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Changes Associated With Tyrosine Phosphorylation During Short-Term Hypoxia in Retinal Microvascular Endothelial Cells In Vitro

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Abstract The occlusion of capillary vessels results in low oxygen tension in adjacent tissues which triggers a signaling cascade that culminates in neovascularization. Using bovine retinal capillary endothelial cells (BRCEC), we investigated the effects of short-term hypoxia on DNA synthesis, phosphotyrosine induction, changes in the expression of basic fibroblast growth factor receptor (bFGFR), protein kinase C (PKCα), heat shock protein 70 (HSP70), and SH2-containing protein (SHC). The effect of protein tyrosine kinase (PTK) and phosphatase inhibitors on hypoxia-induced phosphotyrosine was also studied. Capillary endothelial cells cultured in standard normoxic (pO_2 = 20%) conditions were quiesced in low serum containing medium and then exposed to low oxygen tension or hypoxia (pO_2 = 3%) in humidified, 5% CO_2, 37°C, tissue culture chambers, on a time-course of up to 24 h. DNA synthesis was potentiated by hypoxia in a time-dependent manner. This response positively correlated with the cumulative induction of phosphotyrosine and the downregulation of bFGFR (M_r ~85 kDa). Protein tyrosine kinase inhibitors, herbimycin-A, and methyl 2,5-dihydroxycinnamate, unlike genistein, markedly blocked hypoxia-induced phosphotyrosine. Prolonged exposure of cells to phosphatase inhibitor, sodium orthovanadate, also blocked hypoxia-induced phosphotyrosine. The expression of HSP70, PKCα, and SHC were not markedly altered by hypoxia. Taken together, these data suggest that short-term hypoxia activates endothelial cell proliferation in part via tyrosine phosphorylation of cellular proteins and changes in the expression of the FGF receptor. Thus, endothelial cell mitogenesis and neovascularization associated with low oxygen tension may be controlled by abrogating signaling pathways mediated by protein tyrosine kinase and phosphatases.

Key words: low oxygen tension, neovascularization, signal transduction, tyrosine kinase inhibitors, phosphotyrosine, Western blotting, FGF-receptor, phosphatase inhibitor

Low oxygen tension or hypoxia is associated with a number of pathologic vasoproliferative disorders [Henkind, 1978; Patz, 1982] in which angiogenic factors play a significant role [for reviews see Folkman and Klagsbrun, 1987; D'Amore, 1994]. In conditions such as diabetic retinopathy, biochemical changes imposed by prolonged hyperglycemia cause vessel occlusion and chronic hypoxia which induces retinal neovascularization [Schultz and Grant, 1991]. Although responses to hypoxia may vary depending on the origin of endothelial cells [Tretyakov and Farber, 1993], the process of neovascularization initiated by conditions such as hypoxia is generally modulated by a balance of stimulatory and inhibitory factors; these extracellular modulating factors may occur in different combinations in different disease states [Glaser, 1988]. The ischemia-induced release of angiogenic factors such as bFGF [de Juan et al., 1990a,b; Hanneken et al., 1991; Vender, 1992] and vascular endothelial growth factor (VEGF) [Simorre-Pinatel et al., 1994; Adamis et al., 1993] probably account for the mitogenic activity of culture medium derived from cells exposed to low oxygen tension [Xiao et al., 1993]. The interaction of such ligands with cognate receptors

Abbreviations used: BCA, bicinchoninic acid; bFGF, basic fibroblast growth factor; BRCEC, bovine retinal capillary endothelial cells; BrdU, bromodeoxyuridine; ECL, enhanced chemiluminiscence; FGFR, fibroblast growth factor receptor; PTK, protein tyrosine kinase; RIPA, radioimmunoprecipitation assay.

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leads to the receptor downregulation in cultured endothelial cells [Boes et al., 1991] which has also been linked to hypoxia [Shaul et al., 1991]. Such receptor dynamics occurs by the internalization, endocytosis, and lysosomal degradation of growth factor-receptor complexes [Carpenter and Cohen, 1976; Sorkin and Waters, 1993].

Hypoxia elicits a variety of responses in endothelial cells, suggesting that it is a critical physiological condition. This gamut of responses includes increased cell proliferation and synthesis of IL-1α [Shreeniwas et al., 1992], upregulation of TGFβ [Santilli et al., 1991], increase in glucose transporter [Loike et al., 1992], intracellular calcium [Arnould et al., 1992], prostaglandins [Michiels et al., 1993], membrane-associated proteins [Ogawa et al., 1992], endothelin-1 [Gertler and Ocasio, 1993], and platelet aggregating factor (PAF) [Caplan et al., 1992].

The autocrine release of cytokines such as bFGF and other cell-activating processes which characterize the hypoxic response, are analogous to wound healing mechanisms [Lindner and Reidy, 1992] and seem to reflect a homeostatic adaptive response to hypoxic stress [Weinhouse et al., 1993; Graven et al., 1993].

Although the role of tyrosine kinases in cell proliferation in general is well documented [Isakov et al., 1994], the role of this enzyme in hypoxia-induced responses associated with neovascularization is relatively poorly described. Recently, in Jurkat T-cells, Koong et al. [1994] determined that hypoxia (pO_2 = 0.02%) activates nuclear factor κB via increased tyrosine phosphorylation, and Eckardt et al. [1994], in attempting to define the role of PKC in the oxygen-dependent production of erythropoietin in cultured hepatocytes, speculated that a kinase other than PKC appears to be an essential element of hypoxic signaling. In this paper, we present direct evidence that a time-dependent increase in tyrosine phosphorylation of a number of cellular proteins—concomitant with FGF receptor downregulation, both events attributable to increased protein tyrosine kinase activity—is among the diverse processes associated with the mitogenic signaling pathways of hypoxia in retinal capillary endothelial cells. It seems that hypoxia-induced tyrosine phosphorylation may not only be key in mediating hypoxia-induced neovascularization, but may, depending upon the range of proteins affected, also serve as a plausible paradigm for the activation of cell-transforming oncoproteins, in vivo.

MATERIALS AND METHODS

Primary Cell Culture

Retina obtained from fresh bovine eyes was cultured in Dulbecco's Modified Eagle's medium (DMEM), as described by Capetandes and Gerritsen [1990]. Cells were determined to be endothelial cells based on their morphology and immunological staining for factor VIII [Gerritsen et al., 1987]. Aliquots of early passage bovine capillary endothelial cells (BRCEC) were frozen and used in all experiments.

Incubation Conditions

Equal volumes of cell suspension were seeded in tissue culture plates and incubated in standard tissue culture conditions of 5% carbon dioxide at 37°C. Culture medium comprised 10% fetal calf serum, 90 μg/ml endothelial cell growth supplement, and 1% antibiotic-antimycotic in low glucose DMEM (Gibco, Grand Island, NY). After 24 h, culture medium was replaced with fresh medium and the plates returned to the normoxic chamber until the monolayers were confluent. Hypoxic conditions were attained by supplying 2% oxygen gas (Puritan Bennett, Linthicum Heights, MD) through a rubber tubing to an air-tight transparent plexi-glass chamber (Forma Scientific, Marietta, OH) located in a 37°C chamber. Oxygen measurements of effluent gas were made at regular intervals using a Beckman (Fullerton, CA) oxygen analyzer (model D2). The oxygen tension within the hypoxic chamber stabilized to 3%.

Drug Treatments

Samples to be tested for the effect of protein tyrosine kinase inhibitors on hypoxia-induced phosphotyrosine were quiesced in serum-free medium in normoxia for 2 h and then pretreated with 50 μM herbimycin A, genistein, and methyl 2,5-dihydroxycinnamate for 3 h. Control samples did not receive drug treatments. Following drug treatment, cell monolayer surfaces were rinsed with PBS, culture medium replaced with fresh serum-free medium and all culture plates including controls were finally transferred to and maintained in hypoxia for 24 h.

To test for the effect of phosphatase inhibitor sodium orthovanadate on hypoxia-induced phosphotyrosine, monolayers quiesced in serum-free medium for 2 h were pretreated with 50 μM sodium orthovanadate for 3 h, cell monolayer surfaces were rinsed, fresh serum-free medium

added, and then transferred to hypoxia for 24 h. Control samples were also placed in hypoxic chamber for 24 h but did not receive vanadate pretreatment.

DNA Synthesis

Confluent monolayers cultured in 35 mm Falcon tissue culture plates under normoxia (pO_2 = 20%) were quiesced in low serum (1.5%)-containing medium overnight. Cells were then transferred to serum-free medium and either continuously maintained in normoxic conditions (control) for 24 h or transferred to hypoxia on a time course of 1, 2, 18, 20, and 24 h with each sample set up in triplicates. DNA synthesis was measured by quantitative analysis of incorporated Bromodeoxyuridine (BrdU) (Amersham, Arlington Heights, Ill.). All samples were labeled in BrdU during the last 2 h of hypoxia; the 1 h sample was labeled for 1 h in hypoxia. Cells were fixed and blocked with BSA/Tween and then incubated in anti-BrdU and peroxidase-linked anti-mouse IgG. Color reaction was stopped by adding stop color solution, and then absorbance of supernate was read at 410 nm using an ELISA plate reader, according to Amersham cell proliferation assay protocols.

Sample Preparation

Whole cell lysates were obtained by incubating cells on ice in a 1:2 mixture of lysis buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, 35 μg/ml PMSF, 0.3 μg/ml EDTA, 0.7 μg/ml pepstatin A, 0.5 μg/ml leupeptin, 250 μM sodium orthovanadate, and 50 μM sodium fluoride) and SDS sample buffer (160 mM Tris-HCl, pH 6.8, 4% SDS, 30% glycerol, 5% β-mercaptoethanol, 10 mM DTT, 0.01% bromophenol blue). Lysed cells were harvested by using a cell scraper and transferred into microfuge tubes. Lysate viscosity was reduced by a few passages through a 25 gauge needle followed by centrifugation at 13,000 rpm for 10 min and then boiled for 5 min. Sample concentration was determined by the Pierce (Rockford, IL) BCA method analyzed by SDS PAGE or stored at −20°C.

Immunoprecipitation

Polyclonal anti-FGFR (10 μg) anti-serum (UBI, Lake Placid, NY) was added to whole cell lysates solubilized in 500 μl lysis buffer in a microcentrifuge tube. The mixture was vortexed and incubated at 4°C for 1 h. Protein A-agarose (20 μl) was added, vortexed, and incubated for another 1 h at 4°C with occasional agitation. After washing three times by centrifugation (5 min at 1,000*g*) and resuspension in 100 μl volumes of PBS, PBS/0.5 M NaCl, and lysis buffer, the immunoprecipitate was finally resuspended and boiled in 50 μl SDS sample buffer for 5 min.

Western Immunoblot Analysis

Equal quantities of protein samples were subjected to SDS PAGE and run at constant 200 V for 45 min in 10%, 0.75 mm thick precast acrylamide minigels (Bio-Rad, Richmond, CA) using a Bio-Rad miniprotean apparatus. Broad range biotinylated (6.5–200 kDa) and prestained (18.5–106 kDa) molecular weight protein markers were run simultaneously with the samples and used to determine molecular weight of unknowns. Gels were equilibrated in Towbin's transfer buffer and then electroblotted onto 0.4 μM nitrocellulose membranes (Bioblot-NC®, Costar, Cambridge, MA) using the Bio-Rad minitank blot apparatus. Membranes were blocked in 1% nonfat dried milk and 3% BSA for 1 h and probed with antibody containing 1% BSA for a minimum of 2 h at room temperature or 16 h at 4°C. Blots were washed in tris buffered saline (TBS) and incubated for 1 h at room temperature in a double secondary antibody matrix composed of IgG-horseradish peroxidase (HRP) and streptavidin-HRP and 3% BSA. Blots were washed again, immersed in enhanced chemiluminiscence (ECL) immunodetection reagents, and then exposed to Kodak X-ray film from 15 s to 5 min, as described in Amersham ECL protocols. Coomassie-stained gels were fixed in 45% methanol and 10% acetic acid aqueous solution for 10 min, soaked in saturated picric acid solution briefly (to reduce background and increase sensitivity), stained in 0.25% aqueous Coomassie brilliant Blue (R250) for a minimum of 2 h, and then destained in several washes of 10% acetic acid solution.

RESULTS

DNA Synthesis and Cell Morphology

Exposure of endothelial cells to short-term hypoxia showed an increase in cell proliferation (Fig. 1). Compared to controls, DNA synthesis in hypoxic cells was initially depressed but assummed an exponential rate 18 h after onset of hypoxia. Even though cell activity was main-

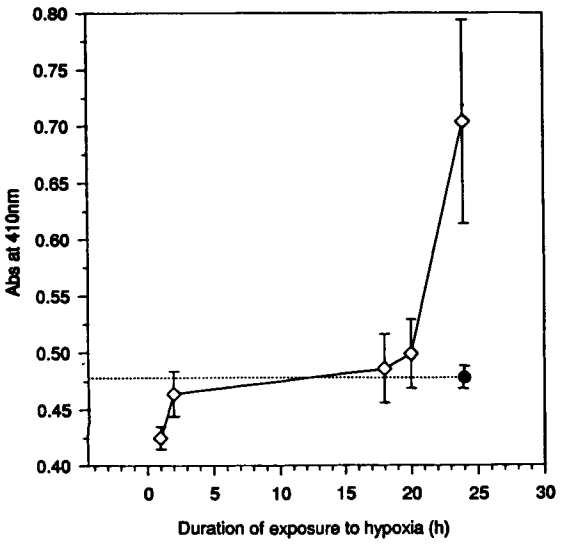

Fig. 1. Effect of hypoxia on DNA synthesis. Cells cultured in 35 mm dishes were quiesced overnight in DMEM containing 1.5% calf serum. Culture medium was replaced with fresh serum-free DMEM medium, and the plates were transferred to the hypoxic chamber starting with the 24 h samples (open diamonds). Cells were labelled in equal volumes of bromodeoxyuridine in the last 2 h of incubation. Control samples (end of dotted line, filled circle) were kept in normoxia. After labeling, cells were immediately fixed and processed for spectroscopic analysis as detailed in Materials and Methods. Values represent the mean ± SD of three replicates, corrected for background absorbance.

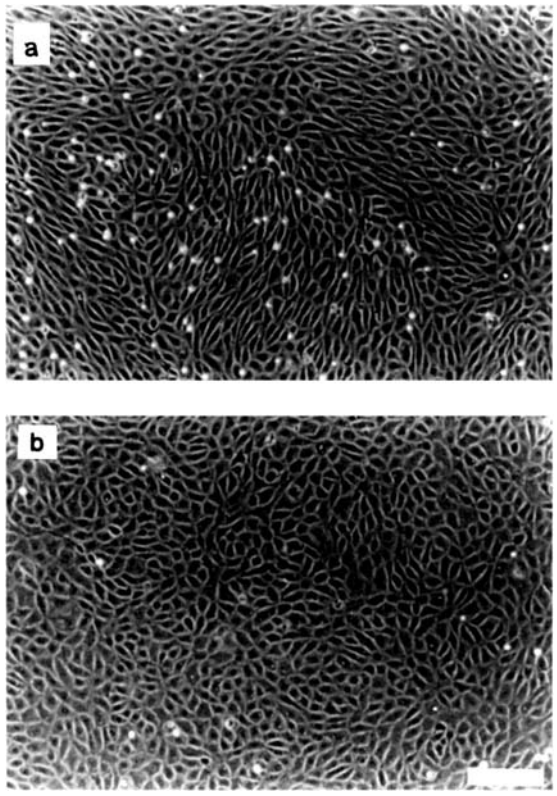

Fig. 2. Phase contrast photomicrograph showing the effect of 24 h exposure to hypoxia on endothelial cell morphology. Cells were cultured in complete DMEM culture medium under standard normoxic culture conditions until confluent. Cells were quiesced in 1.5% serum-containing medium overnight, followed by 24 h incubation in serum-free medium, in normoxia (control, **a**) or in hypoxic chamber (**b**). Bar = 100 μm.

tained, 24 h exposure to hypoxia resulted in a less organized morphological layout of the endothelial cell monolayer (Fig. 2).

Effect of Hypoxia on Phosphotyrosine Induction

Cells incubated on a time course of up to 24 h showed an accumulation of phosphotyrosine that steadily increased with time (Fig. 3). Tyrosine phosphorylation was evident in a number of cellular substrates; ~M_r 210, 200, 116, 76, and 52 kDa. The induction of phosphotyrosine intensified with time especially at 18–24 h of hypoxia.

Effect of PTK Inhibitors on Phosphotyrosine Induction

The effect of protein tyrosine kinase inhibitors was varied (Fig. 4). Control for drug untreated exhibited tyrosine phosphorylation, which was blocked by drug treatment to varying degrees. Herbimycin A and methyl 2,5-dihydroxycinnamate were potent blockers of tyrosine phosphorylation in treated cells. The effect of genistein on tyrosine phosphorylation, however, was minimal; levels of phosphotyrosine in genistein-treated cells were similar to those of controls.

Effect of Phosphatase Inhibitor on Phosphotyrosine Induction

Cells pretreated with vanadate showed a slight increase in phosphotyrosine in the first hour of hypoxia, diminishing markedly afterwards (Fig. 5). The range of substrates that showed tyrosine phosphorylation in vanadate-treated samples, ranged from 200 kDa to less than 30 kDa. In general, accumulation of hypoxia-induced phosphotyrosine was suppressed by phosphatase inhibitor, sodium orthovanadate.

FGFR and the Effect of Hypoxia on Its Downregulation

Coomassie-stained FGFR immunoprecipate was shown to be a monomer corresponding to approximately M_r 85 kDa (Fig. 6). Expression of the FGF receptor declined gradually as the dura-

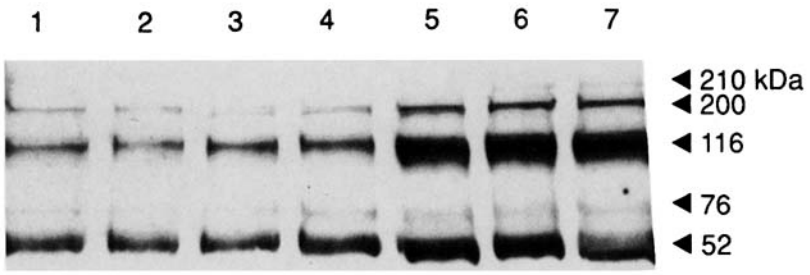

Fig. 3. The effect of hypoxia on phosphotyrosine induction. Endothelial cells cultured in complete medium to confluence were quiesced in 1.5% serum containing medium overnight, transferred to serum-free medium, and then maintained in normoxia for 24 h (control, lane 1), or transferred to hypoxia on a time course of 15 min (lane 2), 1 h (lane 3), 2 h (lane 4), 18 h (lane 5), 20 h (lane 6), or 24 h (lane 7). Equal concentrations of isolated proteins determined by Pierce (Rockford, IL) BCA protein quantitation were loaded on 10% acrylamide SDS PAGE minigels, and electroblotted onto nitrocellulose membrane using the miniprotean apparatus (Bio-Rad, Richmond, CA). Samples were run simultaneously with broad range biotinylated molecular weight markers (Bio-Rad). Blotted proteins were challenged with anti-phosphotyrosine antibody (1:1,000 dilution) (UBI, Lake Placid, NY), avidin-HRP/IgG-HRP secondary antibody mix, and detected by ECL (Amersham, Arlington Heights, IL) on kodak X-ray film.

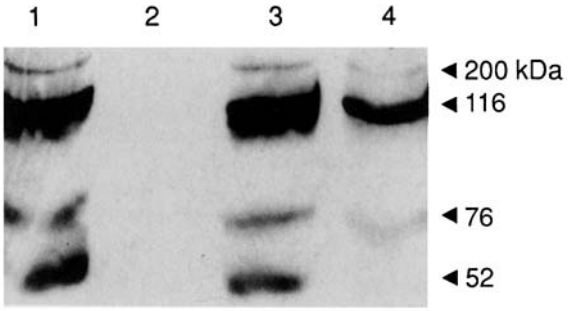

Fig. 4. Effect of protein tyrosine kinase inhibitors on hypoxia-induced phosphotyrosine in endothelial cells. Cells cultured in 35 mm Petri plates in normoxia were quiesced in 1.5% serum containing medium overnight. The cells were then incubated in fresh serum free DMEM medium to which 50 μM herbimycin A (lane 2), genistein (lane 3), or methyl 2,5-dihydroxycinnamate (lane 4) was added and incubated for 2 h. No drug was added to the control sample (lane 1). At the end of drug pretreatment, plates were transferred to hypoxia for 24 h, following which whole cell lysates were subjected to protein isolation, Pierce BCA protein quantitation, SDS PAGE (10% minigel), and electroblotted on nitrocellulose membrane using the miniprotean apparatus (Bio-Rad). Samples were simultaneously run with broad range biotinylated molecular weight markers (Bio-Rad). Electroblotted proteins were challenged with anti-phosphotyrosine primary antibody (UBI), avidin-HRP/IgG-HRP secondary antibody mix, and detected by ECL (Amersham) on Kodak X-ray film.

tion of hypoxia was increased from 1 to 24 h (Fig. 6). This trend of downregulation was more evident in Western blots (upper panel) than in Coomassie Blue stained samples (lower panel).

Expression of PKCα, SHC, and HSP70 Expression

Western immunoblot analysis showed only slight increases (at 20–24 h hypoxia) in the expression of PKCα (Fig. 7a) and HSP70 (Fig. 7c), but no such change was observed for SHC (Fig. 7b). In general, the changes observed in the expression of these molecules (i.e., PKC and HSP70) were not as dramatic as those observed for phosphotyrosine.

DISCUSSION

Low oxygen tension or hypoxia is closely associated with a sequence of events that result in new blood vessel formation. Our studies sought to provide some insights into the signal transduction mechanisms that characterize these events. Since neovascularization induced DNA synthesis (and cell proliferation), we rationalized that the increase in cell proliferation was a direct function of mechanisms associated with normal proliferative activities. Starting with cell morphology, our results indicate that no deleterious change was evident during a 24 h exposure to 3% hypoxia. This outcome is consistent with the fact that cell proliferation continued unabated. The increased DNA synthesis shown here is consistent with hypoxia-induced proliferative activities in endothelial cells. Endothelial cell proliferation has been shown to continue even under severe and prolonged hypoxia [Shreeniwas et al., 1991]. Quiescing cells prior to testing for DNA synthesis ensures a low and synchronized basal level of DNA synthesis, thereby increasing the sensitivity of the assay in detecting the effects of hypoxia.

Since neovascularization is a result of a fine balance between agonists and antagonists [D'Amore, 1994], our results thus seem to indicate that in the short term, hypoxia potentiates the autocrine release of factors whose stimula-

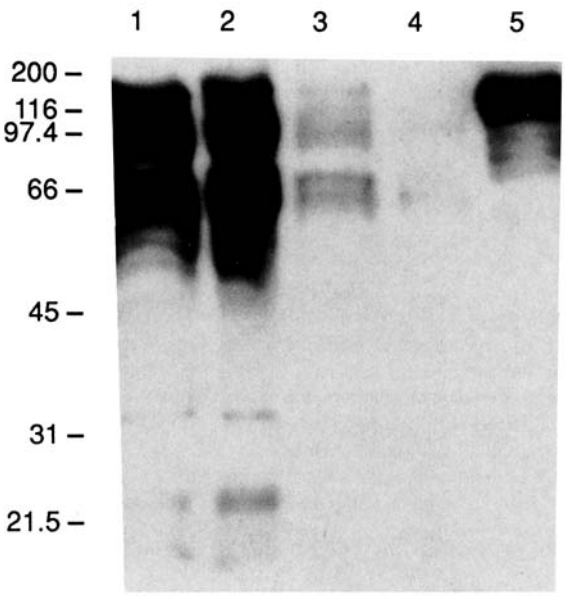

Fig. 5. Effect of sodium orthovanadate pretreatment on hypoxia-induced phosphotyrosine. Cells incubated in normoxia to confluence were quiesced in serum-free medium for 2 h, and then exposed to serum-free medium containing 50 μM sodium orthovanadate for an additional 3 h. Following drug pretreatments in normoxia, cell cultures were transferred to and maintained in hypoxia chamber for 1 h (lane 2), 4 h (lane 3), or 24 h (lane 4). Control samples (lane 1) were also maintained in hypoxia but did not receive any drug treatment. Protein extraction, electroblotting, and SDS PAGE were as detailed in Materials and Methods. Identity of phosphotyrosine was confirmed by a positive control for phosphotyrosine (lane 5). Samples were run together with broad range biotinylated molecular weight markers (Bio-Rad).

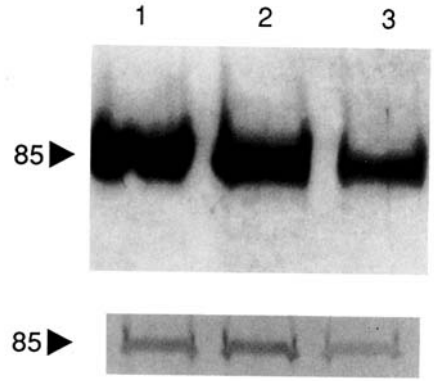

Fig. 6. Changes in FGFR induced by hypoxia. Confluent monolayers were incubated in 1.5% serum-containing medium overnight and then maintained in serum-free normoxic conditions for an additional 24 h (control, lane 1) or transferred to and maintained in hypoxia for 1 h (lane 2) or 24 h (lane 3). Whole cell lysates were subjected to FGFR immunoprecipitation in equal volumes of lysis buffer and polyclonal anti-FGFR (UBI), as detailed in Materials and Methods. Immunoprecipitates were run on a 10% SDS PAGE minigel, using a miniprotean apparatus (Bio-Rad) and electroblotted onto nitrocellulose membrane. Samples were run along with broad range biotinylated molecular weight standards (Bio-Rad). Protein detection was performed by incubation of blocked membranes in a 1:500 dilution of anti-FGFR **(upper panel)**, or stained with Coomassie brilliant blue **(lower panel)**.

tory action supersedes the inhibitory mechanisms which impinge upon the cells during the period of hypoxia. By showing increased tyrosine phosphorylation during hypoxia, this finding is consistent with and supports the fact that protein tyrosine kinase mediates cellular activation processes associated with cell proliferation, growth, and differentiation [Inaba et al., 1992; Isakov et al., 1994] by initiating a cascade of tyrosine phosphorylation of cellular PTK substrates [Hunter and Cooper, 1985; Purushotham et al., 1993; Xiao et al., 1993]. The increased tyrosine phosphorylation is further supported by Koong et al. [1994] in the hypoxia-induced activation of nuclear factor IκBα. This mode of response is generally akin to the activation of a wide range of protective mechanisms directed against ischemic injury [Zhang et al., 1994].

Since the inhibition of tyrosine phosphorylation correlates with the suppression of cell proliferation [Matsiu and Arai, 1993], we tested the effects of selected tyrosine kinase inhibitors, herbimycin A, methyl 2,5-dihyroxycinnamate, and genistein, on hypoxia-induced tyrosine phosphorylation. Herbimycin A and methyl 2,5-dihyroxycinnamate, unlike genistein, blocked hypoxia-induced phosphotyrosine. This result supports our earlier studies [Koroma and de Juan, 1994] in demonstrating that genistein exhibits a relatively low level potency as a PTK inhibitor, and is consistent with other findings [Graber et al., 1992; Sargeant et al., 1993] which indicate that herbimycin A and methyl 2,5-dihydroxycinnamate are relatively more potent tyrosine kinase blockers. The differences in potency of PTK inhibitors may be generally related to differences in the structural properties of specific drugs [Chang and Geahlen, 1992].

It is a widely held notion that vanadate inhibits protein phosphatases thereby causing tyrosine hyperphosphorylation of cellular proteins and changes in the physiological action of phosphoproteins, which affect cell proliferation, gene expression, and transient cell transformation [Itkes et al., 1990]. We sought to test this hypothesis in relation to hypoxia with the expectation that the inhibition of phosphatases will lead to hyperphosphorylation of cellular proteins during hypoxia. To the contrary, our results show

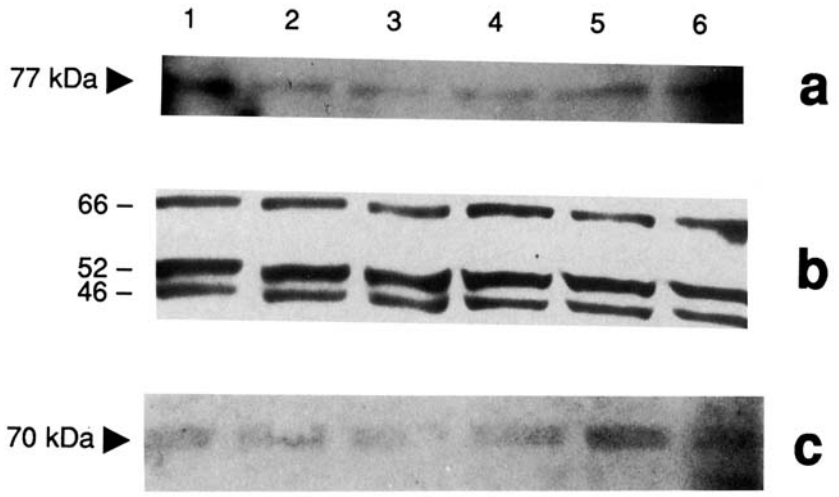

Fig. 7. Western immunoblot showing the effect of hypoxia on PKCα (**a**), SHC (**b**), and heat shock protein 70 (**c**). Monolayers cultured to confluence in 35 mm culture Petri plates were quiesced overnight in DMEM culture medium containing 1.5% calf serum. The medium was replaced with serum-free medium and the culture plates either maintained in normoxia for 24 h (control, lane 1) or transferred to hypoxia for 1 h (lane 2), 2 h (lane 3), 18 h (lane 4), 20 h (lane 5), or 24 h (lane 6). After hypoxia treatment, cells were lysed in 500 μl lysis RIPA buffer in culture plates placed on ice for 20 min. Protein isolation from whole cell lysates proceeded as detailed in Materials and Methods. Equal quantities of protein were run on 10% SDS PAGE minigels, alongside biotinylated molecular weight standards, and then electroblotted onto nitrocellulose, followed by incubation in specific primary antibodies, followed by incubation in IgG-HRP/avidin-HRP secondary antibody mixture. Protein detection was performed by ECL (Amersham) and autoradiography on Kodak X-ray film.

that although vanadate caused a slight increase in phosphorylation during the first 1 h of hypoxia, inhibition of phosphotyrosine occurred afterwards. This result is interesting because it suggests that the normal functioning of phosphatases is related to and probably contingent upon the normal activity of protein tyrosine kinases during hypoxia. This reasoning is supported by the findings of Brady-Kalnay and Tonks [1994], Kanakura et al. [1990], and Vogel et al. [1993], which showed that tyrosine kinases and phosphatases must necessarily work in concert to maintain a fine balance of effector activation required for the regulation of cell growth and differentiation.

Since the activity of tyrosine kinases is linked to growth factor stimulation, we sought to investigate the dynamics of FGFR widely reported to mediate endothelial cell proliferation in vitro and in vivo, as a basis for phosphotyrosine induction in hypoxia. We showed that FGFR, a single band of ~85 kDa, is downregulated during hypoxia. This monomer is probably a variant of FGF-R1β2 reported earlier [Koroma and de Juan, 1994; Xu et al., 1992]. Phenotypic variation of FGFR is widely reported [Johnson et al., 1990; Johnson et al 1991; Hou et al., 1991; Eisemann et al., 1991; Korhonen et al., 1992] and reflective of the multiplicity of characterized FGFs [Gospodarowicz et al., 1986], the diversity of FGF-induced activities [Burgess and Maciag, 1989], and differences in the physiological responses of endothelial cells based on source or origin [Farber and Barnett, 1991; Weinhouse et al., 1993]. The downregulation of FGFR in hypoxia shown in this study is consistent with that reported by Moscatelli [1988] and probably occurs simultaneously with similar changes in other receptors such as those of VEGF, another cytokine strongly associated with hypoxia and neovascularization [Shweiki et al., 1992; D'Amore, 1994]. In general, hypoxia-induced changes in the expression of FGFR may be closely related to the role played by the FGF ligand in wound-healing responses reported by Gadjusek and Carbon [1989].

Regulation of cell proliferation involves a complex pathway that may include a diverse array of signaling molecules such as SHC (*Src* homology region 2-containing protein) and protein kinase C (PKC). In addition, special constitutive and inducible stress proteins such as heat shock protein 70 (HSP70) are known to be associated with environmental stress. To determine the putative roles of these molecules in hypoxia, we evaluated changes in SHC, PKCα, and HSP70 in cells exposed to hypoxia and found no significant changes. With regards to PKC, our results agree

with Eckardt et al. [1994] in showing that kinases other than PKC are more likely mediators in hypoxia-induced cell activation responses. The absence of a strong correlative change in HSP70 also seems to agree with Zimmerman et al. [1991] in suggesting that although hypoxia induces a specific set of stress proteins in endothelial cells, HSP70 as a class may not be involved. Although little is known about SHC in relation to hypoxia, it is known to be a mitogenic signaling molecule [Sasaoka et al., 1994] which exhibits tyrosine phosphorylation under certain types of stimuli [Meyer et al., 1994]. Our results seem to indicate that changes in the expression of SHC may not be involved in hypoxia signaling. Rather, we speculate that changes in SHC may instead relate to changes in the degree of tyrosine phosphorylation of its subunits.

Hypoxia strongly activates endothelial cells [Michiels et al., 1993] via increased phosphotyrosine induction, downregulation of FGFR, and increased DNA synthesis. These responses may indeed be part of a complex pathway regulated by a fine balance between growth promoters and inhibitors [Glaser, 1988; D'Amore, 1994]. Hypoxia seems to create a disequilibrium in favor of cell activation via increased PTK activity. Our results conform to an empirical logic which implies that if hypoxia induces neovascularization [Smith, 1990; Pournaras et al., 1990], and neovascularization correlates with increased cell proliferation [Zhang et al., 1993; Cozzolino et al., 1993], which in turn is associated with phosphotyrosine induction [Koroma and de Juan, 1994; Isakov et al., 1994], then hypoxia can be expected to induce cell proliferation and phosphotyrosine induction. This study concurs with Lindner and Reidey [1992] and Zhang et al. [1994] and suggests that the tendency for hypoxic cells to initiate cell proliferation and neovascularization reflects the repair system typical of bFGF-mediated wound healing [Lindner and Reidy, 1991].

The induction of phosphotyrosine in hypoxia suggests a critical role for protein tyrosine kinase in the pathogenesis of neovascularization in conditions of low oxygen tension. The ability to inhibit phosphotyrosine induction through the use of tyrosine kinase and phosphatase inhibitors presents a potential treatment modality for vascular proliferative diseases associated with ischemia. Increased tyrosine phosphorylation contributes to the cellular transforming activity of specific oncogenes [Ernst et al., 1994]. Thus, we surmise that, depending upon the identities of cellular protein molecules phosphorylated on their tyrosine moieties, hypoxia deriving from microvessel occlusion may be among the factors which trigger such cellular transformations in vivo.

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