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NERF2, a Member of the Ets Family of Transcription Factors, Is Increased in Response to Hypoxia and Angiopoietin-1: A Potential Mechanism for Tie2 Regulation During Hypoxia

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Abstract Vascular endothelial growth factor (VEGF) and angiopoietins regulate endothelial cell survival and migration and are essential for angiogenesis. Considerable progress has been made towards understanding hypoxia-mediated regulation of VEGF and its receptors. In contrast, little is known about the regulation of angiopoietins and their receptors in hypoxic cells. Using RT–PCR, RNAase protection assay, and Western blotting, we found that Tie1 and Tie2 mRNA and protein levels increased in response to hypoxia in human umbilical vein endothelial cells. Previously, we have shown that NERF2, a member of Ets family of transcription factors that is specifically expressed in endothelial cells, binds to the promoter region of Tie2 and transactivates Tie2 expression. In this study, we show that expression of NERF2 was increased under hypoxia and that this increase temporally correlated with the increase in Tie2 expression. Hypoxia-induced expression of NERF2 and Tie2 was blocked by angiopoietin-2, a competitive inhibitor of angiopoietin-1, and by recombinant soluble extracellular domain of Tie2 but not by VEGF-neutralizing antibodies. In addition, angiopoietin-1 directly induced expression of NERF2 in quiescent cells. These novel findings suggest that angiopoietin-1 regulates expression of NERF2 and its own receptor in hypoxic cells. J. Cell. Biochem. 85: 505–515, 2002. © 2002 Wiley-Liss, Inc.

Key words: angiopoietin; Ang1; Ang2; Ets transcription factors; hypoxia; NERF2; Tie1; Tie2

It has been estimated that less than 0.01% of vascular endothelial cells are engaged in cell division in mature blood vessels [Ortéga et al., 1999]. Disease states including wound healing, diabetic retinopathy, coronary and cerebral ischemia, and nearly every type of tumor are associated with increased requirements for oxygen and nutrients. Hypoxia is a potent stimulator of endothelial cell proliferation and survival in vivo and in vitro [Shweiki et al., 1992; Banai et al., 1994]. There is strong evidence to suggest that local production of growth factors, which act selectively on the vascular endothelium, mediate this response in vivo [Knighton et al., 1983; Gospodarowicz et al., 1989; Knighton and Fiegel, 1989]. These factors stimulate vascular endothelial cells to proliferate and migrate into the surrounding tissue, resulting in the formation of new blood vessels from a preexisting vascular network [Holash et al., 1999; Ortéga et al., 1999].

Two families of receptor tyrosine kinases have been identified whose expression is almost exclusively restricted to cells of endothelial

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lineage [Mustonen and Alitalo, 1995; Ortéga et al., 1999]. These are vascular endothelial growth factor (VEGF) and Tyrosine kinase receptors with Immunoglobulin and EGF homology domains (Tie) receptors [Mustonen and Alitalo, 1995]. The ligand for Tie1 has not been identified. Angiopoietin-1 (Ang1) is a ligand for Tie2, which leads to autophosphorylation and intracellular signaling, whereas angiopoietin-2 (Ang2) is a naturally occurring competitive inhibitor of Ang1 and blocks Tie2 phosphorylation [Davis et al., 1996; Maisonpierre et al., 1997]. Although Ang1 does not induce DNA synthesis or endothelial cell division, it stimulates cell survival and migration [Witzenbichler et al., 1998; Fujikawa et al., 1999a; Jones et al., 1999].

Little is known about the regulation of expression of angiopoietins and Tie receptors during pathologic conditions. In bovine microvascular endothelial cells, Ang2 expression is induced under hypoxia, whereas expression of Ang1 and Tie2 are not significantly changed [Mandriota and Pepper, 1998; Oh et al., 1999; Mandriota et al., 2000]. Expression of angiopoietins and Tie is not altered by hypoxia in other bovine endothelial cells [Mandriota and Pepper, 1998]. In contrast, hypoxia induces Tie2 expression in human coronary microvascular, dermal microvascular, and umbilical vein endothelial cells [Willam et al., 2000]. The increase in Tie2 in response to hypoxia was greatest in HCMEC (> 3-fold) and smallest in HUVEC (1.7-fold). These data suggest that the response to hypoxia differs for endothelial cells derived from different tissues.

The initial goal of this study was to determine the effect of hypoxia on expression of angiopoietins, Tie1 and Tie2 in primary human endothelial cells. Our second goal was to examine the effect of hypoxia on expression of NERF2, a member of the Ets transcription factor family found specifically in endothelial cells, which is a known transactivator of *Tie2* gene [Dube et al., 1999; Iljin et al., 1999]. Hypoxia induced an increase in expression of Tie1 and Tie2 that corresponded with an accumulation of Tie proteins in hypoxic cells. Expression of NERF2 was also increased and this increase preceded the accumulation of Tie2. Ang2 and purified recombinant soluble Tie2 receptor (sTie2Fc) blocked the upregulation of NERF2 and Tie2 in hypoxic cells, but had no effect on expression of Tie1. The implications of Ang1 and hypoxia-mediated regulation of Tie 2 expression are discussed.

MATERIALS AND METHODS

Reagents

Purified recombinant Ang1 and Ang2 and soluble extracellular domain of Tie2 receptor (sTie2Fc) were kindly provided by G.D. Yancopoulos (Regeneron Pharmaceuticals, Inc., NY). Ang1 was a modified form of human Ang1 [Maisonpierre et al., 1997]. VEGF neutralizing antibodies were from R&D Systems (Minneapolis, MN). Rabbit polyclonal anti-human Tie1, Tie2, and horseradish peroxidase (HRP)-conjugated goat anti mouse antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti phospho-tyrosine (anti-P-tyr, 4G10) and pan Erk antibodies were from Upstate Biotechnology (Lake Placid, NY), and HRP-conjugated goat polyclonal rabbit antibodies were from New England Biolabs (Beverly, MA).

Cell Culture

Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical cords [Jaffe et al., 1973]. Cells were obtained by pooling three independent donor cords to minimize variability and were used in passage three or four on tissue culture plates coated with 1.5% gelatin (Sigma Chemical Co., St. Louis, MO). HUVEC were grown in complete medium [M199 (Bio Whittaker, Walkersville, MD) supplemented with 20% FCS, bovine brain extract with heparin (Clonetics Corp., San Diego, CA), Glutamine, non-essential amino acids, and antibiotics/antimycotics] as described previously [Namiki et al., 1995]. Human microvascular cells (HMEC) were purchased from ATCC (Rockville, MD) and grown in minimal essential media supplement with 10% FCS.

Hypoxia

HUVEC were grown to > 80% confluency and media was replaced with fresh complete media prior to placing the plates in the hypoxia chambers. Ang2 (96.5 ng/ml), sTie2Fc (10 μg/ml) or anti-VEGF neutralizing antibodies (10 μg/ml) were added as indicated. Plates were placed in humidified hypoxia chambers (Bellco Glass, Vineland, NJ) and flushed for 15 min every 12–14 h with gas mixture containing 1% O_2, 5% CO_2, and 94% N_2 for indicated times at 37°C. At the end of incubation period, the plates

were placed on ice, washed twice with ice-cold PBS and cells were extracted for total RNA or protein analysis.

Growth Factor Stimulation

Cells were grown to 80–90% confluency, placed in a 1:1 mixture of complete media and serum-free media containing 1% BSA for 16 h. Ang1 and VEGF were added at 250 and 100 ng/ml, respectively, for indicated times and incubated at 37°C in 5% CO_2.

Reverse Transcriptase–Polymerase Chain Reaction (RT–PCR)

Cells were washed twice with ice-cold PBS, incubated on ice for 5 min with RNA isolation buffer containing acidic guanidine isothiocyanate (Ambion Inc., Austin, TX or RNAeasy, Qiagen, Valencia, CA), scraped from the plate, vortexed, and stored at −80°C. Total RNA was isolated according to the manufacturers' instructions. First strand cDNA was generated from 5 μg of total RNA using SuperScript II Reverse Transcriptase kit and oligo$(dT)_{12-18}$ priming (Gibco BRL, Grand Island, NY). Glyceraldehyde dehydrogenase (GAPDH) or β-actin, Ang1, Ang2, Tie1, Tie2, VEGF, and Ets transcription factors were amplified by polymerase chain reaction (PCR) using primers specific for human DNA sequences [Fujikawa et al., 1999b]. The NERF1a and NERF1b top strand primer was 5′-GTG TCT CCG GCC GCG GGT-3′, NERF1a bottom strand primer was 5′-GAT GAA TTC AGG ACT TCT-3′, NERF1b bottom strand primer was 5′-CAC AAG GAG GAA CAA ACA C-3′, NERF2 top strand primer was 5′-AGC AGC TTC AAT TGT CTT-3′ and the bottom strand primer was 5′-ATG ACA TCA GCA GTG GTT G-3′. The optimized PCR conditions for Taq polymerase (Promega Corp., Madison, WI) was 94°C for 5 min, followed by 35–40 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min, and 1 cycle at 72°C for 5 min. In some instances, PCR was performed using Advantage polymerase mixture (Clontech Laboratories, Inc., Palo Alto, CA). The optimized PCR conditions was 94°C for 2 min, followed by 35–40 cycles of 94°C for 45 s and 64°C for 1 min 30 s, and 1 cycle at 64°C for 5 min.

RNAase Protection Assay (RPA)

Fifteen μg of total RNA was incubated with excess ^{32}P-labeled anti-sense RNA probe (Angio-1 template set, BD PharMingen, San Diego, CA) at 56°C for 12–16 h. Free probe and other single-stranded RNA were digested by incubation with RNAase A for 45 min at 30°C according to manufacturers' recommendations. After removal of the RNAase A, samples were loaded onto a 5% polyacrylamide gel and separated by electrophoresis. Dried polyacrylamide gel was exposed to radiographic film.

Western Blot Analysis

Cells were grown to >80% confluency and media was replaced with fresh media prior to being placed in the hypoxia chamber. At the indicated times, the cells were washed twice with ice-cold phosphate buffered saline and lysed in HEPES, pH 7.4, containing 1% NP-40, and protease inhibitors [Susa et al., 1992]. Protein concentration was determined by Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) using bovine IgG as a standard. Whole cell lysate (WCL) protein (50 μg) was fractionated by 12% SDS–PAGE and electrophoretically transferred onto nitrocellulose membranes. For immunoprecipitations, cell extracts (400–1,000 μg of total protein) were incubated with primary antibodies for 3 h followed by Protein A/Sepharose for 1 h and washing of the beads five times with lysis buffer and PBS. Membranes were blocked for 1 h at room temperature [7% nonfat milk, 2% BSA in phosphate buffered saline containing 0.2% Tween-20 (PBS-T)] and incubated with anti Tie1 at 1:1,000 or Tie2 at 1:1,000 antibodies overnight at 4°C. Membranes were reacted with appropriate HRP-conjugated secondary antibodies for 40 min at room temperature. Immunoreactive bands were detected using ECL reagent (Pierce, Rockford, IL) as per manufacturer's instructions. Membranes were then probed for total Erk expression by overnight incubation at 4°C with anti-pan Erk antibodies (1:5,000), followed by incubation with HRP-conjugated goat anti rabbit antibodies at 1:3,000 and ECL solution.

Quatitative Analysis

The amount of RT product used in each of the PCR reactions was first adjusted for β-actin or GAPDH. Simultaneous PCR reactions were carried out for β-actin or GAPDH and either Tie1, Tie2, VEGF, NERF isoforms, Ang1, or Ang2. The corresponding PCR products were separated on a 2% agarose gel. Density of the individual bands was measured using NIH

Image 1.3 program. Data were expressed as a ratio of the intensity of a specific band/intensity of the internal control band (β-actin or GAPDH for RT–PCR or L32 for RNAase protection assay). The corrected data was analyzed by ANOVA.

RESULTS

Tie1 and Tie2 mRNA and Protein Levels are Induced by Hypoxia

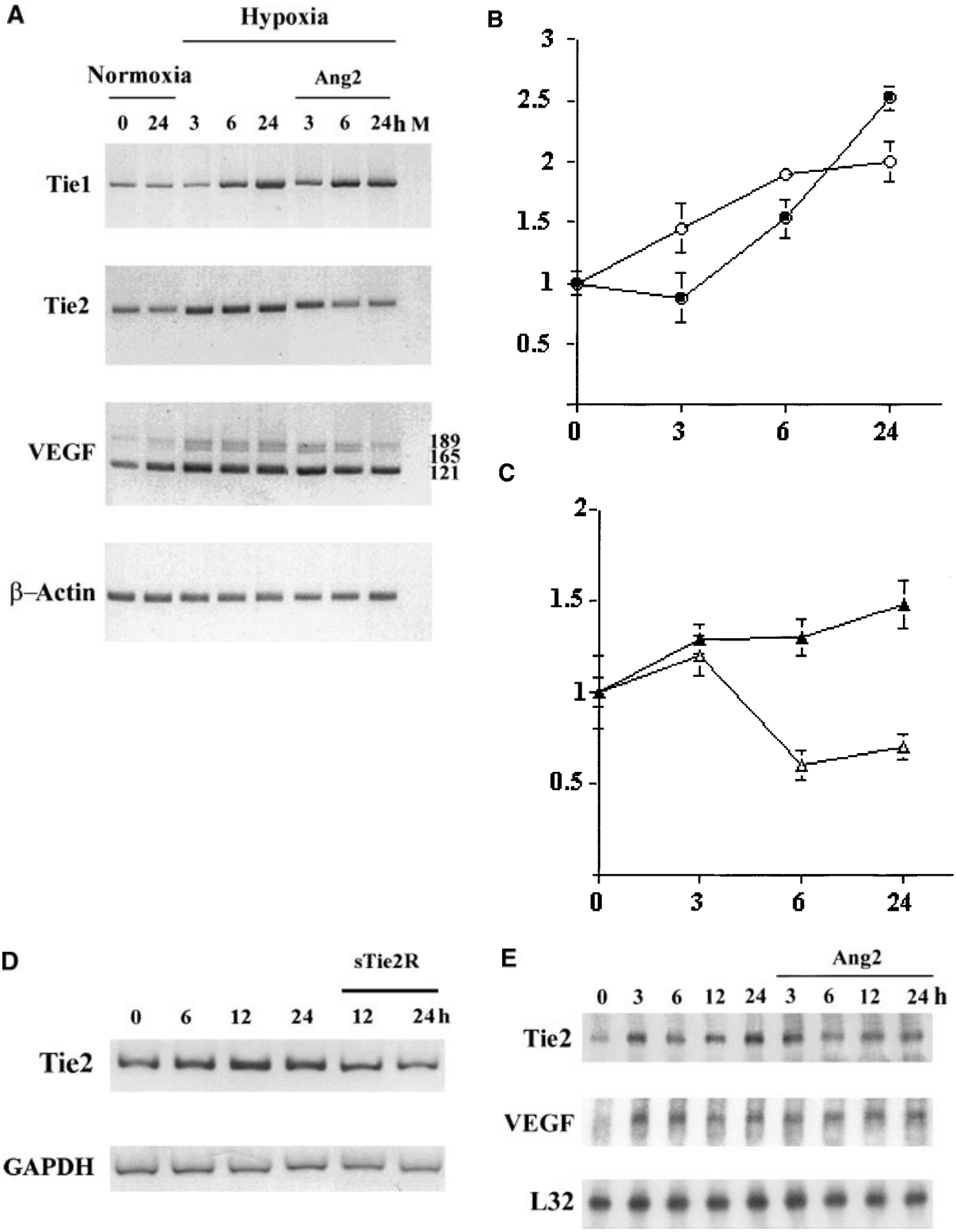
Conflicting data has been reported regarding the effect of hypoxia on expression of Tie1 and Tie2 in endothelial cells from different sources [Mandriota and Pepper, 1998; Oh et al., 1999]. We examined expression of Tie1 and Tie2 in HUVEC exposed to hypoxia for up to 24 h. Cells were cultured under normoxia or 1% O_2 and total RNA was isolated at indicated times. The amount of RNA used for each RT–PCR was normalized for the expression of β-actin. As anticipated, expression of VEGF isoforms increased during hypoxia (Fig. 1A). In addition, Tie1 and Tie2 mRNA increased in response to hypoxia. The increase in Tie2 expression was evident at 3 h with a maximal increase of 1.5–2-fold observed at 12–24 h. The change in Tie1 mRNA was delayed and more gradual, starting at 6 h and continuing for the entire 24-h period. The results were quantified by NIH Image 1.3 and the densities expressed as a ratio of Tie2/β-actin (Fig. 1B). Similar results were obtained in HMEC (data not shown).

To examine whether Ang1-mediated activation of Tie2 plays a role in induction of Tie expression, we used competitive inhibitors of Ang1, i.e., Ang2 and sTie2Fc [Davis et al., 1996; Maisonpierre et al., 1997]. Induction of Tie2 mRNA in hypoxic cells was blocked by Ang2 and sTie2Fc (Fig. 1A,D). The results were quantified by NIH Image 1.3 and the densities expressed as a ratio of Tie/β-actin (Fig. 1C). In contrast, expression of Tie1 and VEGF isoforms were not altered by Ang2 or by sTie2Fc. VEGF-neutralizing antibodies also had no effect on Tie expression under hypoxia (data not shown). The changes in Tie2 mRNA expression in response to hypoxia and Ang2 were confirmed by RNAase protection assay (Fig. 1E).

We then determined whether hypoxia-mediated changes in Tie1 and Tie2 mRNA correlated with protein levels. Significant increases in Tie1 and Tie2 protein levels was observed in cells exposed to hypoxia after 12 h as detected by Western blot analysis of whole cell lysates (Fig. 2A). Similar amounts of protein were loaded on the gels as shown by probing the membranes with antibodies against ERKs1 & 2 (Fig. 2A, lower panel) or p85 subunit of PI 3-kinase (data not shown).

Because competitive inhibitors of Tie2 activation, Ang2, and sTie2Fc, blocked the induction of Tie2, Ang1-mediated activation of Tie2 may play a role in regulation of Tie2 expression in hypoxic cells. To determine whether expression of Tie2 is induced by Ang1, Tie2 expression, we simultaneously examined Ang1-treated quiescent HUVEC grown under normoxia and cells cultured under hypoxia for 16 h. At the end of 16 h, Tie2 expression was induced to similar levels by hypoxia and by treatment with Ang1 (Fig. 2B). Transient autophosphorylation of

Fig. 1. Expression of Tie1 and Tie2 in HUVEC exposed to hypoxia. **A**: Increased expression of Tie2, but not Tie1 or VEGF, is blocked by Ang2 in hypoxic cells. HUVEC were grown to $>80\%$ confluency and then placed under normal O_2 or in hypoxia chambers for the indicated times. Ang2 (96.5 ng/ml) was added where indicated. Total RNA was isolated and RT–PCR analysis was performed using specific primers for human Tie1, Tie2, VEGF, and β-actin. M represents a mock RT–PCR in which the reaction contained no template. The three isoforms of VEGF (corresponding to 121, 165, and 189 amino acids) induced in response to hypoxia are indicated. The amount of RNA used for each assay was adjusted for expression of β-actin and was quantified using NIH Image 1.3 program. **B**: Quantitation of expression of Tie1 and Tie2 under hypoxia. The amount of RNA used for each assay was adjusted for expression of β-actin (as shown in **Panel A**). Expression of Tie1 (closed circles) and Tie2 (open circles) was quantified using NIH Image 1.3 program. The results represent means ± SE from three independent experiments. **C**: Ang2 blocks Tie2 but not Tie1 expression induced by hypoxia. Expression of Tie1 (closed triangles) and Tie2 (open triangles) was quantified using NIH Image 1.3 program. The results represent means ± SE from three independent experiments. **D**: Soluble extracellular domain of Tie2 receptor (sTie2Fc) reduces hypoxia-mediated increase in Tie2 expression. HUVEC were grown as described above and harvested at indicated times. sTie2Fc at 10 μg/ml was added prior to exposure of cells to hypoxia. Total RNA was isolated and RT–PCR analysis was performed using the GAPDH and Tie2 primers described in the Materials and Methods. The amount of RNA used for each assay was adjusted for expression of GAPDH. **E**: Expression of Tie2, but not that of VEGF, was reduced by Ang2 in cells under hypoxia as detected by RNAase protection assay. Cells were grown under normal oxygen tension or placed in hypoxia chambers. Ang2 (100 ng/ml) was added where indicated. RNAase protection assay was carried out using 15 μg of total RNA and the Angio-1 primer set from BD PharMingen, San Diego, CA according to the manufacturer's instructions. Data were quantified using NIH Image 1.3 and adjusted for expression of L32.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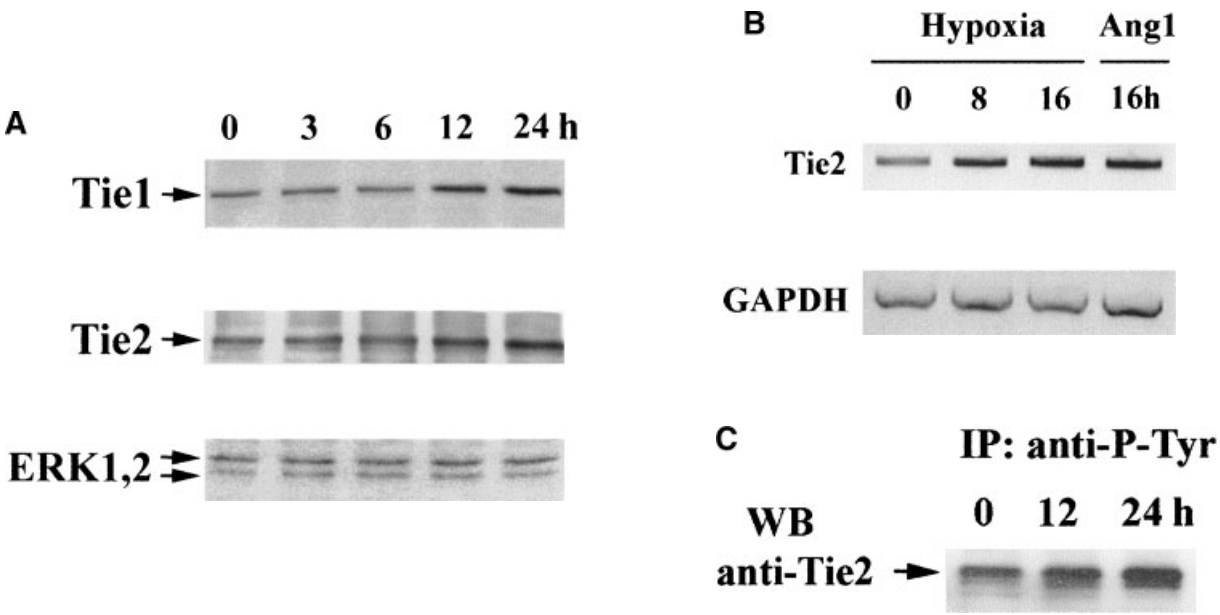

Fig. 2. Tie1 and Tie2 protein expression and Tie2 autophosphorylation are increased in response to hypoxia. **A**: Accumulation of Tie1 and Tie2 proteins in hypoxic cells. Cells were grown to >80% confluency, placed in hypoxia chambers and harvested at indicated times. Western blot analysis was performed on 50 μg of whole cell lysate (WCL) protein using anti-Tie1 and Tie2 antibodies. Equal loading of protein was confirmed by probing the membrane with a pan Erk antibody as indicated on the lower panel. The results shown are representative of three independent experiments. **B**: Hypoxia and Ang1 increase Tie2 expression to comparable levels. Cells were grown in complete M199 media under hypoxia for 8–16 h or under normal oxygen tension (see Fig. 3C) supplemented with Ang1 (100 ng/ml) for 16 h. Expression of Tie2 was determined by RT–PCR after total RNA isolation. Tie2 expression was increased to a similar extent by hypoxia and Ang1. The amount of RNA used for each assay was adjusted for expression of GAPDH. One of three representative experiments is shown. **C**: Tie2 activation in response to hypoxia. Tyrosine phosphorylated proteins from 1 mg of WCL from HUVEC isolated at time 0 and after 12 or 24 h of exposure to hypoxia were immunoprecipitated using anti-phosphotyrosine antibodies. Western blot analysis was performed after SDS–PAGE using a polyclonal anti-Tie-2 antibody.

Tie2 in response to Ang1, which peaks at 10 min, has been extensively documented [for review, see Davis and Yancopoulos, 1999]. We examined whether Tie2 is also activated in hypoxic cells. As previously reported [Wong et al., 1997; Fujikawa et al., 1999b], autophosphorylation of Tie2 was detectable in quiescent cells (Fig. 2C). In contrast to a transient Ang1-mediated activation of Tie2, a prolonged increase in anti-P-tyr immunoprecipitable Tie2 protein was evident for up to 24 h in hypoxic cells (Fig. 2C). Similar results were obtained by probing anti-Tie2 immune precipitates with anti-P-tyr antibodies (data not shown). These data further indicate that, similar to Ang1, hypoxia induces activation of Tie2, which is required for induction of Tie2 expression.

Expression of Ets Transcription Factor, NERF2, is Induced by Hypoxia in Response to Activation of Tie2 and by Ang1

Considerable progress has been made regarding the mechanisms of regulation of VEGF and its receptors; less is known about the regulation of Tie2. NERF2, a member of Ets family of transcription factors, was previously identified as a potent transactivator of *Tie2* gene [Oettgen et al., 1996; Dube et al., 1999]. In addition, two NERF1 isoforms (NERF1a and b) are competitive inhibitors of NERF2 and may modulate the transcriptional effect of NERF2 [Oettgen et al., 1996]. To determine whether Ets transcription factors are involved in the induction of Tie2 under hypoxia, we examined expression of NERF isoforms in hypoxic cells. All three NERF isoforms, particularly NERF1b and NERF2, were induced in response to hypoxia (Fig. 3A). In contrast to NERF 1a and 1b isoforms, the expression of NERF2 was robust, time-dependent, and continued for 24 h.

Because hypoxia stimulates VEGF secretion in endothelial cells, and VEGF is a potent activator of many biological effects, we determined whether VEGF plays a role in induction of NERF isoforms by using VEGF-neutralizing antibodies. VEGF-neutralizing antibodies had

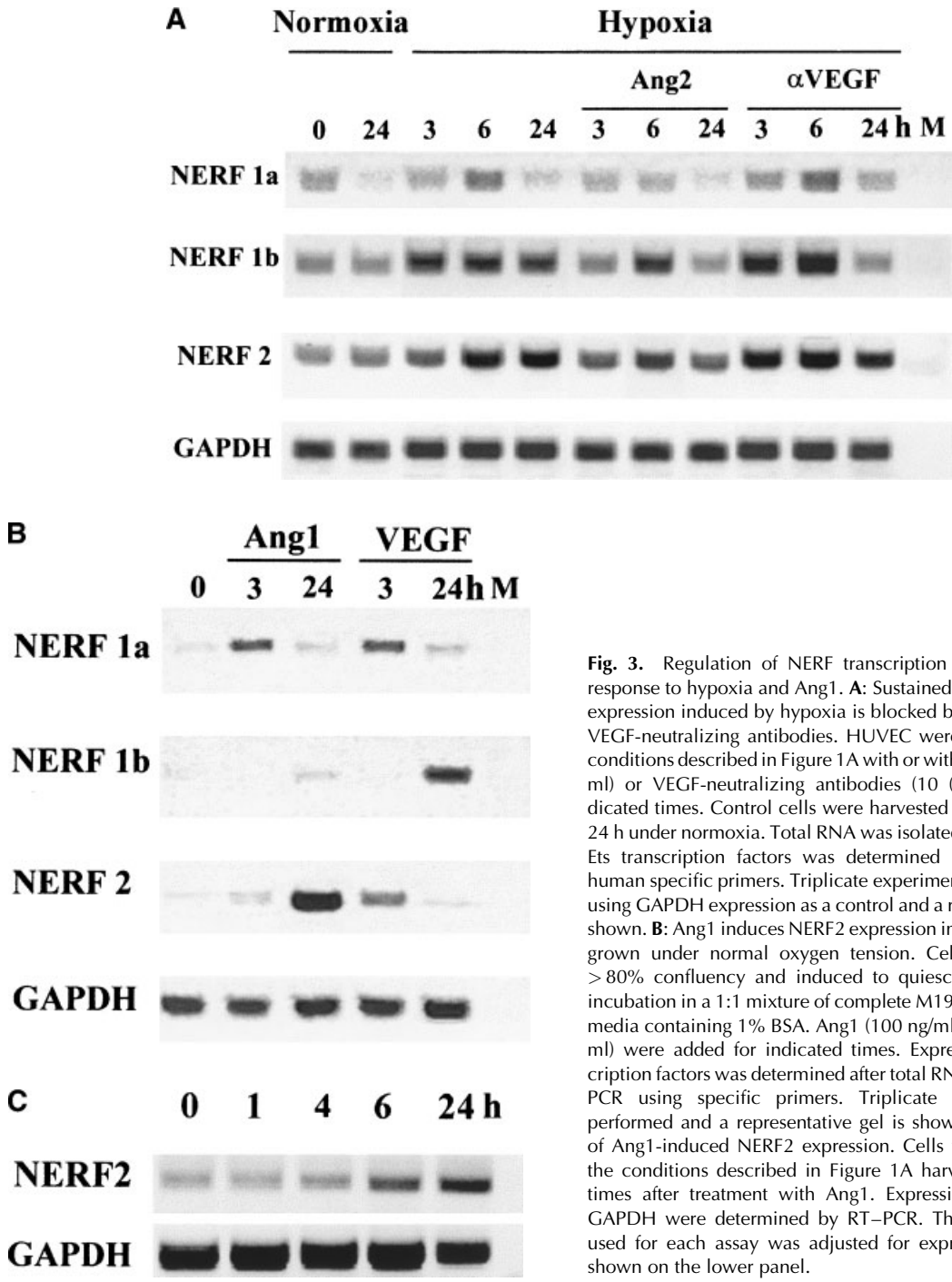

Fig. 3. Regulation of NERF transcription factor isoforms in response to hypoxia and Ang1. **A**: Sustained increase in NERF2 expression induced by hypoxia is blocked by Ang2, but not by VEGF-neutralizing antibodies. HUVEC were grown under the conditions described in Figure 1A with or without Ang2 (96.5 ng/ml) or VEGF-neutralizing antibodies (10 (μg/ml) for the indicated times. Control cells were harvested at time 0 and after 24 h under normoxia. Total RNA was isolated and expression of Ets transcription factors was determined by RT–PCR using human specific primers. Triplicate experiments were performed using GAPDH expression as a control and a representative gel is shown. **B**: Ang1 induces NERF2 expression in quiescent HUVEC grown under normal oxygen tension. Cells were grown to $>80\%$ confluency and induced to quiescence by overnight incubation in a 1:1 mixture of complete M199 media and M199 media containing 1% BSA. Ang1 (100 ng/ml) or VEGF (100 ng/ml) were added for indicated times. Expression of Ets transcription factors was determined after total RNA isolation by RT–PCR using specific primers. Triplicate experiments were performed and a representative gel is shown. **C**: Time course of Ang1-induced NERF2 expression. Cells were grown under the conditions described in Figure 1A harvested at indicated times after treatment with Ang1. Expression of NERF2 and GAPDH were determined by RT–PCR. The amount of RNA used for each assay was adjusted for expression of GAPDH shown on the lower panel.

no effect on induction of NERF1b or NERF2 (Fig. 3A). In contrast, Ang2 led to a significant reduction in NERF1b and NERF2 expression within 6 h and completely blocked the response at 24 h.

The role of Ang1 and VEGF on expression of NERF isoforms was further examined by stimulating HUVEC grown under normal oxygen tension with these growth factors. Low levels of expression for all three NERF isoforms were detected in quiescent cells. Addition of Ang1 resulted in a substantial increase in NERF2 mRNA, which was detectable within 3 h and peaked at 24 h (Fig. 3B). VEGF also weakly

induced NERF2 expression that was apparent only at 3 h, and NERF1b expression at 24 h. Ang1 had no effect on expression of NERF1b. A transient increase in NERF1a expression in response to both growth factors was also observed in some, but not all, experiments. To define the time course of Ang1-mediated induction of NERF2, we determined NERF2 mRNA content at different time points. NERF2 mRNA increased within 4 h and continued to increase for 24 h (Fig. 3C). These data indicate that Ang1 regulates expression of NERF isoforms, specifically expression of NERF2. Taken together, these data suggest that Ang1 and hypoxia induce activation of Tie2, which correlates with a prolonged increase in expression of NERF2 transcription factor.

Ang1, But Not Ang2, Is Induced Under Hypoxia

The results of our experiments indicate that Tie2 and NERF2 expression under hypoxia could involve a balance between endogenous Ang1 and Ang2 levels. Therefore, we examined expression of angiopoietins in cells grown under hypoxia. The expression of Ang1 increased in response to hypoxia with a maximal increase detected at 6 h (Fig. 4A,B). The increase in Ang1 mRNA temporally correlated with activation of Tie2 receptor and NERF2 expression in hypoxic cells. In contrast, Ang2 expression did not change in hypoxic cells. Thus, hypoxia-mediated increase in Ang1 results in Tie2 activation and induction of NERF2 expression. Sustained increase in expression of NERF2 transcription factor may be responsible for induction of Tie2 expression under hypoxia.

DISCUSSION

Hypoxia is a major stimulus for angiogenesis associated with tumor growth, inflammation and vascular remodeling [Holash et al., 1999; Ortéga et al., 1999]. Information on the effects of hypoxia on expression of angiopoietins and Tie receptors is limited and the regulation of their expression appears to depend on the origin of endothelial cells. Lack of Tie2 is lethal in embryonic development because of severe microvascular defects [Dumont et al., 1994; Puri et al., 1995; Sato et al., 1995]. Although Tie2 mRNA declines after birth [Sato et al., 1995], mRNA and protein levels are high in adult quiescent endothelium, significantly increased following balloon-induced injury of carotid artery

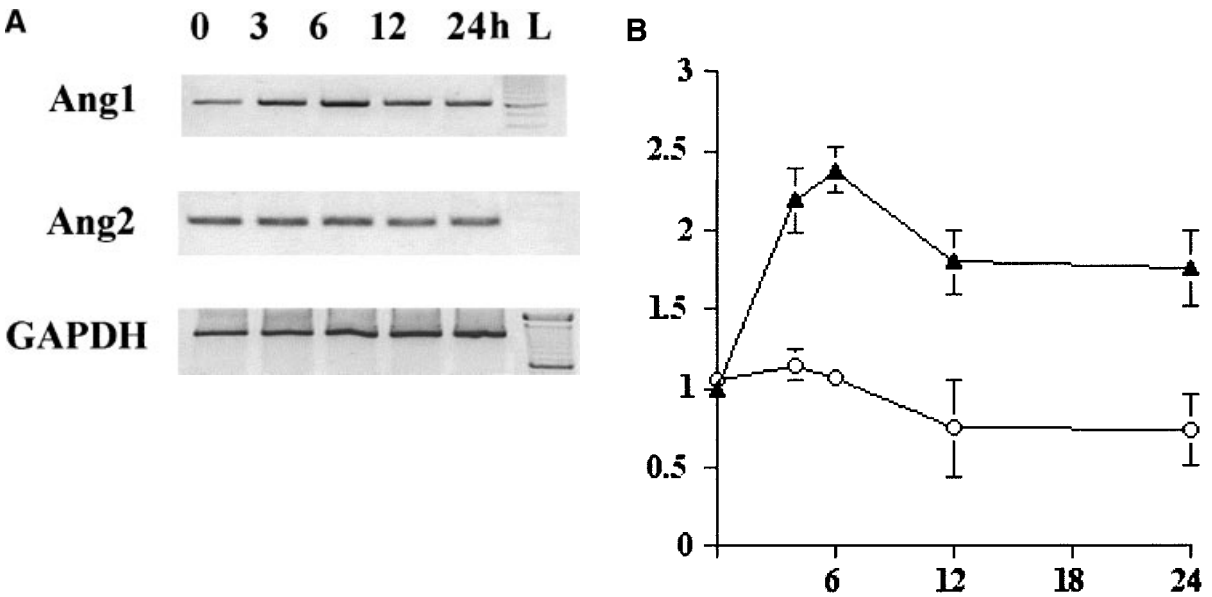

Fig. 4. Hypoxia induces an increase in Ang1 expression. **A**: Ang1, but not Ang2 expression is increased in response to hypoxia. HUVEC were grown under hypoxia as described in Figure 1A for indicated times. Expression of Ang1 and Ang2 was determined by RT–PCR after total RNA isolation. The amount of RNA used for each assay was adjusted for expression of GAPDH. L represents the molecular weight ladder. One of four representative experiments is shown. **B**: Quantitation of Ang1 (closed triangles) and Ang2 (open triangles) mRNA in cells under hypoxia. The amount of RNA used for each RT–PCR assay was adjusted for expression of GAPDH (as shown in **Panel A**). The results are presented as means ± SE from three independent experiments.

[Fujikawa et al., 1999b] and high in metastatic tumors [Kaipainen et al., 1994; Fujikawa and Varticovski, 1997; Wong et al., 1997; Holash et al., 1999]. Tie2 expression is reportedly induced by hypoxia within 2 h in bovine endothelial cells [Oh et al., 1999]. Hypoxia was also associated with a robust increase in Tie2 expression in human coronary microvascular endothelial cells, human dermal microvascular cells and, to a smaller degree, in HUVEC [Willam et al., 2000]. We found a time-dependent increase in Tie2 as well as Tie1 mRNA and protein levels in early passage HUVEC exposed to hypoxia. The increase in Tie2 expression was similar to previously reported data for HUVEC under hypoxia ($\pm$ 1.5-fold) [Willam et al., 2000]. The increase in Tie2 mRNA correlated with accumulation of protein. In addition, an increase in the level of autophosphorylation of Tie2 indicates that the receptor was activated in hypoxic cells.

Ang1 and Ang2 belong to a family of ligands that modulate Tie2 signaling by activation and inhibition, respectively [Davis et al., 1996; Maisonpierre et al., 1997; Valenzuela et al., 1999]. Activation of Tie2 by Ang1 leads to receptor autophosphorylation and activation of signaling pathways including PI 3-kinase, Akt and MAPK, which are required for endothelial cell survival and migration. However, activation of Tie2 does not lead to a mitogenic response [Kontos et al., 1998; Davis and Yancopoulos, 1999; Fujikawa et al., 1999a; Korpelainen et al., 1999] and activation of Tie2 has been reported in quiescent endothelial cells [Wong et al., 1997; Fujikawa et al., 1999b]. Whether Tie2 signaling has a role in regulation of gene expression has not been examined. We found that Tie2 is further activated under hypoxia and that blocking Tie2 activation by Ang2 or by sTie2Fc impaired hypoxia-induced increase in Tie2 mRNA. In contrast, VEGF-neurtalizing antibodies had no effect. These data indicates that activation of Tie2 is required for upregulation of its own mRNA under hypoxia. Further studies are necessary to establish whether Ang1 or other Tie2 ligands, such as Ang4 [Valenzuela et al., 1999], contribute to activation of Tie2 under hypoxia in endothelial cells.

Members of the Ets family of transcription factors regulate expression of several endothelial specific genes, including Flt-1 [Wakiya et al., 1996], vascular endothelial cadherin [Gory et al., 1998], and intercellular adhesion molecule-2 [McLaughlin et al., 1999]. NERF2, an Ets family transcription factor, binds to the promoter region of Tie1 and Tie2 and is a potent transactivator of *Tie2* gene [Dube et al., 1999; Iljin et al., 1999]. We observed a time-dependent increase in NERF2 expression in HUVEC grown under hypoxia, which was evident at 3 h and preceded the upregulation of Tie1 and Tie2 expression. Importantly, Ang2 and sTie2Fc specifically blocked the accumulation of NERF2 and Tie2 mRNAs, but had no effect on expression of Tie1. VEGF-neutralizing antibodies had no effect on expression of *Tie* genes.

The NERF1a and NERF1b isoforms are competitive inhibitors of NERF2 and may modulate the effect of NERF2 as a positive transcriptional regulator of Tie2 [Oettgen et al., 1996]. Both NERF1 isoforms were induced by hypoxia, whereas Ang1 had no effect on NERF1b induction. Importantly, NERF2 expression was induced by Ang1 in cells grown under normal oxygen tension as well as by hypoxia, and was blocked by treatment of hypoxic cells with Ang1 inhibitors. In addition, induction of NERF2 expression by Ang1 and hypoxia correlated with the increase in Tie2 mRNA. Thus, Ang1 induces NERF2 expression, which may be, at least in part, responsible for transcriptional activation of *Tie2* gene and accumulation of Tie2 mRNA and protein. Enhanced Tie2 expression in bovine endothelial cells in response to supplemental Ang1 has been previously described [Mandriota and Pepper, 1998]. It is not known whether other hypoxia-inducible transcription factors, for example endothelial PAS-1 [Tian et al., 1997] play a role in hypoxia induced upregulation of *Tie* genes.

In addition to smooth muscle cells, fibroblasts, and other cells which are closely associated with the vasculature, Ang1, and Ang2 are expressed in endothelial cells [Witzenbichler et al., 1998; Davis and Yancopoulos, 1999; Oh et al., 1999]. Increased expression of Ang2 in response to hypoxia and VEGF was detected in bovine adrenal cortex microvascular cells but not observed in other endothelial cells [Mandriota and Pepper, 1998]. Another study reported that, in bovine retinal and aortic endothelial cells, expression of Ang2 increased in response to VEGF and hypoxia, whereas Ang1 expression was high and did not change in response to VEGF or hypoxia [Oh et al., 1999]. The difference between these studies may be due to the origin of the endothelial cells as well as the

experimental protocols. As previously reported [Witzenbichler et al., 1998; Oh et al., 1999], we detected Ang1 mRNA in cells growing under normal oxygen tension. Ang1 expression increased in response to hypoxia, whereas Ang2 expression was not altered. The increase in Ang1 mRNA occurred as early as 4 h and correlated with the induction of NERF2 and Tie2 mRNA levels and protein. We did not measure secreted Ang1 or Ang2 because of lack of suitable antibodies. Induction of Ang1 in hypoxic cells suggest an autocrine loop in which Ang1-mediated increase in NERF2 expression leads to transcriptional activation of Tie2 in response to hypoxia. Another example of an autocrine loop induced by hypoxia is expression and secretion of VEGF in endothelial cells by stabilization of mRNA, which leads to activation and increase in surface expression of VEGF receptors on the same cells [Shweiki et al., 1992; Mustonen and Alitalo, 1995; Namiki et al., 1995; Stein et al., 1995; Brogi et al., 1996]. Because Tie2 signaling does not lead to DNA synthesis, the induction of NERF2 may be mediated by a similar mechanism. Thus, activation of endothelial cell Ang1 plays an important role in regulation of expression of its receptor during hypoxia and angiogenic response. The precise mechanism of Tie2 regulation under hypoxia will require further studies.

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