A Hypoxia-Independent Hypoxia-Inducible Factor–1 Activation Pathway Induced by Phosphatidylinositol-3 Kinase/Akt in HER2 Overexpressing Cells

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Abstract
HER2 overexpression, a known prognostic factor in many human cancers, can activate phosphatidylinositol-3 kinase (PI-3K)/Akt pathways and plays an important role in mediating cell survival and tumor development. Hypoxia-inducible factors (HIFs) promote angiogenesis and energy metabolism and thereby enhance tumor growth and metastasis. HIFs, composed of α and β subunits, are activated in most human cancers, including those that overexpress HER2. Previous reports have suggested that increased PI-3K/Akt or decreased PTEN activity may activate the HIF pathway in various tumors, but the detailed mechanism is still not completely understood. Here we reported an interaction between the HIF and PI-3K/Akt pathways in HER2-overexpressing cancer cells. Our results indicate that HER2 overexpression, which results in constitutively active Akt, turns on HIF-1α independently of hypoxia, and this activation is weaker than that under hypoxic condition. Further investigation showed that Akt is required for the hypoxia-independent HIF activity. The PI-3K/Akt pathway did not affect the HIF-1α binding with its E3 ligase von Hippel-Lindau but enhanced the binding affinity between the HIF-1α and β subunits. Furthermore, we found that Akt interacts with HIF-1/3 and regulates HIF activity. Our results indicated that HER2 can induce HIF activation via the activation of Akt suggesting that activation of HER2/Akt pathway may promote angiogenesis independent of hypoxia, which may have important implications for the oncogenic activity of HER2 and Akt. (Cancer Res 2005; 65(8): 3257-63)

Introduction
The HER2 gene, also known as neu or erbB2, encodes a 185-kD transmembrane receptor tyrosine kinase that has partial homology with other members of the epidermal growth factor receptor family (1, 2). Amplification or overexpression of HER2 gene has been found in ~30% of human breast and ovarian cancers and is associated with increased tumor grade and shorter overall survival rate suggesting that HER2 overexpression most likely plays a critical role in the development of human cancers (2, 3). Activated HER2 phosphorylates many downstream molecules that in turn activate a variety of signaling cascades, including the phosphatidylinositol-3 kinase (PI-3K)/Akt (1, 2, 4). The oncogenic roles of PI-3K and Akt depend on their ability to induce multiple effects on cell survival, cell cycle progression, and cell proliferation. Many targets of the PI-3K/Akt signaling pathway have been identified, and these targets may underlie the ability of this regulatory cascade to promote cell survival and tumor growth and progression (1, 2, 4–6).

Pathologic angiogenesis, a hallmark of cancer, is regulated by a balance between proangiogenic and antiangiogenic molecules (7). Without blood vessels, cancer cannot grow beyond a critical size or metastasize. Hypoxia is the strongest stimulus for triggering the angiogenic switch for pathologic angiogenesis and vascular remodeling in tumors. Recent discoveries have shown that hypoxia-inducible factors (HIFs) are the key players in angiogenesis through activation of proangiogenic growth factors, including vascular endothelial growth factor (VEGF) and others (8–10). The HIF DNA binding complex is a heterodimer of α and β subunits, both of which are basic helix-loop-helix procedure periodic acid-Schiff (PAS) transcription factors. In cells replete with oxygen, HIF-1α is hydroxylated at proline residues by its prolyl hydroxylases, which is recognized by the von Hippel-Lindau (VHL), and degraded by the VHL-dependent ubiquitin-proteasome pathway. When oxygen tension is limited, the prolyl hydroxylation is inhibited and the HIF-1α subunit is stabilized and translocated to the nucleus, where it dimerizes with the β subunit (8, 11). The β subunit of HIF-1α is regulated by ARNT, which is critically involved in a range of transcriptional systems, is dispensable for HIF-1 DNA binding and transcription (12, 13). Similar to HIF-1α, HIF-1β deficiency is also embryonically lethal, indicating that the heterodimeric HIF-1 complex is a nonredundant master regulator of oxygen homeostasis (14, 15).

Recent studies have shown that HIF is up-regulated in a broad range of cancers, including those overexpressing HER2, and its expression correlates with tumor grade and vascularity (9, 16). Several groups had reported that HIF is activated upon the activation of different oncogenes and the inactivation of tumor suppressor genes, such as PTEN. It was showed that PTEN suppressed hypoxic accumulation of HIF-1α protein and expression of its target gene VEGF. This process involved modulation of Akt, although Akt was not directly linked to phosphorylation of HIF-1α (4, 17). The PI-3K/Akt and HIF pathways share many common features including both can induce VEGF to promote angiogenesis and enhance the glucose metabolism, and also enhance tumor malignance and metastasis. However, how PI-3K/Akt is involved in the HIF pathway regulation and under what conditions and to which extent PI-3K/Akt regulates HIF activity are still controversial (18, 19). Here, we used HER2-overexpressing cells as a model to investigate the potential relation between the...
PI-3K/Akt and HIF pathways. We found that HER2 overexpression can induce HIF activity under normoxic condition. This hypoxia-independent HIF activity requires Akt, which interacts with HIF-1α and enhances the binding affinity between the HIF-1α and β subunits, resulting in HIF activation.

Materials and Methods

Cell culture and materials. All cell lines were grown in DMEM/F12 supplemented with 10% fetal bovine serum. The dominant-negative Akt transfectants of HER2/3T3 and MDA-MB-453 cells have been described previously (4, 5). The cells were transfected by the liposome method (20). The inhibitors AG825, wortmannin, LY294002, rapamycin, MG132, and anti-FLAG-HIF-1α antibodies were kindly provided by Dr. Frank S Lee of University of Pennsylvania School of Medicine (21); FLAG-HIF-1α plasmids were kindly provided by Dr. Y Fujii-Kuriyama from Tohoku University, Japan, respectively (22, 23).

Akt, Ser271 in HIF-1α was done according to the manufacturer’s protocol (Clontech Inc., Palo Alto, CA). Ser271 phosphorylated Akt, p70, and Thr389 phosphorylated p70 antibodies were from New England Biolabs (Beverly, MA), HIF-1α (NB-100-105 and NB-100-125) antibodies were from Novus (Littleton, CO). HA-HIF-1α and VHL-FLAG plasmids were kindly provided by Dr. Frank S Lee of University of Pennsylvania School of Medicine (21); FLAG-HIF-1α; and glutathione S-transferase (GST)-HIF-1α were kindly provided by Dr. Gary H. Perdew and Dr. Y Fujii-Kuriyama from Tohoku University, Japan, respectively (22, 23).

Constructs of hypoxia-inducible factor-1. Site-directed mutagenesis was done according to the manufacturer’s protocol (Clontech Inc., Palo Alto, CA). Ser271 in HIF-1α was replaced by either Ala or Asp by using the following primers: for S271A, 5′-CGAATTGAGGTGTGGCGATAGCTCTGTGGACCC-3′ and for S271D, 5′-CGAATTGAGGTGTGGCGCTAGCTCTGTGGACCC-3′. These sequences of constructs were verified by automated sequencing.

Immunoprecipitation and Western blot. Immunoprecipitation and Western blotting were done as described previously (20).

Transient transfection. Luciferase assay was done using the wild-type hypoxia response luciferase (HRE-luc) or mutant hypoxia response luciferase (mut/HRE-luc). Transfection assay was described before (20).

Northern blot analysis. Northern blot was done as described before (24).

Results

PI-3K/Akt is required for HER2-mediated hypoxia-inducible factor–1 transcription activity and vascular endothelial growth factor expression. To study the effect of Akt on HER2-mediated HIF-1 transcription activity and VEGF mRNA expression, we used a model system consisting of NIH 3T3 cells, HER2/3T3 cell (NIH 3T3 cells transformed with HER2), and dominant-negative Akt 3T3 cells (HER2/3T3 cells stably transfected with dominant-negative Akt; refs. 4, 5). To exclude the possibility that the HIF response element (HRE)–driven luciferase reporter effect is not HIF dependent but rather caused by the general transcription and translation induced by the Akt pathway, we adopted the established method of using the ratio of wide-type (wt) HRE to mutant (mut) HRE as an index to measure HIF transcriptional activity (4, 21).

In vitro kinase assay and in vitro direct protein-binding assay. GST proteins were purified; in vitro kinase assay and in vitro binding assays were done as described before (5, 20).

Figure 1. PI-3K/Akt pathway activates the HIF pathway and increases VEGF mRNA expression in HER2-overexpressing breast cancer cell lines. A, effect of HER2 and Akt on HIF-1α transcriptional activity. NIH 3T3, HER2-transformed 3T3 cells, and dominant-negative Akt (Dn-Akt) stable transfect in HER2-transformed NIH 3T3 cells were cotransfected with wt HRE-Luc or mut HRE-Luc. pTK-Renilla was used as an internal control. All cells were treated under 1% O2 for 6 hours before the total RNA preparation. Equal (20 μg) RNA samples were fractionated by gel electrophoresis and transferred to nitrocellular membranes and serially probed for 32P-labeled human VEGF and GAPDH.
together, the results indicated that the Akt pathway is required for HER2-mediated HIF transcriptional activity, which can be detected under both hypoxic and normoxic conditions.

To further confirm the above notion, we investigated the effect of Akt on the expression of HIF-1 target gene, VEGF, by Northern blot analysis. Similar results were observed. Under the normoxic condition, the dominant-negative Akt could almost totally block VEGF mRNA expression in both NIH 3T3/HER2 and MDA-MB-453 cells. Moreover, inhibition of the Akt pathway by dominant-negative Akt also blocked the mRNA expression of VEGF under the hypoxic condition but not to the same extent as under normoxic conditions (Fig. 1C and D).

**The HER2/PI-3K/Akt pathway is involved in regulating the hypoxia-inducible factors protein level.** To study the requirements of HER2/PI-3K/Akt pathways in HER2-induced up-regulation of HIF protein level, we used the inhibitors for HER2 tyrosine kinase (AG825), PI-3K (wortmannin and LY294002), and FRAP (rapamycin), FKBP-rapamycin-associated protein, also known as mammalian target of rapamycin, which is a known downstream target of Akt kinase involved in protein synthesis, to treat cancer cell lines overexpressing HER2. The relative effects of these inhibitors on the activity of HER2, PI-3K/Akt, and FRAP were examined by phosphorylation of the tyrosine residue of HER2, phosphorylation of Akt at residue Ser473, and phosphorylation of p70S6k at residue Thr389. Under the normoxic condition, growth factor insulin-like growth factor 1 (IGF-1) stimulation could modestly increase HIF protein accumulation compared with serum starvation (Fig. 2A and B, left, lanes 1 and 2). All the inhibitors could almost completely block the growth factor–induced HIF protein accumulation under the normoxic condition, even at the lowest effective dose concentration. However, under the hypoxic condition (Fig. 2A and B, right), the effects of these inhibitors on the HIF-1α protein level were less profound. In the MDA-MB-453 cells, AG825, LY294002, and wortmannin modestly inhibited the hypoxia-induced...
HIF-1α protein expression in a dose-dependent manner, whereas rapamycin had less effect on the HIF-1α protein expression induced by hypoxia (Fig. 2A, right). In the SKBR3, all four inhibitors only slightly blocked HIF-1α protein induction by hypoxia even at their higher effective concentrations (Fig. 2B, right). Comparing between lane 1 from right and left, Fig. 2A and B, it showed that HIF-1α protein level increased significantly under the hypoxic condition, in good agreement with the previous studies. In addition, there exists a hypoxia-independent pathway, in which the PI-3K/Akt pathway is involved in the HER2-induced up-regulation of HIF protein level. The basal level of HIF-1α protein under the normoxic condition is much lower than that under hypoxic condition (Fig. 2A and B, lane 1 between left and right) and can be enhanced in response to the IGF treatment (Fig. 2A and B, lanes 1 and 2, left). However, the IGF treatment does not have significant effect on the high basal level of HIF-1α under the hypoxic condition (Fig. 2A and B, lane 1 between left and right). Rapamycin inhibited HIF-1α protein level under the normoxic condition (Fig. 2A and B, lanes 2 and 6, left) but virtually had no effect under hypoxic conditions (Fig. 2A and B, lanes 9 and 10, right). Together, the results suggest that under normoxic condition, the growth factor induced HIF-1α expression requires HER2/Akt activities. However, the hypoxia-induced HIF-1α expression, which is much stronger than the growth factor-induced expression, is much less dependent on the HER2/Akt pathway.

To further support the phenomenon observed in the chemical inhibition of the HER2/PI-3K/Akt pathway, we used mouse embryonic fibroblasts (MEF) that either do not have both alleles of Akt (Akt−/−) or only one functional allele of Akt (Akt+/−) to observe the effect of null/partial Akt on HIF-1α protein expression. Under normal oxygen concentration and a serum stimulation, both Akt−/− and Akt+/− MEF cells showed much lower HIF-1α expression level than the wild-type MEF; however, the Akt−/− MEF cells had the lowest HIF-1α protein (Fig. 2C). Again, under the hypoxic condition, the enhanced HIF 1-α expression was not significantly affected by the status of Akt. The HIF-1α downstream target gene Glut-1 protein level showed a similar pattern (Fig. 2C). This phenomenon indicated that Akt is required for expression of HIF-1α under normoxic condition and the hypoxia-induced HIF-1α expression is not sensitive to Akt activity.

**PI-3K/Akt enhances the binding affinity of hypoxia-inducible factor–1α with hypoxia-inducible factor–1β but not with von Hippel-Lindau.** It is well known that under the normoxic condition, HIF-1α is recognized by VHL complex and targeted to the proteasome degradation. We therefore examined whether PI-3K/Akt could affect the binding affinity between HIF-1α and VHL. No change in the binding affinity between HIF-1α and VHL was observed by coimmunoprecipitation experiments, under either the normoxic or the hypoxic condition (Fig. 3A) suggesting that the PI-3K pathway does not affect the binding of VHL and HIF-1α protein.

As mentioned before, the HIF-1α level could also be affected by the β subunit. It is known that HIF-1α acquired a new conformational state upon dimerization with HIF-1β, rendering HIF-1α more resistant to proteolytic digestion in vitro (12, 15). Moreover, in HIF-1β mutant (G316A) hepatoma cells or HIF-1β−/− ES cells, HIF-1α stability in the nucleus is lowered in the absence of HIF-1β, and accordingly, there is lack of the HIF-1 DNA-binding and HIF-1 mediated gene activation (13, 14). We therefore investigated whether the binding affinity between the α and β subunits of HIF could be affected by the PI-3K/Akt pathway. The binding affinity between the two subunits of HIF was repressed following the treatment with the PI-3K inhibitor wortmannin under both normoxic and hypoxic conditions (Fig. 3B) suggesting that PI-3K/Akt may regulate the HIF pathway through HIF-1β.

**Akt interacts with hypoxia-inducible factor–1β and regulates the hypoxia-inducible factor transcriptional activity.** Because the PI-3K/Akt activity enhances the binding affinity between the α and β subunits of HIF-1 and because the interaction between the two subunits is mediated by the HLH-PAS domain, we examined the HIF sequences and found a potential conserved Akt phosphorylation site inside the HLH-PAS domain of HIF-1β suggesting that Akt might bind and phosphorylate HIF-1β (Fig. 4A). We therefore immunoprecipitated the endogenous Akt with an anti-Akt antibody and detected endogenous HIF-1β by Western blotting with an anti-HIF-1β antibody, in the MDA-MB-453 cell line and its dominant-negative Akt stable transfectant (Fig. 4B). After HEK293 cells transfected with FLAG-tagged HIF-1β and HA-tagged constitutively active Akt or dominant-negative Akt, we detected HIF-1β by Western blotting with an anti-HIF-1β antibody following the immunoprecipitation with an anti-HA antibody to precipitate the Akt. Following immunoprecipitation with an anti-FLAG antibody, we also detected Akt by Western blotting with an anti-Akt antibody (Fig. 4C). Moreover, using the GST-protein pull-down assay, we found that the GST-Akt protein associated with the in vitro translated HIF-1β labeled with [35S]-methionine (Fig. 4D). These data suggest that Akt and HIF-1β are physically associated with each other.

![Figure 3](https://example.com/figure3.png)

**Figure 3.** PI-3K/Akt affect HIF-1α interaction with HIF-1β, but not with VHL. A, PI-3K/Akt does not affect HIF-1α interaction with VHL. HA-tagged HIF-1α (10 μg) and Flag-tagged VHL (10 μg) were cotransfected into HEK293 cells. After 48 hours, the transfected cells were treated with 100 nmol/L wortmannin or hypoxia. HIF-1α was immunoprecipitated with an anti-HA antibody and VHL was blotted with an anti-FLAG antibody. B, PI-3K/Akt affects HIF-1α interaction with HIF-1β. HA-tagged HIF-1α (10 μg) and Flag-tagged HIF-1β (10 μg) were cotransfected into HEK293 cells. After 48 hours, the transfected cells were treated with 100 nmol/L wortmannin or hypoxia. Cells were lysed after 48 hours, and Akt was immunoprecipitated with an anti-HA antibody. HIF-1β was detected with an anti-FLAG antibody after transfer to a nitrocellulose membrane.
The above results suggest that Akt interacts with HIF-1β. To test whether Akt may phosphorylate HIF-1β, we first carried out coimmunoprecipitation experiments using an Akt-substrate antibody that can specifically detect potential Akt phosphorylation site "RXRXX(S/T)." A potential Akt phosphorylation site on HIF-1β was indeed detected and this phosphorylation disappeared after wortmannin treatment, which blocks the PI3K/Akt activity. Next we examined whether the predicted Ser271 was the Akt phosphorylation site. The immunocomplex kinase assay was done using the purified GST-HIF-1β and GST-HIF-1βS271D as substrates. We found that the wild-type protein, but not the mutant, was phosphorylated by the Akt kinase (Fig. 5B). In addition, the GST-HIF-1βS271D protein enhanced the binding ability with the in vitro translated HIF-1β labeled with [35S]-methionine (Fig. 5C). Finally, we tested the HIF transcription activity by the HRE reporter lucerase assay in the MDA-MB-453 and SKBR-3 cell lines and found that under the normoxic condition, the HIF-1β mutant S271D, which mimics the phosphorylation status, increased the HRE response in both cell lines and increased malignancy (2, 25). HER2 signaling also enhances VEGF expression, which is a major mediator in tumor angiogenesis (26). We showed here that HER2-mediated VEGF expression under normoxic conditions could be almost totally blocked by dominant-negative Akt. Dominant-negative Akt also could partially block VEGF mRNA expression under hypoxic conditions by 20% to 50%. The major transcription factors mediating VEGF expression are HIFs, which also activate numerous other molecules to promote angiogenesis and energy metabolism and thereby enhance tumor growth and metastasis (8, 9). HIF activation also occurs in HER2 overexpressing cancer cells. Previous reports have suggested that increased PI3K/AKT or decreased PTEN activity can activate the HIF pathway in various tumors (17, 26–29). We further investigated the relationship and functional role between the HIF and PI3K/Akt pathways in a HER2-overexpressing cancer model. Our results indicate that HER2 up-regulates HIF-1α activity in the absence of hypoxic stimulation and that the activated HIF-1α protein and its activity can be repressed by the genetic and chemical inhibition of the PI3K/Akt pathway. Under hypoxic conditions, however, the effect of PI3K/Akt pathway on the enhanced HIF-1α protein level seems to be less significantly and cell type dependent, consistent to the previous findings (18, 19).
Two factors may regulate HIF-1α protein modification and degradation. One well-known factor is hydroxylation and degradation, which is inhibited by hypoxia and contributes to major HIF-1α stability. Another, lesser-known factor is the interaction with β-subunit. Because HIF-1α stability in the nucleus is much lower in HIF-1α mutant (G316A) hepatoma cells and HIF-1β−/− ES cells, the heterodimerization was proposed to be required for stable association within the nuclear compartment, and HIF-1α acquired a new conformational state upon dimerization with HIF-1β, rendering HIF-1α more resistant to proteolytic digestion (12–15).

In support of this notion, we found that Akt could phosphorylate HIF-1α, enhance the HIF-1β binding ability with HIF-1α, and increase the transcriptional activity.

It is worthwhile to mention that the current study is also clinically relevant. Herceptin, a monoclonal antibody against HER2, when used to treat the HER2 overexpressing breast tumor, can inhibit the expression of proangiogenic factors including VEGF, resulting in a reduction in the tumor vasculature and a decrease in tumor size (30). Furthermore, phosphorylated Akt was effectively reduced by Herceptin treatment, which also led to the dramatic inhibition of VEGF (31). Our findings suggest that HER2-induced HIF activation requires the activation of Akt and may contribute to HER2-mediated angiogenesis, and that oncogenic signaling such as HER2 and Akt may activate HIF pathway to prompt the HIF-mediated angiogenesis process in the absence of hypoxia. This hypoxia-independent, HER2/Akt-induced HIF-1α expression may allow the HER2/Akt-activated tumors to possess certain degree of angiogenesis activity, even in the absence of hypoxia, and therefore enhance their ability for tumor progression. Taken together, these data suggest that HER2-mediated phosphorylation of Akt contributes to HIF-mediated VEGF secretion and that by inhibiting HER2 or Akt phosphorylation, VEGF secretion and angiogenesis is inhibited. Moreover, our result provides a mechanism for the oncogenic activity of HER2 and the PI-3K/Akt pathway in tumor angiogenesis and metastasis.

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