Modulation of insulin-like growth factor (IGF) and IGF binding protein biosynthesis by hypoxia in cultured vascular endothelial cells

M Tucci, K Nygard, B V Tanswell, H W Farber1, D J Hill and V K M Han

MRC Group in Fetal and Neonatal Health and Development, Department of Paediatrics, Obstetrics and Gynaecology, Biochemistry and Anatomy, Lawson Research Institute, University of Western Ontario, London, Ontario, Canada N6A 4V2 and 1The Pulmonary Center, Boston University School of Medicine, Boston, Massachusetts 02118, USA

Abstract

Endothelial cells (EC) are hypoxia-tolerant and their capacity to proliferate in low oxygen tension is essential to maintain vascular endothelium integrity. The present study addresses whether hypoxia alters the expression of insulin-like growth factor (IGF) and IGF binding protein (IGFBP) genes in bovine aortic EC (BAEC) and bovine pulmonary artery EC (BPAEC). EC were cultured in normoxic (21%) conditions and exposed to 0% oxygen for 24, 48, or 72 h; some cells were reoxygenated by exposure to 21% oxygen for 24 or 48 h following hypoxia. IGF-I peptide and mRNA levels were very low in both cell types, and decreased further with exposure to hypoxia. Ligand blotting showed that both cell types synthesized 24 kDa (IGFBP-4), 30 kDa (IGFBP-5 and/or IGFBP-6), 43 kDa and 48 kDa IGFBPs (IGFBP-3 glycosylation variants). IGFBP-4 was the predominant IGFBP expressed by both cell types and did not change with exposure to hypoxia. Hypoxia caused a significant increase in IGFBP-3 secretion in BPAEC but not in BAEC. IGFBP-3 stable mRNA levels in BPAEC were increased correspondingly. IGFBP-5 was expressed only in BAEC and decreased with exposure to hypoxia. IGFBP-6 mRNA expression was low and increased in both cell types with exposure to hypoxia. These results demonstrate that EC IGFBP baseline expression as well as its expression in hypoxia vary in different vascular beds and suggest that the IGFBPs may be the dominant paracrine regulators of proliferation of EC as well as maintenance of endothelium integrity during hypoxia.

Introduction

Endothelial cells (EC) occupy a strategic location in the vasculature where they play an important role in the maintenance of vascular integrity and the passage of growth mediators and cytokines into the extravascular space. Hypoxemia is one of the most frequent and common insults to the vascular endothelium. Several previous studies have demonstrated that EC possess remarkable tolerance and adaptation to hypoxic conditions (Farber & Rounds 1990, Ogawa et al. 1990, Shreeniwas et al. 1991, Zimmerman et al. 1991, Graven et al. 1993, Tucci et al. 1996). These studies have shown that cultured vascular EC tolerate acute hypoxia (0% oxygen) for periods up to five days and chronic hypoxia (3% oxygen) for periods up to several months, continue to divide at rates dependent on the origin of the EC as well as the ambient oxygen concentration to which they are exposed, and demonstrate no evidence of significant cellular damage despite exposure to these seemingly adverse hypoxic conditions (Farber & Rounds 1990, Zimmerman et al. 1991, Graven et al. 1993, Tucci et al. 1996, 1997). The mechanisms underlying this hypoxia tolerance as well as the factors affecting EC proliferation during hypoxia are not fully understood.

are known to affect several metabolic processes (King et al. 1985, Bar et al. 1987a, 1988a,b), are involved in potential interplay between vascular endothelium and subendothelial components (Hansson et al. 1987, 1989, Delafontaine et al. 1991, Khorsandi et al. 1992, Nakao et al. 1992, Taylor et al. 1993) and may play a role in the intrinsic growth of normal and diseased blood vessels (King et al. 1985, Nicosia et al. 1994).

In other cell types and biological systems, exposure to decreased oxygen alters the expression of the genes encoding IGF system peptides. Numerous studies have described modifications of various IGF system peptides following hypoxic-ischemic brain injury (Klempt et al. 1992, 1993, Lee et al. 1992, Beilharz et al. 1993, Lee & Bondy 1993, Stephenson et al. 1995, Johnston et al. 1996, Sandberg et al. 1996), hypoxia induced by reduced uterine blood flow (McLellan et al. 1992, Price et al. 1992, Owens et al. 1994, Asano et al. 1995), hypoxia-induced pulmonary hypertension (Perkett et al. 1992, Dempsey et al. 1994, Townsend & Stenmark 1995), myocardial ischemia (Reiss et al. 1994, Buerke et al. 1995, Kluge et al. 1995) and acute tubular necrosis (Noguchi et al. 1993, Tsao et al. 1995). In experimental vascular injury, a context in which initial EC proliferation occurs in relative hypoxia, studies have shown induction of aortic IGF-I mRNA in the vascular wall after balloon denudation (Cercek et al. 1990, Khorsandi et al. 1992) while immuno-histochemical studies have described increased IGF-I and IGF-II protein accumulation during repair of injured arterial intima (Hansson et al. 1987, 1989, Levinovitz et al. 1992). However, little is known about the effect of hypoxia on the IGF system in vascular EC. This understanding is critical as many clinical situations require ongoing EC proliferation despite adverse hypoxic conditions in order to allow for tissue repair.

Based on observations that exposure to hypoxia alters the expression of the IGF system peptides and our studies demonstrating changes in EC proliferation during exposure to hypoxia (Farber & Rounds 1990, Tucci et al. 1997), we hypothesized that hypoxia alters the rate of EC cellular division by altering the expression of IGFs and/or IGFBPs. In the current study, we found that exposure of cultured EC to hypoxia produces alterations in baseline expression of IGFs and IGFBPs that are dependent on the vascular bed from which they originated. These in vitro studies carried out in cultured EC eliminate confounding interactions between EC and non-endothelial components, and allow delineation of hypoxia-associated changes in the IGF system in isolated vascular endothelium. The findings suggest that locally expressed IGFs and IGFBPs play a role in the altered proliferation of EC exposed to acute hypoxia, with IGFBPs being the dominant paracrine regulators.

This work was presented in part at the May 1996 meeting of the Pediatric Academic Societies (The American Pediatric Society and the Society for Pediatric Research) and has been published in abstract form (Tucci et al. 1996).

Materials and Methods

Materials

Human recombinant IGF-I was purchased from IMCERA Bioproducts (Terre Haute, IN, USA) and human recombinant IGF-II from Bachem Co. (Torrance, CA, USA). IGF-I and IGF-II were iodinated using the Chloramine-T method as previously described (Hill 1990). Tissue culture plastic ware was obtained from Falcon Plastics (Los Angeles, CA, USA), Costar (Cambridge, MA, USA) and United Scientific (Quincy, MA, USA).

Endothelial cell culture

Bovine aortic and pulmonary arterial endothelial cells (BAEC and BPAEC respectively) were isolated from freshly excised calf aortas and pulmonary arteries as previously described (Zimmerman et al. 1991, Graven et al. 1993, Tucci et al. 1997). Animals used for these experiments were male calves 4-8 weeks of age. EC were obtained by lightly scraping the intimal surface of longitudinally opened vessels and seeded initially into 35 mm plastic Petri dishes with growth media containing minimal essential medium (MEM; Gibco BRL, Grand Island, NY, USA) supplemented with 15% heat-inactivated bovine calf serum (BCS; Hyclone Laboratories, Logan, UT, USA), 1 mM sodium pyruvate (Gibco BRL), 10 000 U/ml penicillin and 10 µg/ml streptomycin (Sigma Chemical Co, St Louis, MO, USA). Cultures were maintained in a humidified incubator at 37°C in 5% CO2. EC purity was assured by the typical cobblestone appearance on phase-contrast microscopy, the uptake of fluorescent acetylated-low density lipoprotein and the presence of immunofluorescence for von Willebrand factor (Farber & Rounds 1990, Zimmerman et al. 1991, Graven et al. 1993, Tucci et al. 1997). BAEC and BPAEC used in these experiments were derived from at least 3 different primary lines subcultured for the study between passages 4 and 12 and were of the same passage number from the same animal for individual experiments.

Exposure to hypoxia

BAEC and BPAEC were seeded into P-100 tissue culture dishes and used at 75-80% confluence. At this time, culture medium was removed and EC were incubated in serum-free medium (SFM) and subjected to the appropriate O2 condition. Control cells were maintained in 21% O2 throughout the experimental protocol. All hypoxic cells were exposed to 0% O2, 5% CO2, and balanced...
N\textsubscript{2} in humidified sealed chambers (Billups-Rothenburg, Del Mar, CA, USA) at 37 °C for 24–72 h. In reoxygenation studies, cells were exposed to 0% oxygen for 24 h, then returned to 21% oxygen for 24 or 48 h. For all hypoxic conditions lasting longer than 24 h, chambers were gassed every 24 h.

At the end of the experimental protocol, conditioned media were collected and centrifuged at 3000 g to remove cellular debris. One milliliter aliquots of all samples were stored in −70 °C for IGF RIA and the remaining conditioned media were concentrated by ultrafiltration using Centricon-10 concentrators pre-treated with 5% Tween-20 (Amicon, Beverly, MA, USA). Cells from the same cultures were used for total RNA extraction and DNA quantification.

Western ligand blot analysis
Ligand blot analysis was performed by a modification of the method of Hossenlopp et al. (1986) as previously described (Bradshaw & Han 1993). Conditioned medium, 250 µg/lane, was subjected to electrophoresis on a non-reducing 8–12% gradient sodium dodecyl sulfate (SDS) polyacrylamide gel and proteins were then transferred electrophoretically to a 0.2 µm nitrocellulose membrane (Schleicher and Schuell, Keene, NH, USA). The membrane was washed in Tris–NaCl pH 7.4, 0.5 mg/ml sodium azide, and 3% Nonidet P-40 (Sigma) and in membrane was washed in Tris–NaCl pH 7.4, 0.5 mg/ml (Schleicher and Schuell, Keene, NH, USA). The membrane was then washed sequentially in Tris–NaCl pH 7.4, 0.1% Tween 20, and 1% BSA, overnight at 4 °C. The membrane was then washed sequentially in Tris–NaCl pH 7.4 and 0.1% Tween 20 (2 × 15 min) and Tris–NaCl pH 7.4 (3 × 15 min). The membrane was air dried and exposed to X-ray film (XAR, Kodak Laboratories, Rochester, NY, USA) with intensifying screens at −70 °C for 3 to 14 days. Total protein concentrations of the conditioned media were determined by the Bio-Rad Protein assay (Bio-Rad Laboratories Ltd, Richmond, CA, USA). Relative levels of IGFBPs were quantified by densitometry (Personal Densitometer SI, Molecular Dynamics, Sunnyvale, CA, USA).

Western immunoblotting analysis
To identify which IGFBP species was secreted by EC, a modified Western blot method was employed as previously described (Phillips et al. 1993). Briefly, conditioned media were separated on 8–12% SDS polyacrylamide gels and transferred to nitrocellulose membranes. After transfer, the nitrocellulose membranes were washed with PBS buffer for 10 min, non-specific binding sites blocked by incubation in PBS containing 5% (w/v) BSA, and then incubated with primary antisera at 4 °C for 18 h. Antibodies used were rabbit polyclonal anti-IGFBP-4 (1:500 dilution; UBI, Lake Placid, NY, USA), rabbit polyclonal anti-human IGFBP-5 (1:500 dilution; UBI), and rabbit anti-human IGFBP-6 (1:500 dilution; Austral Biologicals, San Ramon, CA, USA). After incubation, the nitrocellulose membranes were washed with PBS buffer and incubated with biotinylated goat anti-rabbit immunoglobulin (1:500 dilution; Vector Laboratories, Burlingame, CA, USA) for 1 h at 23 °C. Following a further wash, the membrane was incubated with avidin and biotinylated peroxidase for 1 h at 23 °C, washed with PBS buffer followed by 50 mmol Tris buffer/l (pH 7.5) at room temperature. Immunoreactive proteins were visualized using freshly prepared 3–3'-diaminobenzidine tetrahydrochloride (1.89 mmol/l) containing 0.03% (v/v) hydrogen peroxide. The reaction was quenched by washing in excess 50 mmol Tris buffer/l.

Immunoprecipitation
For some samples, immunoprecipitation was performed in order to identify IGFBP species with very low expression (Harlow & Lane 1988). Antibodies used were the same as for Western immunoblotting analysis. Briefly, the appropriate lyophilized antibody was resuspended as directed and 1 µl added to 49 µl Heps binding buffer (1:50 dilution). A 10 µl aliquot of the antibody suspension was added to 90 µl concentrated conditioned media (final antibody dilution of 1:500), vortexed, and incubated overnight at 4 °C to allow antibody–antigen binding. The suspension was then added to 50 µl protein A sepharose suspended in 20 mM sodium phosphate buffer, pH 7.0 (Pharmacia Canada Inc., Baie d’Urfé, Québec, Canada) and incubated at 4 °C for 3–5 h. Sepharose-bound antigen–antibody complexes were spun down at 3000 r.p.m. and eluted from the sepharose by boiling in Laemmli buffer. The supernatant containing the IGFBP of interest was collected and processed for Western ligand blotting as described above.

DNA quantification
DNA quantification was performed by the fluorometric DNA assay described previously (Brunk et al. 1979). Briefly, EC monolayers were rinsed with PBS, precipitation was performed with 12.5% trichloroacetic acid (Fisher Scientific, Nepean, Ontario, Canada) and DNA was solubilized in 0.1 M NaOH (Fisher Scientific). Duplicate 100 µl aliquots of DNA suspension were used for quantification after addition of 0.2 M HCl, 2 mM EDTA and HOESCHT 33258 dye (Sigma-Aldrich, Oakville, Ontario, Canada) and measurements were obtained by fluorescence spectrophotometry (Hitachi F-4010 fluorescence spectrophotometer, Tokyo, Japan).

RNA preparation and Northern blot analysis
Total RNA was prepared from EC using the single-step method of RNA isolation by guanidinium
thiocyanate-phenol-chloroform extraction (Chomczynski & Sacchi 1987) and Northern blotting was performed as described previously (Han et al. 1988). Briefly, total RNA (20 µg/lane) was denatured and subjected to electrophoresis in a 1% agarose gel containing 2·2 M formaldehyde. The RNAs were transferred to a Zeta-Probe nylon membrane (Bio-Rad Laboratories) by the capillary transfer technique. Following transfer, the blots were baked and hybridized at 42 °C overnight with 32P-labeled ovine IGF-I, IGF-II, and IGFBP-1 to -6 cDNA probes (2 × 106 c.p.m./µl buffer containing 5 × SSPE (0·75 M NaCl, 44 mM NaH2PO4·2H2O, 5 mM EDTA, pH 7·4), 7% SDS, and 5 µg/ml denatured salmon sperm DNA). The cDNA inserts were labeled with 32P-dCTP (ICN, St Laurent, Québec, Canada) to specific activities of 1–2 × 109 c.p.m./µg by the random priming technique using an oligo-labeling kit (Pharmacia Canada Inc.). Blots were washed twice (30 min each) in 1 × SSC (0·15 M NaCl, 15 mM sodium citrate, pH 7·0)-0·1% SDS at 42 °C, twice (30 min each) in 0·1 × SSC-0·1% SDS at 42 °C, air-dried and subjected to autoradiography using intensifying screens at −70 °C. Subsequently, blots were stripped between hybridizations by washing twice in 0·01 × SSC and 0·5% SDS for 30 min each at 80 °C. Consistency in loading and transfer of total RNAs in each lane was checked by probing the blots with a radiolabeled cDNA for 18S ribosomal RNA (a gift from Dr David Denhardt, Rutgers University, NJ, USA). Autoradiograms were quantified using densitometry and/or phosphor-imaging (Personal Densitometer SI and Phosphorimagier SI, Molecular Dynamics, Sunnyvale, CA, USA).

Radioimmunoassay

The IGF-I and IGF-II concentrations in conditioned medium were determined by a modification of a previously described RIA method following separation of IGFs from IGFBPs using acid-gel chromatography (Hill 1990). Briefly, 1 ml samples of conditioned medium were dried in a vacuum centrifuge (SpeedVac Savant concentrator, Savant, Farmingdale, NY, USA), resuspended in 500 µl 0·2 M acetic acid and loaded onto a Sephadex G50 column. One milliliter fractions were collected and IGF-containing fractions were pooled for each conditioned medium sample, lyophilized, resuspended in radioimmunoassay buffer and subjected to RIA. Recovery of IGF-I and IGF-II from the column was determined by the addition of 200 000 c.p.m. 125I-IGF-II and was greater than 90%.

Data analysis

All experiments were performed at least three times and each experimental condition performed in triplicate. To compensate for loading differences among RNA samples in Northern blot analyses, densitometric analysis of the hybridizing mRNA bands were equalized to the corresponding 18S ribosomal RNA bands. In ligand blot analyses, signal intensity of IGFBPs as quantified by densitometry was adjusted per µg DNA. The percentage change in mRNA and protein expression for each hypoxic and reoxygenation condition was then compared with the control condition (21% oxygen) and the data expressed as the percentage change relative to control. All data are presented as the mean ± s.e.m. ANOVA was used for time-course analysis and comparisons performed using the Bonferroni multiple comparisons test. Differences were considered significant at P < 0·05.

Results

IGF-I and IGF-II expression and release

IGF-I mRNA levels in both BAEC and BPAEC exposed to 21% or 0% oxygen were low. IGF-I mRNA was relatively more abundant in BPAEC than BAEC with regard to the expression of 9·0 kilobase (kb), 8·0 kb and 4·2 kb IGF-I transcripts. The 8·0 kb transcript decreased (to 25% of control level) with exposure to hypoxia and increased within 24 h of reoxygenation (to 50% of control level) in BPAEC. Although the mRNA levels were lower, a similar modulation occurred in BAEC (Fig. 1). The expression of IGF-II mRNAs was lower than that of IGF-I in both cell types (data not shown).

IGF-I was detectable only in the conditioned media of normoxic and reoxygenated EC (levels were below 1 ng/ml); IGF-I was not detectable in the conditioned media of hypoxic EC. IGF-II was also detectable in some conditioned media used for these studies; its expression was low (below 2 ng/ml) and showed no variation with exposure to hypoxia.

IGFBP expression and secretion

Ligand blot analyses demonstrated that BAEC and BPAEC secreted four IGFBP species (Fig. 2); the 24 kDa protein was confirmed as IGFBP-4, the 43 kDa and 48 kDa proteins were unglycosylated and glycosylated IGFBP-3, and the 30 kDa protein was consistent with either IGFBP-5 and/or IGFBP-6. IGFBP-4 was the most abundant IGFBP in both BAEC and BPAEC. The identity of IGFBP-3 and IGFBP-4 was confirmed by Western immunoblotting with specific antisera (data not shown); IGFBP-5 identity was confirmed by immuno-precipitation (Fig. 3). Northern blots probed sequentially with IGFBP-1 to -6 cDNAs demonstrated expression of IGFBP-3, -4, -5 and -6 mRNAs. Neither the protein, nor the mRNA for IGFBP-1 and IGFBP-2 was detected (Figs 4 and 5).

IGFBP-3 Both cell types consistently expressed two glycosylation variants of IGFBP-3 (calculated sizes 43 kDa
and 48 kDa); however, IGFBP-3 levels were greater in normoxic BPAEC than BAEC conditioned media (Fig. 3). IGFBP-3 release into the media of BPAEC increased 2–3-fold within 24 h of exposure to hypoxia while, in BAEC, no significant change occurred (Fig. 3). A single 2·4 kb IGFBP-3 mRNA was consistently expressed by both cell types; with exposure to hypoxia, its expression increased 3–4-fold in BPAEC while it decreased in BAEC (Fig. 4).

**IGFBP-4** The production of IGFBP-4 in both BAEC and BPAEC remained unchanged with exposure to hypoxia (Fig. 2). Northern analysis revealed a similar abundance of IGFBP-4 (2·3 kb) mRNA expression in both cell types during normoxia. With exposure to hypoxia, IGFBP-4 mRNA abundance decreased significantly in BAEC, while no change was observed in BPAEC (Fig. 4). During reoxygenation, BAEC IGFBP-4 mRNA levels returned to control levels within 24 h, while the mRNA levels in BPAEC remained the same.

**IGFBP-5** Ligand blot analyses of conditioned media from both BAEC and BPAEC revealed equivalent amounts of an approximately 30 kDa IGFBP consistent with either IGFBP-5 or -6 (Fig. 2). Immunoblotting and immunoprecipitation using antihuman IGFBP-5 antiserum confirmed that this 30 kDa IGFBP is IGFBP-5 (Fig. 3). In addition, the fact that IGFBP bound $^{125}$I-IGF-I and $^{125}$I-IGF-II with similar affinity in ligand blots indicated that this IGFBP is unlikely to be IGFBP-6. A single IGFBP-5 mRNA (6·0 kb) was expressed in both normoxic cell types but was less abundant in BPAEC than in BAEC. Interestingly, the IGFBP-5 mRNA abundance was significantly diminished in the more confluent cultures. With exposure to hypoxia, the expression of IGFBP-5 mRNA decreased in BAEC and returned to control levels with reoxygenation (Fig. 5). The IGFBP-5 levels were low to undetectable in BPAEC.

**IGFBP-6** Whether a component of the 30 kDa IGFBP constitutes IGFBP-6 could not be confirmed by immunoblotting because of the lack of a specific antiserum. Northern analysis for IGFBP-6 revealed weak expression of a 1·3 kb hybridizing band in both cell types. With exposure to hypoxia, IGFBP-6 mRNA expression exhibited a 2– to 3-fold increase that returned to control levels with reoxygenation (Fig. 5).

**Discussion**

In this study we have demonstrated that exposure to acute hypoxia causes cultured vascular EC to alter the expression of IGF and IGFBP genes. These include (a) a reduction in IGF-I gene expression, (b) EC type-specific changes in response to hypoxia for some IGFBPs and (c) the reversibility of hypoxia-induced changes of both IGF and IGFBPs. Since IGFs and IGFBPs interact with other components of the vasculature including mural cells such as smooth muscle cells, pericytes and fibroblasts, a paracrine interaction between EC and mural cells via IGF and IGFBP may be an important component of vascular endothelial and mural cell response to hypoxia.

IGF-I is secreted by many cell types including vascular EC (Kern et al. 1989, Delafontaine et al. 1991, Gajdusek et al. 1993). While some have reported that the major portion of EC IGF-I is derived from exogenous sources (Gajdusek et al. 1993), several have also demonstrated the existence of a low de novo synthesis of IGF-I by vascular
Figure 2 Western ligand blot analysis of conditioned media from BAEC (lanes 1–6) and BPAEC (lanes 7–12) maintained in 21% oxygen, exposed to hypoxia (0% oxygen) or exposed to hypoxia followed by reoxygenation. (a) Autoradiograph of a representative Western ligand blot showing control 21% oxygen (C), exposure to 0% oxygen for 24 h, 48 h and 72 h, and reoxygenation for 24 h (R24) and 48 h (R48) after exposure to 0% oxygen for 24 h. Ovine fetal serum (+C) is used as a positive control. Four bands were identified: 24 kDa (IGFBP-4), 30 kDa (IGFBP-5 and/or -6), 43 and 48 kDa IGFBP-3 glycosylation variants. (b) Densitometric analysis (mean from three separate experiments performed in triplicate) of changes in IGFBP-3 levels in conditioned medium. Relative levels of IGFBP-3 were quantified by densitometry, normalized per µg DNA and are expressed as the change relative to control (21% oxygen) for each cell type. (c) Similar analysis for the 30 kDa ligand (IGFBP-5 and/or -6). (d) Similar analysis for IGFBP-4. *P<0·05 (compared to controls 21% oxygen).
EC, as we have shown in this study (Kern et al. 1989, Delafontaine et al. 1991, Gajdusek et al. 1993). Cultured EC express high-affinity IGF receptors which are probably involved in the processing of IGF-I by vascular endothelium (Bar & Boes 1984, Bar et al. 1989, Boes et al. 1991). In both EC types, exposure to hypoxia caused downregulation of IGF-I mRNA expression. As IGF-I is a mitogen for EC and other vascular components (King et al. 1991). In both EC types, exposure to hypoxia caused downregulation of IGF-I mRNA expression. As IGF-I is a mitogen for EC and other vascular components (King et al. 1991, Hansson et al. 1987, 1989, Khorsandi et al. 1992, Nakao et al. 1992, Grant et al. 1993), this reduced IGF-I synthesis may affect autocrine/paracrine regulation of endothelium integrity and regeneration as well as regulation of IGF-I availability within mural components of the vasculature. Interestingly, baseline IGF-I expression was more pronounced in BPAEC than BAEC, an observation which is not attributable to differences in confluency (Gajdusek et al. 1993) and which suggests differential processing and/or utilization of IGF-I by EC from different vascular origins.

The biological actions of endogenous or exogenous IGF-I are modulated by EC IGFBPs and alterations in the biosynthesis of these IGFBPs will influence IGF actions. Cultured EC express mRNAs for IGFBP-2 through -6 and secrete substantial quantities of IGFBPs into the culture medium (Boes et al. 1992, Moser et al. 1992, Yang et al. 1993). In addition, IGFBPs synthesized by EC are involved in the mobilization and transfer of IGFs across the EC barrier (Gajdusek et al. 1993, Taylor et al. 1993). Marked variability has been described in the expression of the various IGFBPs that is dependent on numerous factors including the vascular origin of the EC (Moser et al. 1992), the culture milieu and the confluency of the cell cultures used (Hayzer et al. 1995, Delafontaine et al. 1996). The IGFBP profile of EC in this study is similar to that described previously. To date IGFBP-1 has not been shown to be expressed in macrovascular EC (Moser et al. 1992), nor in our BAEC and BPAEC. IGFBP-2 mRNA is detectable in large vessel EC using reverse transcription-PCR (Bar et al. 1987), but neither IGFBP-2 mRNA nor protein was detected in our cells. Both EC types expressed IGFBP-3 as described (Hayzer et al. 1995). IGFBP-4 was the predominant IGFBP expressed by both types of EC (Boes et al. 1992, Moser et al. 1992, Yang et al. 1993). Although a small amount of a 30 kDa glycosylation variant of IGFBP-4 has been described (Moser et al. 1992), no 30 kDa band corresponding to IGFBP-4 was seen in either BAEC or BPAEC. Low expression of IGFBP-5 and -6 has already been reported in large vessel EC; their expression is apparently higher in microvessels (Moser et al. 1992). Our findings concur with this observation and demonstrate low expression of both these binding proteins in vascular EC.

Under normoxic conditions, IGFBP-3 inhibits growth of actively proliferating EC; confluent growth-arrested (contact-inhibited) EC show marked induction of IGFBP-3 gene expression, while actively proliferating EC have lesser IGFBP-3 gene expression and protein production (Delafontaine et al. 1996). In our study, hypoxic BPAEC, despite subconfluent cell densities, increased IGFBP-3 gene expression whereas BAEC at the same densities did not. This vascular bed-dependent response suggests that BPAEC, which are exposed to relatively lower oxygen tension in vivo than BAEC, are perhaps more hypoxia tolerant and need a lesser proliferative impetus under hypoxic conditions than their aortic counterparts.

With exposure to hypoxia, IGFBP-4 protein levels were unchanged in both cell types while mRNA levels showed a striking decrease in BAEC. That IGFBP-4 gene expression exhibits an origin-dependent differential response to hypoxia suggests that EC from systemic (aortic) vascular beds are more susceptible to downregulate this generally inhibitory IGFBP in order to promote IGF-I action. In contrast, pulmonary EC, which may be more hypoxia tolerant given their in vivo environment, require a lesser proliferative stimulus. Interestingly, while total protein production in EC decreases by up to 75% with exposure to hypoxia (Farber & Round 1990), levels of IGFBP-4 protein remained unchanged in both EC types suggesting that this binding protein plays an important role within the vasculature. A potential explanation for unchanged protein synthesis despite decreased mRNA expression is that hypoxia alters proteolytic processing of IGFBP-4 by various proteases (Kamyar et al. 1994, Gockerman & Clemmens 1995, Parker et al. 1995) leading to increased stability of secreted IGFBP-4 or that hypoxic modulation of one IGFBP may be regulating the availability of other IGFBPs (Fowlkes et al. 1995, Boes et al. 1996, Donnelly & Holly 1996, Noll et al. 1996).

IGFBP-5 has the unique property of adhering tightly to fibroblast extracellular matrix and may be an important factor in regulating IGF-I actions (Parker et al. 1996).
Figure 4  Northern analysis of total RNA (20 μg/lane) extracted from BAEC (panels A–C) and BPAEC (panels D–F) maintained in 21% oxygen, exposed to hypoxia (0% oxygen) or exposed to hypoxia followed by reoxygenation. All blots were probed sequentially with $^{32}$P-labeled bovine IGFBP-3, ovine IGFBP-4 and 18S rRNA cDNAs. All samples were analyzed in duplicate. Fetal sheep liver RNA is used as a positive control (+C). Panel A: representative Northern blot of total RNA from BAEC cultures exposed to control 21% oxygen (C), to 0% oxygen for 24 h, 48 h and 72 h, and to reoxygenation for 24 h (R24) and 48 h (R48) after exposure to 0% oxygen for 24 h. Panel B: densitometric analysis (mean from three separate experiments performed in triplicate) of changes in IGFBP-3 mRNA in BAEC. Relative levels of IGFBP-3 mRNA expression were quantified by densitometry, normalized by comparison with 18S and expressed as the change relative to control (21% oxygen). Panel C: similar analysis for IGFBP-4 in BAEC. Panel D: representative Northern blot of total RNA from BPAEC cultures exposed to the same conditions as panel A. All blots were probed sequentially with the same $^{32}$P-labeled used for BAEC in panel A. Panel E: analysis for IGFBP-3 in BPAEC similar to that performed in BAEC. Panel F: analysis for IGFBP-4 in BPAEC similar to that performed in BAEC. *P<0.05 (compared to controls 21% oxygen).
Moreover, in other cell types IGFBP-5 mRNA expression is enhanced by ischaemia/reperfusion in myocardium (Kluge et al. 1995) and is upregulated coordinately with IGF-I expression in injured astrocytes after hypoxic-ischemic brain injury (Lee et al. 1996). IGF-I upregulates IGFBP-5 synthesis by transcriptionally activating the IGFBP-5 gene in aortic smooth muscle cells (Duan et al. 1996) or by protecting IGFBP-5 from proteolysis (Cheng et al. 1996). In our study, we have observed that the capacity to synthesize IGFBP-5 differed according to the vascular bed of origin; although IGFBP-5 protein levels in the conditioned media were comparable in both cell types, IGFBP-5 mRNA expression was markedly lower in BPAEC suggesting that the latter cells may have either more efficient translation of IGFBP-5 mRNA or less IGFBP-5 degradation. IGFBP-5 mRNA expression decreased in BAEC with exposure to hypoxia. Nonetheless, its predominance in BAEC and its decrease with exposure to hypoxia in both cell types taken with its demonstrated upregulation in non-endothelial cell types exposed to hypoxic stress (Kluge et al. 1995, Lee et al. 1996) suggests a potential role for IGFBP-5 in the transport of IGFs from the vascular compartment to subendothelial target tissues.

Little is known about IGFBP-6 expression in the vasculature. While some have described IGFBP-6 production in vascular smooth muscle cells (Boes et al. 1996), no studies have reported hypoxia-induced IGFBP-6 modulation. In both our EC types, IGFBP-6 mRNA expression was very low and increased with exposure to hypoxia. Potential explanations for this observation include modulation of endogenous IGFBP-6 gene expression and/or altered mRNA stability with exposure to hypoxia.

While biosynthesis of IGFBPs is affected by exposure to hypoxia in other cell types or biological systems, no previous study has described IGFBP production by the vascular endothelium during hypoxia. In the ovine fetus subjected to hypoxia, IGF and IGFBP levels in the serum...
and their gene expression in various tissues are altered (McLellan et al. 1992, Price et al. 1992, Owens et al. 1994, Asano et al. 1995). After hypoxic–ischemic injury in the central nervous system, IGF-I, IGFBP-2, IGFBP-3 and IGFBP-5 are induced at sites of injury and recovery (Lee et al. 1992, Klempt et al. 1992, 1993, Beilharz et al. 1993, Lee & Bondy 1993, Stephenson et al. 1995, Johnston et al. 1996, Sandberg et al. 1996). The involvement of the IGF system has also been described in hypoxia-induced pulmonary hypertension studies in the newborn (Perkett et al. 1992, Dempsey et al. 1994, Townsend & Stenmark 1995), in acute tubular necrosis (Noguchi et al. 1993, Tsao et al. 1995) and in ischemic myocardium (Reiss et al. 1994, Buerke et al. 1995, Kluge et al. 1995, Moromisato et al. 1996). While most of these studies demonstrate hypoxia-induced alterations of the IGF system in organ systems, it is critical to understand how individual components within these systems and more particularly within the vasculature are affected by hypoxia in order to delineate how they might then interact in vivo.

Our study showed that hypoxia modulated IGF and IGFBP gene expression in EC and that, for IGF-I and some components of the IGFBP system, this modulation was vascular bed dependent. The fact that IGFBPs were affected more than the IGFs during exposure to hypoxia suggests that any proliferative/regenerative EC response is more dependent on IGFBPs. More importantly, given that IGF-I is a progression factor exerting its control at stage G1 of the cell cycle (O’Keefe & Pledger 1983, Pardee 1989), hypoxia-induced modulation of the IGFBPs which alter IGF bioavailability, might influence EC proliferation and regeneration after injury. Although we did not demonstrate a direct link between the modulation of the IGF system and EC hypoxia tolerance, our findings suggest that IGF/IGFBPs are involved in the maintenance of vascular endothelium integrity and may contribute to endothelium regeneration within the pulmonary and systemic circulations after cellular injury. IGF and IGFBPs may also originate from subendothelial components such as smooth muscle cells and fibroblasts. Therefore, the maintenance of endothelial integrity during hypoxia may be mediated by EC IGFBPs or by IGF/IGFBPs from other components of the vasculature.

Acknowledgements

M T was supported by a Research Fellowship bursary from the Medical Research Council of Canada and by the Pooled Research Trust Fund of the Lawson Research Institute. H W F was supported by a Career Investigator Award from the American Lung Association and a grant from the National Institutes of Health (HL-45537). V K M H and D J H were supported by a Group grant in Fetal and Neonatal Health and Development from the Medical Research Council of Canada. This work was in part supported by the grant from the Ontario Thoracic Society. We wish to thank Michelle Gregory for her expert technical assistance.

References


Bar RS & Boes M 1984 Distinct receptors for IGF-I, IGF-II, and insulin are present on bovine capillary endothelial cells and large vessel endothelial cells. Biochemical and Biophysical Research Communications 124 203–209.


Townsend SF & Stenmark KR. 1995 Developmental and hypoxic regulation of pulmonary artery adventitial fibroblast insulin-like growth factor binding proteins (IGFBPs). *Pediatric Research* **71A**.


Tucci M, Nygaard K, Farber HW & Han VKM 1996 Modulation of IGF and IGFBP expression by hypoxia in cultured vascular endothelial cells (EC). *Pediatric Research* (Abstract 2333) 39R2A.


Received 30 April 1997
Revised manuscript received 22 September 1997 Accepted 22 October 1997


M TUCCI and others · Hypoxia modulates IGF and IGFBP in endothelial cells