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 protein domains. 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-1 (also called ARNT), which is critically involved in a range of transcriptional systems, is indispensable 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 shown 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 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 (M2) were purchased from Sigma (St Louis, MO). The anti-FLAG antibodies were from Novus (Littleton, CO). HA-HIF-1α and VHL-FLAG plasmids 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′-CGAATGAGGTGTGGCGCTAGCTCT-3′ and 5′-TCGAAGTGACCC-3′ and for S271D, 5′-CGAATGAGGTGTGGCGCTAGCTCT-3′ and 5′-TCGAAGTGACCC-3′. The 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). As expected, HER2/3T3 cells induced higher HIF-1 transcriptional activity than did the parental NIH 3T3 cells. The fold increased was more profound under the 20% O2 concentration condition, although the basal level is higher under the hypoxic condition (Fig. 1A). The Akt pathway is known to be constitutively activated in HER2/3T3 cells, and when this pathway was blocked by dominant-negative Akt, the HIF activity was lower (Fig. 1A). This result was also supported by the treatment of a specific PI-3K inhibitor, wortmannin, which produced a similar lower HIF-1 activity in HER2/3T3 cells (Fig. 1A). To further extend our observation to other cells, we also did similar experiments using another HER2-overexpressing breast cancer cell line, MDA-MB-453, and its stable dominant-negative Akt transfectant. Consistently, the dominant-negative Akt transfectant showed reduction in HIF transcriptional activity in the MDA-MB-453 cells (Fig. 1B). Taken together, our results strongly support the notion that PI-3K/Akt is required for HER2-mediated hypoxia-inducible factor–1 transcription activity and vascular endothelial growth factor expression.
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. 1 C 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. 2 A 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. 2 A 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, AKT+/C0 embryonic fibroblasts (MEF) that either do not have both alleles of Akt (Akt+/−) or only one functional allele of Akt (Akt+/−,−/−) were cotransfected into HEK293 cells. After 48 hours, the transfected cells were treated with 100 nmol/L wortmannin or hypoxia. HIF-1α protein expression induced by hypoxia (Fig. 2A, right) was immunoprecipitated with an anti-HA antibody and VHL was blotted with an anti-FLAG antibody (Fig. 4A). Moreover, in HIF-1α 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.

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.** 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 motifs of HIF-1α and other known Akt substrates. Consensus Akt phosphorylation target sequence (top). Numbers on the right, positions of the final residues in each case. B, immunoprecipitation (IP) of endogenous Akt and detection of endogenous HIF-1α. Endogenous Akt was immunoprecipitated from 1,000 μg of MDA-MB-453 cell lysates with a specific sheep antibody against Akt or with control immunoglobulin G. After transfer to a nitrocellulose membrane, endogenous HIF-1α was detected with a specific mouse antibody against HIF-1α and Akt in HEK293 cells. Immunoprecipitation of HIF-1α and detection of Akt: HA-tagged DN-Akt or CA-Akt (10 μg) and Flag-tagged HIF-1α (10 μg) were cotransfected into HEK293 cells. Cells were lysed after 48 hours, and Akt was immunoprecipitated with an anti-HA antibody. After transfer to a nitrocellulose membrane, HIF-1α was detected with an anti-Flag antibody. Coimmunoprecipitation of Akt and Western blotting of HIF-1α; HIF-1α was immunoprecipitated with an anti-Flag antibody and Akt was detected with an anti-Akt antibody. In *in vivo* binding of Akt with HIF-1α. The GST-Akt fusion protein was incubated with an *in vitro*-translated HIF-1α labeled with 35S-methionine in immunoprecipitation buffer, recovered on glutathione beads, and analyzed by SDS-PAGE.

**Figure 4.** Akt interacts with HIF-1α. A, HIF-1α has a potential Akt phosphorylation site: comparison of the amino acid sequences of the Akt-phosphorylation motifs of HIF-1α and other known Akt substrates. Consensus Akt phosphorylation target sequence (top). Numbers on the right, positions of the final residues in each case. B, immunoprecipitation (IP) of endogenous Akt and detection of endogenous HIF-1α. Endogenous Akt was immunoprecipitated from 1,000 μg of MDA-MB-453 cell lysates with a specific sheep antibody against Akt or with control immunoglobulin G. After transfer to a nitrocellulose membrane, endogenous HIF-1α was detected with a specific mouse antibody against HIF-1α and Akt in HEK293 cells. Immunoprecipitation of HIF-1α and detection of Akt: HA-tagged DN-Akt or CA-Akt (10 μg) and Flag-tagged HIF-1α (10 μg) were cotransfected into HEK293 cells. Cells were lysed after 48 hours, and Akt was immunoprecipitated with an anti-HA antibody. After transfer to a nitrocellulose membrane, HIF-1α was detected with an anti-Flag antibody. Immunoprecipitation of Akt and Western blotting of HIF-1α; HIF-1α was immunoprecipitated with an anti-Flag antibody and Akt was detected with an anti-Akt antibody. In *in vivo* binding of Akt with HIF-1α. The GST-Akt fusion protein was incubated with an *in vitro*-translated HIF-1α labeled with 35S-methionine in immunoprecipitation buffer, recovered on glutathione beads, and analyzed by SDS-PAGE.

**Discussion**

The *HER2* overexpression is one of the most important genetic alterations in breast cancer, associated with high tumor grade and shorter overall survival rate (1–3). The functionality of *HER2* is critically mediated mainly by PI-3K/Akt. Many targets of the PI-3K/Akt signaling pathway promote cell survival, tumor progression, 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 PI-3K/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 PI-3K/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 PI-3K/Akt pathway. Under hypoxic conditions, however, the effect of PI-3K/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-1β 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 clinical 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 with 18 U.S.C. Section 1734 solely to indicate this fact.

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