-70 SemiPhor apparatus (Hoefer Scientific Instruments, San Francisco, CA), and subjected to ligand or immunoblot analysis. IGFBP-3 was quantified by ligand blot analysis (Robinson and Rosenzweig, 2004).
Microscopy Confluence. ARPE-19 cells grown on glass cover-slips were treated as indicated and fixed with 3% paraformaldehyde for 20 min at room temperature, and peroxidase activity was quenched with a 30-min incubation at room temperature in PBS containing 1% H2O2. Cells were permeabilized, and nonspecific binding was blocked by incubation for 4 h at 4°C in PBS containing 3% bovine serum albumin and 0.1% Triton X-100. After three 5-min washes with PBS containing 0.1% Triton X-100, cells were incubated overnight at 4°C with an immunohistochemistry specific polyclonal antibody for phospho-Akt (Ser-473) at a dilution of 1:50 in PBS in blocking buffer. Cells were subsequently washed three times for 5 min each in blocking buffer and incubated with 5 μg/ml Alexa Fluor 488 secondary antibody (Molecular Probes) in blocking buffer for 2 h at room temperature in total darkness. After one 5-min wash in blocking buffer, and two 5-min washes in PBS, coverslips were incubated for 10 min with Slowfade Light Antifade Kit equilibration buffer (Molecular Probes). Coverslips were mounted on slides in Slowfade Antifade reagent with glycerol buffer and sealed with nail polish. Slides were viewed on a Leica DMLB epifluorescence microscope under a 64× objective with a 10× eyepiece (640× total magnification), and images were captured with a C5810 color-chilled CCD camera (Hamamatsu Corporation, Bridgewater, NJ) in Adobe Photoshop, version 5.0 (Adobe Systems, San Jose, CA).

Construction of Northern Blot Probes. 32P-Labeled VEGF probe was derived from the full-length coding region for VEGF165 that was cloned into pCRII TA vector (Invitrogen) in the multiple cloning site. The construct was digested overnight at 37°C with EcoRI to yield the 4-kb pCRII plasmid backbone and 500-bp VEGF165 insert. 32P-Labeled IGFBP-3 probe was obtained from the construct pCEP4/IGFBP-3 (Invitrogen) in the multiple cloning site (Wood et al., 1988). The construct was digested overnight at 37°C with HindIII and XhoI to yield the 10.4-kb plasmid backbone and the 3.4-kb HIF-1 α cloning site. The construct was digested overnight at 37°C with PstI and NotI to yield the 10.4-kb plasmid backbone and the 3.4-kb HIF-1 α insert.

VEGF, IGFBP-3, and HIF-1 α cDNAs were gel-purified and desalted on P-30 Bio-Gel spin columns. 32P-Labeled probes were subsequently made using the prepared VEGF, IGFBP-3, and HIF-1 α cDNAs, the Prime-it II Random Primer labeling kit, and 32P-labeled cDNA synthesis. Probes were purified from unincorporated [32P]cDNA using P-30 spin columns.

Northern Blots. Two confluent dishes of ARPE-19 cells per treatment were serum-starved for 24 h before treatment in fresh serum-free medium with IGF-1 and CoCl2. After a 6-h incubation, cells were lysed, and RNA was isolated using the RNAqueous kit according to the manufacturer’s instructions. The RNA concentration was determined spectrophotometrically at A260. RNA from each sample (3 μg) was solubilized in formaldehyde load dye containing 10 μg/ml ethidium bromide (provided in the RNAqueous kit), heated for 15 min at 65 to 70°C, loaded on a MOPS-formaldehyde-1% agarose gel, and electrophoresed until the 18S and 28S ribosomal RNA bands were satisfactorily separated. RNA was subsequently transferred to the NC-Probe membrane by overnight capillary transfer. After a 4-h prehybridization in ULTRAhyb at 42°C, membranes were hybridized at 42°C overnight in ULTRAhyb with the 32P-labeled probe. Membranes were washed with standard saline citrate buffer, sealed in plastic wrap, and exposed to Kodak XAR-film against Kodak lighting screens at ~80°C. Blots were subsequently stripped in five consecutive 10-min washes of stripping solution (1 mM EDTA, 0.1% SDS) at 68°C to allow for subsequent rehybridizations.

Construction of VEGF Promoter Truncations. The VEGF reporter constructs used in transient transfection assays contain sequences derived from the human VEGF promoter driving expression of firefly luciferase. The 2.65 kb (bp −2361 to +298 relative to the transcription start site) VEGF promoter in the 5.6-kb pGL2 basic vector (Mukhopadhyay et al., 1997), herein denoted pGL2VEGF2.65, was used for the construction of two 5′ deletion and one HRE-excised mutations (see Fig. 6G). The truncated and HRE-excised promoter-reporter plasmids were generated on the basis of convenient restriction sites located within the VEGF promoter. Details on the construction of these truncations are presented as supplemental data.

Transfection. To assay the transcriptional activity of HIF-1 α, we used the enolase (ENO1) promoter in pGL2 basic p2.1 vector, containing a 68-bp ENO1 promoter fragment encompassing a HIF-1 α binding site upstream of the luciferase gene (Semenza et al., 1996). Subconfluent ARPE-19 cells in 12-well plates were transfected with 500 μl of serum-free medium containing 1 μl of Fugene 6 transfection reagent, 100 ng of p2.1, and 50 ng of pRL-SV40 control plasmid. Mixtures were first incubated at room temperature for 30 min before addition to the cells. After 24 h, cells were preincubated in 500 μl of fresh serum-free medium containing either 10 μg/ml CsA or vehicle alone (1 μl/ml ethanol) for 1 h. Cells were subsequently treated with 100 nM IGF-1 or 100 μM CoCl2 in 500 μl of fresh serum-free medium containing either 10 μg/ml CsA or 1 μl/ml ethanol per well.

The transcriptional activity of the full-length and truncated VEGF promoter constructs was assayed as above, except that each well was transfected with 500 μl of serum-free medium containing 1.2 μl of Fugene 6 transfection reagent, 10 ng of pRL-SV40 control plasmid, and 200 ng of pGL2VEGF 2.65, pGL2VEGF 1.46, pGL2VEGF 0.76, pGL2VEGF 1.2 + 0.76, or pGL2 basic control vector. After 24 h, cells were treated with 100 nM IGF-1 or 100 μM CoCl2 in 500 μl of fresh serum-free medium.

After an 18-h incubation, cells were lysed in 100 μl of passive lysis buffer provided with the Dual-Luciferase Reporter Assay System per well. Cells were scraped and centrifuged for 10 min at 19,000g, and 20 μl of supernatant per sample was loaded onto a 96-well plate and processed for luciferase activity in the Victor2 1420 Multilabel Counter (PerkinElmer Life Sciences, Downers Grove, IL) using firefly and Renilla luciferase buffers provided with the Dual-Luciferase kit.

Results

VEGF and HIF-1 α mRNA Expression. We first examined the effects of IGF-1 and CoCl2 treatment on HIF-1 α and VEGF mRNA expression in serum-starved ARPE-19 cells (Fig. 1). As a control, we measured IGFBP-3 mRNA expression. IGFBP-3 expression was previously reported to be enhanced in response to the presence of HIF-1 α (Felser et al., 1999). IGF-1 increased VEGF and IGFBP-3 mRNA levels (Fig. 1, A and B), whereas CoCl2 had no effect. Likewise, IGF-1 treatment led to increased HIF-1 α message levels with CoCl2 having no effect on HIF-1 α mRNA levels (Fig. 1C).

HIF-1 α Protein Expression. CsA is a potent immunosuppressive agent that destabilizes HIF-1 α by promoting hydroxylation of Pro-402 and Pro-564 within its oxygen-dependent degradation domain (D’Angelo et al., 2003). IGF-1 and the prolyl hydroxylase inhibitor dimethyl fumarate (DMF) increased HIF-1 α protein expression, whereas CoCl2 had no effect. Likewise, IGF-1 treatment led to increased HIF-1 α message levels with CoCl2 having no effect on HIF-1 α mRNA levels (Fig. 1C).

VEGF Secretion. As shown in Fig. 2B, IGF-1 and, to a lesser extent, CoCl2 significantly increased VEGF secretion.
while only partially (~20%) reducing IGF-1-stimulated VEGF secretion. ARPE-19 cells secrete a single species of IGFBP, IGFBP-3, which resolves into four differentially glycosylated species ranging from 28 to 45 kDa in size on SDS gels (Slomiany and Rosenzweig, 2004a). Treatment of ARPE-19 cells with IGF-1 and, to a lesser extent, with CoCl₂ increased IGFBP-3 secretion (Fig. 2C). Strikingly similar to the results found when assaying VEGF secretion, treatment with CsA resulted in the abrogation of CoCl₂-stimulated IGFBP-3 secretion, although only partially (~50%) reducing IGF-1 stimulated IGFBP-3 secretion.

**HIF-1 Activity.** To determine the effect of CsA treatment on HIF-1 transcriptional activity, ARPE-19 cells were co-transfected with the p2.1 HIF-1 reporter plasmid and the pRL-SV40 control plasmid and treated as described above. As shown in Fig. 2D, IGF-1 and CoCl₂ treatment each stimulated HIF-1 transcriptional activity. Addition of CsA reduced HIF-1 transcriptional activity by both stimuli to control levels. Taken together, these results suggested that CsA was ineffective at blocking CoCl₂-induced increases in Hif-1α expression.
tein expression and that the Hif-1α accumulated was transcriptionally inactive. On the other hand, CsA treatment blocked IGF-1-stimulated Hif-1α protein accumulation and HIF-1 transcriptional activity. Based on these findings, we postulated that IGF-1-stimulated VEGF and IGFBP-3 expression occurring in the presence of CsA is mediated through a HIF-1-independent mechanism.

**Role of PI3K in IGF-1-Stimulated VEGF Secretion.**

We next examined the effect of PI3K/Akt pathway inhibition on IGF-1-stimulated Hif-1α protein expression and VEGF secretion. As shown in Fig. 3A, IGF-1-stimulated Akt phosphorylation was inhibited by the addition of the PI3K inhibitor, LY294002 (Stein and Waterfield, 2000). It should be noted that subsequent reprobes of IGF-1-stimulated ARPE-19 lysates failed to demonstrate immunoreactivity for phospho-ERK (data not shown). LY294002 treatment also blocked IGF-1-stimulated Hif-1α protein accumulation (Fig. 3B) as well as basal and IGF-1-stimulated VEGF secretion (Fig. 3C) and IGFBP-3 secretion (Fig. 3D). Results similar to those shown in Fig. 3 were obtained with the PI3K inhibitor, wortmannin (data not shown). It should also be noted that immunohistochemical staining of ARPE-19 cells revealed the lack of an effect of CsA treatment on IGF-1-stimulated pAkt abundance or localization. In contrast, addition of either 50 μM LY294002 or 200 nM wortmannin reduced pAkt staining to control levels (data not shown).

**Role of PI3K in CoCl2-Stimulated VEGF Secretion.**

Based on the known effects of PI3K inhibitors on vesicular transport (Jones and Howell, 1997), we examined the effect of PI3K inhibition on CoCl2 treatment to rule out this effect as a cause for decreased IGF-1-stimulated VEGF and IGFBP-3 secretion. Immunoblot analysis of whole cell lysates for pAkt confirmed the PI3K-independent nature of CoCl2 action in comparison to IGF-1 (Fig. 4A). Immunoblot analysis of cell lysates for Hif-1α expression revealed strong Hif-1α protein staining in response to CoCl2 stimulation; neither LY294002 nor wortmannin affected Hif-1α protein levels (Fig. 4B). Analysis of VEGF secretion showed that wortmannin addition did not alter CoCl2-stimulated VEGF secretion. However, LY294002 significantly decreased VEGF secretion (Fig. 4C). These results are consistent with the selective ability of PI3K inhibitors to disrupt vesicular transport (Jones and Howell, 1997). Both inhibitors effectively blocked IGF-1-stimulated Akt phosphorylation. Our findings suggest that wortmannin was less effective at inhibiting PI3K(s) activity associated with vesicular trafficking.

**Effect of Rapamycin on VEGF Secretion.**

To establish the involvement of the mTOR/p70s6k pathway in IGF-1-stimulated VEGF and IGFBP-3 expression, we treated cells with rapamycin. As shown in Fig. 5A, rapamycin treatment selectively inhibited Akt phosphorylation without affecting Hif-1α expression or VEGF or IGFBP-3 secretion (Fig. 5B and 5C). These results are consistent with the selective ability of rapamycin to disrupt vesicular transport (Jones and Howell, 1997). Both inhibitors effectively blocked IGF-1-stimulated Akt phosphorylation. Our findings suggest that rapamycin was less effective at inhibiting PI3K(s) activity associated with vesicular trafficking.

![Fig. 3. Effect of LY294002 on IGF-1-stimulated Akt phosphorylation, Hif-1α expression, and the secretion of VEGF and IGFBP-3. Confluent ARPE-19 cells were serum-starved for 24 h and pretreated with LY294002 and CsA for 2 h before treatment with IGF-1 in fresh serum-free medium. A, after 15 min, cells were lysed, and 100 μg of protein was probed for phospho-Akt, Akt, phospho-44/42 MAPK, MAPK, and β-actin. B, after 4 h of stimulation, cells were processed for Hif-1α and β-actin. After 12 h of incubation, conditioned medium was analyzed for VEGF (C) and IGFBP-3 (D). Error bars, mean ± S.D. in secretion between duplicate wells of cells. Significant differences were observed (+, p < 0.05; ++, p < 0.01; +++, p < 0.005). A through D each are representative of three experiments. EtOH, ethanol.](image-url)
ulated VEGF secretion, we used the selective mTOR inhibitor rapamycin. IGF-1 (Fig. 5A), but not CoCl₂ (Fig. 5B), stimulated Hif-1α protein expression was completely inhibited by rapamycin treatment. Rapamycin treatment also decreased IGF-1-stimulated VEGF (Fig. 5C) and IGFBP-3 (Fig. 5D) secretion by ~50 and ~25%, respectively.

VEGF Promoter Truncation Analysis. Up to this point, our findings indicated that IGF-1-stimulated VEGF and IGFBP-3 secretion occurred in the absence of Hif-1α protein expression and HIF-1 transcriptional activity. To confirm these observations, ARPE-19 cells transfected with the pGL2 basic control vector, full-length pGL2VEGF 2.65, and the deletion mutations defined (Fig. 6G) were treated with IGF-1 (100 nM) or CoCl₂ (100 μM) for 18 h. IGF-1 and CoCl₂ each significantly stimulated full-length VEGF promoter activity (Fig. 6, A and B). Deletion of the region upstream of the HRE in the VEGF promoter (pGL2VEGF 1.46 construct) had no effect on CoCl₂ sensitivity but reduced the sensitivity to IGF-1 (Fig. 6, A and E). Removal of the upstream region and HRE (pGL2VEGF 0.71) reduced basal promoter activity and lacked sensitivity to either CoCl₂ or IGF-1 treatment (Fig. 6, A and C). Interestingly, when the region upstream of the HRE was added back (pGL2VEGF 1.2 + 0.71), IGF-1 sensitivity was restored (Fig. 6, A and D). These results indicated that the HRE is essential for CoCl₂-activated VEGF transcription and is partially responsible for IGF-1-induced VEGF promoter activity. The region upstream of the HRE also appeared to confer IGF-1 sensitivity in the absence of the HRE.

Discussion

As expected, treatment of cells with the hypoxia-mimetic CoCl₂ had no effect on Hif-1α message levels. On the other hand, IGF-1 stimulated an ~8-fold induction of Hif-1α mRNA expression, similar to the increase observed in HCT116 human colon carcinoma cells by Fukuda et al. (2002). These findings differed from those of Treins et al. (2002), who showed that Hif-1α mRNA levels in ARPE-19 cells remained unchanged in response to insulin treatment. These differences underscore the notion that multiple mechanisms regulate Hif-1α protein levels; they may be increased by de novo biosynthesis or decreased by von Hippel-Lindau (pVHL) directed proteasomal degradation. Abrogation of Hif-1α degradation resulting from physiologic or chemical hypoxia (CoCl₂) affects Hif-1α protein accumulation (Fig. 3A) (Slomiany and Rosenzweig, 2004a; Brahimi-Horn et al., 2005; Slomiany et al., 2006). Consistent with this observation, CoCl₂ treatment led to a more significant increase in HRE-dependent luciferase activity at the enolase promoter than IGF-1 treatment (Fig. 2D).

Although the signaling cascade leading to IGF-1-induced Hif-1α expression and VEGF secretion is still incompletely understood, it is well established that the VEGF promoter contains a hypoxia response element that is activated by the binding of HIF-1 (Levy et al., 1995). However, quantification of VEGF message and protein levels revealed a much larger induction by IGF-1 compared with CoCl₂. This was surprising, given that hypoxia is the major stimulus of VEGF expression (Levy et al., 1995). Because CoCl₂ stimulated a much greater accumulation of Hif-1α, contributing to greater HIF-1 formation than IGF-1, direct correlations of Hif-1α protein levels as an index of VEGF expression cannot be made. Instead, our findings suggest that IGF-1 increases the transcriptional efficiency of HIF-1 and that additional transcription factors probably participate in VEGF expression.

To further address this possibility and determine whether IGF-1-induced VEGF secretion was solely HIF-1-dependent, we used CsA as an inhibitor of Hif-1α stability (D’Angelo et al., 2003). We observed that CsA treatment inhibited IGF-1-stimulated Hif-1α protein expression and
HIF-1 reporter activity, at the same time only partially reducing VEGF secretion. These findings point toward the presence of HIF-1-independent mechanisms of IGF-1-stimulated VEGF expression.

Treatment with CsA did not seem to affect CoCl₂ induced HIF-1α protein accumulation; however, it reduced HIF-1 transcriptional activity and VEGF secretion to basal levels. The discordance between HIF-1α protein expression and HIF-1 activity may be explained on the basis of an understanding of CoCl₂ and CsA action. The mechanism by which CoCl₂ stabilizes HIF-1α involves inactivation of heme moiety-containing prolyl hydroxylases, which under normoxic conditions hydroxylate prolines 402 and 564 of HIF-1α, marking it for ubiquitination by pVHL (Epstein et al., 2001). In addition, cobalt binds directly to the pVHL-binding site, allosterically inhibiting the binding of pVHL to HIF-1α, preventing its ubiquitination (Yuan et al., 2003). Both actions of CoCl₂ lead to increased HIF-1α levels. In contrast, CsA increases HIF-1α hydroxylation, marking it for pVHL binding, ubiquitination, and degradation (D'Angelo et al., 2003). Thus, we speculate that application of both reagents resulted in stabilized, hydroxylated HIF-1α that was transcriptionally inactive.

To date, a number of studies have examined HIF-1α translation in different cell lines. Insulin-induced HIF-1α protein expression in ARPE-19 cells was PI3K-dependent (Treins et al., 2002), whereas insulin- and IGF-1-stimulated VEGF expression in NIH 3T3 cells was reported to occur via different signaling pathways (Miele et al., 2000). In that study, insulin stimulated PI3K/Akt and IGF-1 stimulated ERK/MAPK. Fukuda et al. (2002) reported that IGF-1-induced HIF-1α synthesis in HCT116 human colon cancer cells was both PI3K- and MAPK-dependent. In the present study, ARPE-19 cells treated with IGF-1 exhibited a significant increase in pAkt based on immunoblot and immunocytochemical staining; no ERK activation was detected. As expected, inhibition of PI3K completely blocked Akt phosphorylation and HIF-1α accumulation in IGF-1-treated cells. We did observe a difference in inhibitor efficacy; LY294002 reduced VEGF secretion to control levels, whereas wortmannin reduced secretion by ~30%.

It is well established that CoCl₂-induced HIF-1α protein accumulation occurs independently of PI3K signaling (Epstein et al., 2001). As expected, wortmannin had no effect on CoCl₂-induced VEGF secretion. However, LY294002 inhibited CoCl₂-stimulated VEGF accumulation in conditioned medium. It has been shown that treatment of mammalian cells with PI3K inhibitors causes lysosomal enzyme missorting and disruption of secretory vesicle formation (Jones and Howell, 1997). This may explain the observed decrease in protein secretion. Our results suggest that at the concentrations used, LY294002 has a broader range of action than wortmannin, providing more effective inhibition (Fukuda et al., 2002; Treins et al., 2002). Therefore, on the basis of our findings with wortmannin, we conclude that in addition to regulation in a PI3K-dependent manner, IGF-1-stimulated VEGF expression also occurs in a PI3K- and HIF-1-independent manner.

We cannot rule out the possibility that LY294002- and wortmannin-reduced secretion was because of mTOR inhibition (Brunn et al., 1996). Fukuda et al. (2002) demonstrated mTOR involvement (PI3K/Akt/mTOR pathways) in IGF-1-stimulated HIF-1α expression with the resultant activation of
mTOR (FRAP, RAFT1) is a Ser/Thr protein kinase that controls a variety of metabolic, transcriptional, and translational processes leading to cell growth/proliferation. mTOR inhibition by rapamycin blocks the phosphorylation and activation of p70\textsuperscript{60k} and promotes 4E-BP1 dephosphorylation, thereby increasing 4E-BP1 binding to eIF4E and decreasing cap-dependent mRNA translation (Beretta et al., 1996).
dition of rapamycin to ARPE-19 cells completely inhibited IGF-1-stimulated Hif-1α accumulation and partially reduced VEGF (~40%) secretion. This suggests that IGF-1-stimulated Hif-1α expression was mTOR-dependent, whereas IGF-1-stimulated VEGF expression was only partially mTOR- and HIF-1-dependent. The inability of rapamycin to abrogate CoCl2-induced increases in Hif-1α accumulation adds further support for a role of mTOR in IGF-1-stimulated increases in Hif-1α expression.

In summary, our findings demonstrate that IGF-1 stimulates Hif-1α transcription and translation, with CoCl2 causing a far greater accumulation of Hif-1α protein and HIF-1 transcriptional activity. Compared with CoCl2, IGF-1 caused a greater increase in VEGF mRNA levels and VEGF protein secretion. CoCl2-stimulated VEGF secretion was entirely HIF-1-dependent, whereas IGF-1-stimulated increases in Hif-1α expression were both PI3K/Akt/mTOR- and HIF-1-dependent and independent.

HIF-1 is the principal regulator of hypoxia-induced VEGF expression. However, evidence of alternative transcription factors mediating stress and cytokine-stimulated VEGF expression is accumulating (Milanini-Mongiat et al., 2002; Abdelrahim and Safe, 2005; Brahimi-Horn et al., 2005; Xu et al., 2005). Based on the present results demonstrating IGF-1-stimulated VEGF secretion in the absence of Hif-1α protein accumulation and HIF-1 promoter activity (p2.1 construct), we tested regions of the VEGF promoter other than the HRE for IGF-1 responsiveness. Cells transfected with the full-length VEGF promoter construct exhibited significant increases in promoter activity in response to IGF-1 and CoCl2 (Fig. 6, A and B). Significantly greater promoter activity was obtained with CoCl2 compared with IGF-1 using the p2.1 HIF-1 reporter. This correlated with the abilities of IGF-1 and CoCl2 to increase Hif-1α protein levels. There was no significant difference between these two stimuli when the full-length VEGF reporter was used. These data underscore the notion that transcription factor(s) other than HIF-1 regulate IGF-1-stimulated VEGF promoter activity.

Deletion of the region upstream of the HRE (~2366 to –1166) nearly abrogated (p < 0.1) IGF-1 sensitivity, which was restored after its splicing to the remaining construct lacking only the HRE (pGL2VEGF 1.2 + 0.71); CoCl2 sensitivity, which was by comparison not significantly affected by deletion of the region upstream of the HRE (p < 0.5), was lost upon deletion of the HRE (pGL2VEGF 0.71) and did not return upon splicing of the upstream region (pGL2VEGF 1.2 + 0.71). These findings indicate that even though IGF-1-stimulated HIF-1 activity at the HRE (representing less than half the intensity of CoCl2), the region upstream of the HRE plays a greater role in IGF-1 responsiveness, functioning in the absence of an active HRE to confer HIF-1-independent IGF-1 sensitivity. The signaling cascades and transcription factor(s) involved in IGF-1-mediated HIF-1-independent VEGF promoter activity remain to be elucidated. Recent findings indicate that Stat3 (Xu et al., 2005) and Sp1/Sp4 (Abdelrahim and Safe, 2005) may be key factors regulating VEGF expression. Given the therapeutic success of VEGF antagonists in the treatment of angiogenesis-based diseases including CNV and cancer (Ng and Adamis, 2005), future therapeutics could take advantage of inhibiting VEGF expression based on these pathways.

Acknowledgments

We thank Dr. David T. Kurtz for intellectual input and reagents, Dr. Robert Gourdie and Jane Jourdan for microscopy support, and Drs. Debarbrata Mukhopadhyay, Greg L. Semenza, and Jian-Xing Ma for constructs.

References


Address correspondence to: Dr. Steven A. Rosenzweig, Department of Cell and Molecular Pharmacology and Experimental Therapeutics, Medical University of South Carolina, 173 Ashley Ave., Charleston, SC 29425. E-mail: rosenzs@musc.edu