Tie2 Receptor Expression Is Stimulated by Hypoxia and Proinflammatory Cytokines in Human Endothelial Cells

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Abstract—The tyrosine kinase receptor Tie2 (also known as Tek) plays an important role in the development of the embryonic vasculature and persists in adult endothelial cells (ECs). Tie2 was shown to be upregulated in tumors and skin wounds, and its ligands angiopoietin-1 and -2, although they are not directly mitogenic, modulate neovascularization. To gain further insight into the regulation of Tie2, we have studied the effect of hypoxia and inflammatory cytokines, two conditions frequently associated with neoangiogenic processes, on Tie2 expression in human ECs. Exposure to 1% O_2 led to a time-dependent significant rise of Tie2 protein levels in human coronary microvascular endothelial cells (HCMECs) and dermal microvascular ECs (HMEC-1) (3.2- and 2.5-fold within 24 hours), which was reversible after reoxygenation, and induced a less marked increase in human umbilical vein ECs (HUVECs; 1.7-fold). Hypoxia-conditioned medium and d-deoxyglucose did not change Tie2 expression, but desferrioxamine and cobalt, which are known to mimic hypoxia-sensing mechanisms, induced Tie2 at ambient oxygen tensions. Tumor necrosis factor-α induced Tie2 in a time- and dose-dependent fashion in all 3 EC types (HUVEC, 2.3-fold; HMEC-1, 2.8-fold; and HCMEC, 3.0-fold; 10 ng/mL; 24 hours). Enhanced expression was also found after exposure to interleukin-1β (1 ng/mL). Changes in Tie2 protein levels were paralleled by changes in mRNA expression. In accordance with these in vitro findings, immunohistochemistry revealed focal upregulation of Tie2 in capillaries at the border of infarcted human and rat myocardium. In conclusion, the data show that hypoxia and inflammatory cytokines upregulate Tie2, which may contribute to the angiogenic response in ischemic tissues. (Circ Res. 2000;87:370-377.)

Key Words: receptor, tyrosine kinase • Tie2 • hypoxia • cytokines • endothelium

Endothelial receptor tyrosine kinases (RTKs) play a central role in establishment, maintenance, and structural adaptation of the vasculature.1,2 On the basis of their structure, the 5 RTKs so far identified can be divided into 2 families. One consists of the RTKs Flk-1 and Flt-1, which, together with their cognate ligand vascular endothelial growth factor (VEGF), are required for blood vessel development during both embryogenesis and conditions of postnatal neovascularization.3,4

Two structurally related tyrosine kinases with immunoglobulin and epidermal growth factor homology domains (Tie1 and Tie2) have more recently been described and constitute a second group of RTKs restricted mainly to endothelial cells (ECs) and their progenitors (for review, see References 2 and 5). Although signaling partners for Tie1 are yet unknown, angiopoietin (Ang) -1 and -2 have been identified as ligands of the Tie2 receptor.6,7 During embryonic development, the expression of Tie2 is transcriptionally activated in EC precursors and maintained throughout embryonic endothelium. Unlike the VEGF receptor system, Tie receptors are not required for vasculogenesis but appear essential to support functions of the more mature endothelium.1,2,5 Tie2−/− mice lack proper hierarchical organization of the vasculature and die during embryogenesis.8,9 Although the expression of Tie2 is downregulated postnatally, it persists in quiescent adult ECs, and tyrosyl phosphorylation in the normal vasculature suggests an active role in the maintenance of blood vessels.10 In addition, Tie2 is upregulated in capillaries during neovascularization processes, including skin wounds,10 and tumors.11,12

Although the Tie2 ligands Ang-1 and Ang-2 do not produce a mitogenic response in cultured ECs,6 they were shown to modulate VEGF-induced postnatal neovascularization.13 Blocking Tie2 activation using recombinant soluble receptor antagonists prevents tumor growth,14 which supports an important role of Tie2 in tumor angiogenesis. Transcriptional regulatory regions of the Tie2 gene that drive angioblast- and endothelium-specific expression have been characterized.15–17 However, despite growing evidence of the functional relevance of Tie2, very little is known about the signals regulating the expression of this receptor.
A system of hypoxia-inducible transcriptional gene activation, which operates through the accumulation of hypoxia-inducible transcription factors (HIFs), has been found to control the activity of several growth factors, including VEGF (for review, see References 18 and 19). Transfection of 293 cells with an expression vector for the hypoxia-inducible transcription factor endothelial PAS protein-1 (EPAS-1, also known as HIF-2) stimulated transcription from a reporter gene composed of the promotor and intron 1 enhancer of the Tie2 gene. Although this observation raises the intriguing possibility that EPAS-1 or related factors mediate hypoxia-induced activation of the Tie2 gene, this hypothesis has not yet been confirmed.

Apart from hypoxia, the release of inflammatory mediators is a further characteristic of tissues undergoing neovascularization, such as wounds, infarctions, or tumors. Cytokines, including tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), promote angiogenesis in animal models, although they do not directly stimulate EC growth. TNF-α and IL-1β were recently reported to downregulate Ang-1, which provided the first evidence for their influence on the Tie2 receptor pathway.

To further explore mechanisms of Tie2 regulation, we have studied its expression in bovine aortic and human umbilical vein and small-vessel ECs, including primary cultures of cardiac microvascular ECs, and report here that hypoxia, TNF-α, and IL-1β upregulate Tie2 in human ECs in a time-dependent fashion. We propose that these mechanisms contribute to the angiogenic response in ischemic myocardium and other organs.

**Materials and Methods**

**Cell Culture**

Human coronary microvascular endothelial cells (HCMECs) were obtained from explanted human hearts as described previously. Written consent was obtained from all patients, and tissue sampling was approved by the local ethics committee. Human umbilical vein endothelial cells (HUVECs) were prepared as described. Human dermal microvascular ECs transfected with the SV-40 large T promotor (HMEC-1), originally prepared from human neonatal foreskins, were kindly provided by R. Bicknell. Bovine aortic endothelial cells (BAECs) that were studied for comparison were gained by treating bovine aortas with collagenase and dispase as described.

ECs were grown on 0.25% gelatin-coated dishes in medium 199, supplemented with 10% FCS (GIBCO), penicillin (100 U/mL), streptomycin (100 μg/mL), glutamine (2 mmol/L), and 10 ng/mL EC growth factor.
(HCMECs, HUVECs, and BAECs) or 0.5 ng/mL epidermal growth factor and 1 mg/mL hydrocortisone (HMEC-1).

Cells were routinely incubated at 37°C in 5% CO₂ balance room air. For experiments, the second to third passage of HUVECs, BAECs and HCMECs was used. Sixteen to 24 hours before exposing cells to different oxygen tensions, as described previously,²⁷ ECs were washed and incubated with medium containing reduced FCS concentrations (1% for HMEC-1 and 10% for HUVECs, BAECs, and HCMECs) and no further supplementation with endothelial or epidermal growth factor and cortisone. Immediately before starting the experiments, reduced FCS medium was changed again. In some experiments, the following agents were added with this final change of medium: TNF-α (1 to 100 ng/mL), IL-1β (1 ng/mL), desferrioxamine (DFO; 75 μmol/L), cobaltous chloride (75 μmol/L), D-deoxyglucose (1 to 5 mmol/L), cycloheximide (10 μg/mL), and actinomycin D (0.1 μg/mL) for 4 to 48 hours.

Cellular viability was tested microscopically using trypan blue exclusion and by measuring the lactate dehydrogenase release using a kit from Sigma.

Myocardial Infarction Model in Rats
The left anterior descending coronary artery was occluded in male 10- to 15-week-old Wistar rats as described.³⁰ Animals were euthanized after 3 weeks, and blocks of heart tissue were frozen in isopentane precooled in liquid nitrogen and stored at −80°C.

Determination of Tie2 mRNA
Expression of Tie2 mRNA was assessed by RNase protection using a 189-bp fragment corresponding to the human mRNA coding region between 897 and 1085 bp of the Tie2 gene and normalized for expression of U6 small nuclear RNA (see online-only data supplement for details, available at http://www.circresaha.org).

Immunoblot Analysis
Immunoblotting for Tie2 was performed after electrophoresis of cell extracts using a polyclonal rabbit antibody against Tie2 (Santa Cruz Biotechnologies) (see online-only data supplement for details, available at http://www.circresaha.org).

Immunohistochemistry
Immunohistochemistry for Tie2 and von Willebrand factor (vWF) was performed on cryosections of rat myocardium and paraffin-embedded myocardium from a patient who died 8 days after myocardial infarction (see online-only data supplement for details, available at http://www.circresaha.org).

Statistics
Results are expressed as mean±SEM. Mann-Whitney U test and Kruskal-Wallis test were used for 2-group and multiple-group comparisons between experimental and control groups. P<0.05 was considered significant.

An expanded Materials and Methods section can be found in an online data supplement available at http://www.circresaha.org.

Results
Hypoxia, Cobalt, and DFO Stimulate Tie2 Protein Levels
In accordance with previous work,²⁵ Tie2 was detectable by immunoblotting under baseline conditions in BAECs and all 3 adult human EC types investigated. To test for the effect of hypoxia on Tie2, EC cultures were incubated at 1% O₂ for up

![Figure 3. A and B, Effects of hypoxia (H; 1% O₂), DFO (75 μmol/L), cobalt chloride (COB; 75 μmol/L), or TNF-α (10 ng/mL) on Tie2 protein levels in BAECs (24 hours of exposure under each condition). N indicates normoxia.](http://circres.ahajournals.org/)

![Figure 4. Effect of TNF-α (A through C) and IL-1β (D) on Tie2 protein expression in HUVECs (A and D), HCMECs (A and D), and HMEC-1 (A through D). Values in panel A are mean±SE (n=3) of signal intensity in immunoblots as derived by densitometry. *Significant difference from protein levels in cells unexposed to cytokines.](http://circres.ahajournals.org/)
to 48 hours. As shown in Figure 1, exposure to low oxygen as compared with continued exposure under normoxia resulted in a time-dependent, significant increase of Tie2 protein levels in HCMECs that was on average 3.1- and 3.9-fold after 24 and 48 hours, respectively. Hypoxic exposure of different human EC types revealed a slightly less pronounced increase in HMEC-1 and no statistically significant increase in HUVECs (Figure 2A). In contrast to the results with human ECs, hypoxia did not, however, change Tie2 expression in BAECs (Figure 3).

The hypoxia-induced stimulation in human microvascular ECs was found to be reversible, as shown in Figure 2C; Tie2 expression in HCMECs exposed to 1% O2 for 24 hours declined gradually after reexposure to 20% O2 and returned to baseline after 24 hours.

In view of the evidence indicating that cobalt and DFO can mimic hypoxic signaling in other systems, cells were also incubated in the presence of 75 μmol/L cobalt chloride (COB; 75 μmol/L) and DFO for 24 hours at 20% O2. As illustrated on representative blots in Figure 2B (lanes 5 and 6), both DFO and cobalt led to a stimulation of Tie2 protein expression in HCMECs and HMEC-1 that was similar to the hypoxic response, with a slight increase detectable after 4 hours and a further increase until 24 hours. Further experiments revealed that the stimulation by TNF-α was dose-dependent; whereas 1 ng/mL enhanced Tie2 signals in some but not all experiments, 10 ng/mL led to a significant increase in all 3 EC types (Figures 4A and 4C). As illustrated in Figure 4D, exposure to IL-1β (1 ng/mL) also increased Tie2 levels.

To further investigate whether the effect of hypoxia on Tie2 expression is a direct cellular one or is mediated by substances released into the medium, HMEC-1 were incubated for 24 hours at 20% O2 in cell culture medium harvested from cells exposed to 1% O2 for 24 hours. This exposure to hypoxia-conditioned medium did not change Tie2 protein levels (data not shown).

We also studied the effect of the antimetabolite d-deoxyglucose to assess the influence of substrate deprivation during hypoxia. Substrate deprivation, mimicked by addition of 1 to 5 mmol/L d-deoxyglucose to HMEC-1 incubated at 20% O2, did not affect Tie2 protein levels (data not shown).

**TNF-α and IL-1β Stimulate Tie2 Protein Levels**

To investigate the effect of proinflammatory cytokines on Tie2 expression, cells were incubated in the presence of 1 to 100 ng/mL TNF-α under normoxic standard cell culture conditions. All 3 human EC types responded to TNF-α with an increase in Tie2 protein levels (Figure 4). As shown in Figure 4B, the time course of TNF-α stimulation resembled the hypoxic response, with a slight increase detectable after 4 hours and a further increase until 24 hours. Further experiments revealed that the stimulation by TNF-α was dose-dependent; whereas 1 ng/mL enhanced Tie2 signals in some but not all experiments, 10 ng/mL led to a significant increase in all 3 EC types (Figures 4A and 4C). As illustrated in Figure 4D, exposure to IL-1β (1 ng/mL) also increased Tie2 levels.
In BAECs, in contrast to human ECs, Tie2 levels were not enhanced by TNF-α (Figure 3).

Hypoxia- and Cytokine-Induced Increases in Tie2 Protein Expression Are Paralleled by Changes at the mRNA Level

Figure 5B shows an autoradiogram of a representative RNase protection assay, and Figure 5A summarizes the results of RNA analysis that were performed to test for changes in Tie2 gene expression in response to cytokines, hypoxia, and cobalt. Hypoxia, cobalt, and TNF-α increased steady-state mRNA levels of Tie2 in human ECs, and changes at the mRNA level roughly paralleled the observed changes in protein expression, although they were somewhat less marked. The protein synthesis inhibitor cycloheximide had no significant effect on hypoxia-induced Tie2 mRNA levels (Figure 6B).

Addition of actinomycin D (0.1 μg/mL) abrogated the induction of Tie2 mRNA (Figure 6B) and protein (Figure 6A) during a subsequent incubation at 1% O₂ or at 20% O₂ in the presence of TNF-α. Lactate dehydrogenase release in cells treated with actinomycin D or cycloheximide increased from 15% to 30% and 24% (normoxia) and from 29% to 38% and 31% (hypoxia), respectively.

Stimulation of Tie2 by TNF-α and Hypoxia Is Not Additive

To test for a potential interaction of cytokines and hypoxia on Tie2 regulation, human ECs were exposed to 1% O₂ in the presence of TNF-α. Although in some experiments the presence of TNF-α tended to enhance Tie2 mRNA expression under hypoxic conditions (lane 4 versus lane 2 in Figure 7B), there was overall no consistent and significant additive effect of hypoxia and 1 ng/mL TNF-α (Figure 7A) or 10 ng/mL TNF-α (data not shown). Similar results were obtained for protein expression (data not shown).

Tie2 Expression Is Focally Enhanced After Myocardial Infarction In Vivo

To address the question of an in vivo correlate of the observed changes in Tie2 in response to hypoxia and cytokines, we have investigated Tie2 expression by immunohistochemistry in rat and human heart tissue after myocardial infarction. In accordance with previous work, Tie2 was detectable in...
arterial and venous vessels of different diameter throughout normal myocardium (Figures 8A, 8C, and 8E), and costaining for vWF confirmed the endothelial localization in capillaries and larger vessels (Figure 8A). In peri-infarct tissue from rat and human infarcted myocardium, staining for Tie2 was far less homogenous and focally appeared more intense in the vicinity of tissue necrosis (Figures 8D and 8F). As expected, these areas also showed a marked inflammatory cell infiltrate. Costaining for Tie2 and vWF in peri-infarct rat myocardium (Figures 8G and 8H) also revealed a predominately vascular pattern of Tie2 expression (Figure 8H), but some isolated, possibly mononuclear infiltrating cells were also positive.

**Discussion**

Angiogenesis is required for embryonic development, wound healing, and other repair processes. Hypoxia and inflammation are 2 frequent characteristics of tissues undergoing neovascularization, and we show in this study that both are independently capable of inducing the expression of the Ang receptor Tie2. The underlying mechanisms seem to be widely preserved in the human vasculature. Thus, hypoxia and the proinflammatory cytokines TNF-α and IL-1β tend to influence Tie2 expression in all 3 types of human ECs investigated, although the amplitude of modulation in response to hypoxia was more marked in dermal and cardiac microvascular cells and did not reach statistical significance in HUVECs (Figure 2). In contrast, the cytokine response was more uniform in microvascular and umbilical vein ECs (Figure 4). In all 3 cell types, changes in Tie2 protein expression roughly paralleled changes at the mRNA level (Figure 5), indicating that the latter is an important determinant of the observed regulation.

The effect of hypoxia on Tie2 expression appears to depend, however, on the origin of the ECs. In previous studies, Oh et al. did not find hypoxia to influence Tie2 mRNA expression in bovine retinal or aortic ECs, and Mandriota and Pepper described a downregulation of Tie2 mRNA to 62% in bovine adrenal microvascular ECs exposed to hypoxia. In view of these discrepancies with our results in human ECs, we have also studied BAECs for comparison. As reported by Oh et al. and Mandriota and Pepper, in these bovine cells we did not find a hypoxic stimulation (Figure 3). Whether these differences in response are related to species or reflect differences in the oxygen sensitivity of different vascular beds remains to be determined.

The effect of hypoxia observed in human ECs appears to be a direct cellular one rather than one mediated through autocrine or paracrine signals, because we found no effect of hypoxia-conditioned medium on Tie2 expression. Several characteristics of this response were determined that have previously been described for a widely operative system of cellular oxygen sensing involving the accumulation of a family of HIFs. As described for several other genes regulated through this system, the effect of hypoxia on Tie2 expression appeared not to be due to substrate deprivation and could be mimicked by the addition of cobalt and DFO (Figure 2). Both are believed to interfere with the role of iron in the hypoxia-sensing mechanism, although the underlying reactions have not been clarified. Actinomycin D abrogated the hypoxic response, indicating that the induction is at least partially due to transcriptional activation. These data complement the findings by Tian et al. and Schlaeger et al., who reported that overexpression of EPAS-1/HIF-2 can activate transcription of a Tie2-driven reporter gene. In contrast, however, to several other genes regulated by HIF, experiments in the presence of cycloheximide suggested that hypoxic induction of Tie2 mRNA does not require ongoing protein synthesis (Figure 6).

With respect to the effects of cytokines on Tie2 expression, Ristimäki et al. have reported no change of Tie2 expression on HUVECs after exposure to IL-1β and TNF-α for 6 hours, which does not necessarily contradict our findings, showing stimulation of Tie2 in response to more prolonged exposure to these cytokines (Figure 4). Mandriota and Pepper have previously detected a moderate, 1.6-fold induction by basic fibroblast growth factor, which is somewhat less than the response to TNF-α and IL-1β observed in this study.

Interestingly, although the mechanisms by which hypoxia and TNF-α stimulate Tie2 are likely to be dissimilar, we were unable to demonstrate an additive effect by exposing cells to TNF-α under hypoxic conditions (Figure 7). In ischemic tissues, in vivo effects of hypoxia and inflammation cannot be dissected in immunohistochemical studies, and both mechanisms may have contributed to focal upregulation of Tie2 in peri-infarctus myocardium, illustrated in Figure 8.

The pathophysiological consequences of upregulation of Tie2 certainly depend on the function of Ang signaling. Although both Ang-1 and Ang-2 bind to Tie2 with high affinity, only Ang-1 causes receptor autophosphorylation in ECs. Because of its paracrine expression pattern and the observed defects in genetically altered mice, it has been proposed that Ang-1 maintains vessel structures and influences the assembly of perivascular cells in vascular remodeling. Recent studies on the stabilizing effects of Ang-1 on the HUVEC network support this notion. Because Tie2 phosphorylation by Ang-1 can be inhibited by an excess of Ang-2 and the phenotype of mice overexpressing Ang-2 resembles Ang-1 or Tie2 knockouts, Ang-2 is believed to serve as a physiological antagonist for Ang-1.

Interestingly, 2 factors reported here to stimulate Tie2, hypoxia and IL-1β, have recently been found to downregulate Ang-1 whereas Ang-2 is stimulated by hypoxia. In concert, these effects of hypoxia and cytokines on the local balance of Ang-1/Ang-2 may disrupt the interactions between ECs and their microenvironment by inhibiting Ang-1–induced Tie2 signaling and thereby make ECs more responsive to angiogenesis initiators. In this situation, enhancing Tie2 signaling by upregulation of the receptor may in fact counterbalance the proangiogenic stimuli exerted by hypoxia and cytokines. However, somewhat contradicting this hypothesis, Ang-1 but not Ang-2 gene transfer was found to enhance collateral vessel formation in the rat ischemic hind limb model, and such an effect would be promoted by the upregulation of Tie2 in response to ischemia that is demonstrated in this study. In addition, it is also possible that Ang-1 and Ang-2 activate Tie2 in fundamentally different
ways, stimulating different signaling pathways and resulting in different biological responses.\textsuperscript{36}

Further potential interaction has to be considered between the Tie2 and the VEGF pathway. Although Ang-1 and Ang-2 are not directly mitogenic, both augment the formation of new vessels when coadministered with VEGF.\textsuperscript{14} Hypoxia induces VEGF expression by transcriptional activation via HIF-1 and by post-transcriptional stabilization of its mRNA.\textsuperscript{4} Several cytokines, including IL-1\textbeta, stimulate VEGF expression in vitro.\textsuperscript{4} The VEGF receptor Flk-1 is also induced by hypoxia, although possibly indirectly.\textsuperscript{37,38} and by IL-1\textbeta.\textsuperscript{25} Furthermore, VEGF induces endothelial Ang-2 mRNA levels.\textsuperscript{21} Existing evidence therefore suggests that both hypoxia and inflammatory mediators invoke an orchestration of several endothelial growth factors and receptors that may play different, but interacting roles to achieve an adaptation of the vasculature under conditions of impaired perfusion, including wound healing and tumor growth.

The relevance of hypoxia signaling alone for tumor angiogenesis has been demonstrated in experimental models of tumors lacking a functional HIF complex, which are less vascularized and grow significantly slower.\textsuperscript{39,40} In view of our findings and work showing that inhibition of Tie2 signaling prevents tumor growth,\textsuperscript{14} it is tempting to speculate that reduced expression of Tie2 may contribute to growth inhibition in HIF-deficient tumors.

Moreover, the question arises whether the effect of hypoxia on Tie2 expression may also be relevant during embryonic development. Mice lacking HIF-1, EPAS (HIF-2), or their vascular endothelial growth factors and receptors that may play different, but interacting roles to achieve an adaptation of the vasculature under conditions of impaired perfusion, including wound healing and tumor growth.

In summary, this study has identified hypoxia and inflammatory cytokines as stimuli of Tie2 expression in human ECs. Because both factors are closely associated with active angiogenesis in pathological conditions and developmental vascularization, they may be important regulators of the Ang receptor pathway.

Acknowledgments

This work was supported by university research funds, the German Academic Exchange Service, and the German Research Foundation (DFG, EC 87 3-1). The technical assistance of S. Schulz is gratefully acknowledged.

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Circ Res. 2000;87:370-377
doi: 10.1161/01.RES.87.5.370

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