Hypoxia Activates a Platelet-derived Growth Factor Receptor/Phosphatidylinositol 3-Kinase/Akt Pathway That Results in Glycogen Synthase Kinase-3 Inactivation

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Abstract

Hypoxia initiates numerous intracellular signaling pathways important in regulating cell proliferation, differentiation, and death. In this study, we investigated the pathway that hypoxia uses to activate Akt and inactivate glycogen synthase kinase-3 (GSK-3), two proteins the functions of which are important in cell survival and energy metabolism. Severe hypoxia (0.01% oxygen) initiated a signaling cascade by inducing the tyrosine phosphorylation of the platelet-derived growth factor (PDGF) receptor within 1 h of treatment and increasing receptor association with the p85 subunit of phosphatidylinositol 3-kinase (PI 3-K). Hypoxia-induced signaling also resulted in PI 3-K-dependent phosphorylation of Akt on Ser-473, a modification of Akt that is important for its activation. This activation of Akt by hypoxia was substantially diminished in cells that possessed mutations in their PDGF receptor-PI 3-K interaction domain. In addition, Akt activation by hypoxia was resistant to treatment with the growth factor receptor poison suramin but was sensitive to treatment with the PI 3-K inhibitor wortmannin. Activation of Akt by hypoxia resulted in the phosphorylation of GSK-3α and GSK-3β at Ser-9 and Ser-21, two well-documented Akt phosphorylation sites, respectively, that are inactivating modifications of each GSK-3 isoform. In support of the phosphorylation data, GSK-3 activity was significantly reduced under hypoxia. In conclusion, we propose that hypoxia activates a growth factor receptor/PI 3-K/Akt cascade that leads to GSK-3 inactivation, a pathway that can impact cell survival, proliferation, and metabolism.

Introduction

The cellular response to decreased oxygen is important both in normal development and tumor progression. Previous studies have indicated that these responses are attributable to the modulation of unique intracellular signal transduction pathways. Recently, the identification of a transcription factor, HIF-1, has advanced the understanding of hypoxia-induced intracellular signaling pathways (1–5). Studies of HIF-1 knockout embryos indicate that HIF-1 plays a significant role in angiogenesis and tumor growth and is also necessary for proper embryonic development, presumably through the transcriptional activation of genes involved in angiogenesis, glycosylation, and tissue remodeling (6–9). Because regulation of HIF-1 appears to be in large part attributable to posttranslational modifications of the HIF-1α subunit, which results in its stabilization, the list of proteins (iron chelators, kinases, heat shock protein 90, antioxidants, and ubiquitin) that may bind or somehow modify HIF-1 specifically under a hypoxic microenvironment is rapidly expanding (10–15). This knowledge may provide potential targets for intervention in treatment of cancers, strokes, coronary artery disease, and peripheral vascular disease.

In previous studies, we described a hypoxia-induced PI 3-K/Akt/HIF-1 pathway, leading to the activation of vascular endothelial growth factor (16). These studies suggested that Akt or protein kinase B was one of the protein kinases that lay downstream of the phospholipid products of PI 3-K and transduced the signal induced by hypoxia that results in HIF-1α stabilization in some cell types. Recently, it has been shown that 3’-phosphorylated phosphoinositides target Akt to the plasma membrane, an event that leads to the phosphorylation of Akt at two residues, Thr-308 and Ser-473, by phosphatidylinositol 3-kinase (3, 4, 5)P3-dependent protein kinases (17–20). Phosphorylation of these critical residues in turn releases Akt from an inhibited conformation, thereby activating its kinase function. Some of the downstream targets of Akt are important in regulating metabolic functions such as glycolysis, glucose uptake, and glycosylation. These targets include GSK-3α (21, 22), glucose transporter 4 (23, 24), and 6-phosphofructose 2-kinase (25). Other substrates of Akt are involved with its antiapoptotic function and include CED-3, c-Myc, Fas, nuclear factor-κB, and more recently, p53 (26–30).

Although a variety of signaling molecules in addition to PI 3-K (Src, Ras, and mitogen-activated protein kinase) have been shown to be modulated by hypoxia and reported to be involved with HIF-1 activation, (11, 16, 31, 32), how these molecules are initially activated by hypoxia is currently unknown. Because many of the signaling molecules activated by hypoxia are also activated by growth factors, we hypothesize that hypoxia may induce the activation of growth factor receptors and their associated pathways. The general scheme of growth factor receptor activation proceeds through a series of well-documented steps. The growth factor ligand binds to the extracellular domain of a heterodimeric receptor. The binding of the ligand results in dimerization of the receptor and autophosphorylation of the receptor at specific residues in its intracytoplasmic domain. Several Src homology 2-containing signaling molecules bind to these phosphorylated tyrosines and activate diverse downstream signal transduction pathways, the most studied of which are the mitogen-activated protein kinase and the PI 3-K pathways.

In contrast to ligand-stimulated growth factor receptor activation, UV irradiation, osmotic stress, and heat shock have been reported previously to activate growth factor receptors through ligand-independent mechanisms (33–35). Rosette and Karin (33) showed that UV light and osmotic stress caused aggregation of growth factor receptors that resulted in the activation of the JNK signaling pathway. Their study tested the hypothesis that physical stress could cause changes in the cell membrane, resulting in clustering and activation of growth factor receptors and downstream signaling cascades. Huang et al. (36) showed that UV treatment caused an accumulation of ROS which in turn activated several growth factor receptors, most notably the EGFR. Recent publications have proposed that hypoxia induces a mitochondria-dependent accumulation of ROS, suggesting the neces-
Hyoxia-induced PDGFRβ-PI 3-K/Akt/GSK-3 cascade.

In this study, we investigated the activation of growth factor receptors, PI 3-K, and Akt in inactivation of GSK-3 by hypoxia. We showed that within 1 h of treatment with hypoxia, PDGFRβ was phosphorylated on tyrosine residues, leading to the activation of PI 3-K. Hypoxia also resulted in Akt activation, which was partially dependent on intact interaction between PDGFRβ and PI 3-K. Akt activation by hypoxia was blocked by wortmannin but was not blocked by suramin, suggesting that this pathway was activated in a growth factor ligand-independent but PI 3-K-dependent manner. Finally, hypoxia treatment resulted in the phosphorylation and inactivation of GSK-3, a downstream target of Akt. These studies have led us to propose that hypoxia initiates a PI 3-K/Akt/GSK-3 signaling cascade through ligand-independent activation of growth factor receptors.

Materials and Methods

Cell Cultures and Treatments. HT1080 cells (human fibrosarcoma) were cultured in a medium supplemented with 10% FCS. ATW+ and FF740/51 cells (canine kidney epithelial cells), graciously provided by J. Cooper (Fred Hutchinson Cancer Center, Seattle, WA), were cultured in DMEM supplemented with 10% FCS and under 500 µg/ml G418 selection. Cells were cultured in a well-humidified 95% air, 5% CO2 incubator at 37°C. For hypoxia treatments, 5 × 10^5 cells were plated on glass dishes and incubated overnight in serum-containing media for each cell line. The following day, the cells at ~80% confluence were cultured in serum-free medium overnight. Hypoxia treatments were performed in chambers (Anaerobic Systems) gassed with a mixture of 95% nitrogen, 5% carbon dioxide (Praxair), resulting in an oxygen level at 0.01%. Suramin and hydrogen peroxide from Sigma were dissolved in water. Wortmannin (Sigma) was dissolved in DMSO.

Immunoblotting and Immunoprecipitations. After treatment (within the hypoxia chamber for the hypoxia-treated cells), cells were washed in PBS and lysed in buffer containing 137 mM sodium chloride, 20 mM Tris-hydrochloric acid (pH 7.5), 1 mM magnesium chloride, 1 mM calcium chloride, 10% glycerol, 1% NP40, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 µg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate. Lysates were vortexed and centrifuged, and the protein concentration of the supernatant was quantitated using the bicinchoninic acid protein assay (Pierce Biochemicals). For immunoprecipitations, 500–750 µg of lysate were incubated with antibody to phosphotyrosines (Transduction Laboratories) or PDGFRβ (Santa Cruz), and these immunocomplexes were collected with Protein A/G Plus Agarose (Santa Cruz). After several washes in 1% NP40 in PBS, 0.5 mM lithium chloride in 0.1 mM Tris-hydrochloric acid (pH 7.5), and TNE buffer (10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 1 mM EDTA), the immunoprecipitates along with whole-cell lysates were subjected to SDS-PAGE, transferred to polyvinylidene difluoride membrane (Amersham), and immunoblotted with antibodies to phosphotyrosines, PDGFRβ, p85 (UBI), phosphospecific Akt S473 (New England Biolabs), phosphospecific GSK-3β (QBC/BioMol), phosphospecific GSK-3α (UBI), or phosphospecific ERK1/2 (New England Biolabs). The bands were visualized using the ECF reagents (Amersham/Vistra) per the manufacturer’s protocol and quantitated on a PhosphorImager (Molecular Devices) with ImageQuant software.

Results

Hypoxia Treatment Resulted in Tyrosine Phosphorylation of PDGFRβ, Association of p85 with PDGFRβ, and Activation of PI 3-K. Several groups have shown a ligand-independent activation of growth factor receptors by stresses such as UV irradiation and heat shock. Although most of the previous studies focused on EGFR, we chose to investigate PDGFRβ as the target for hypoxia-induced signaling because of its involvement in angiogenesis (42) and glioma tumorigenesis (43). Using immunoprecipitation techniques, we demonstrated that hypoxia induced the tyrosine phosphorylation of the PDGFRβ within a 30–60-min treatment in HT1080 cells (Fig. 1A). Hydrogen peroxide treatment also resulted in a similar activation of PDGFRβ. We probed the PDGFRβ immunoprecipitates with PDGFRβ to show that equal amounts of PDGFRβ existed in the cells and that equal amounts were immunoprecipitated (Fig. 1A). A 60-min treatment with hypoxia resulted in a 2.5-fold increase in tyrosine phosphorylation of PDGFRβ as compared with control treatment in room air (Fig. 1B). We also found that hypoxia caused a 3-fold increase in the tyrosine phosphorylation of EGFR within 60 min of treatment (data not shown).

In addition to the tyrosine phosphorylation of PDGFRβ, hypoxia stimulated the association between the p85 subunit of PI 3-K and PDGFRβ (Fig. 1A) and resulted in subsequent PI 3-K activation. Using phosphotyrosine immunoprecipitates with which all tyrosine phosphorylated, activated growth factor receptors were potentially isolated, we performed PI 3-K assays on extracts of ATW+ cells (TRMP cells that normally lack PDGFRβ but stably transfected with...
wild-type PDGFRβ) to determine the effect of hypoxia and oxidative stress by hydrogen peroxide on PI 3-K activity. Although both hypoxia and hydrogen peroxide increased PI 3-K activity, hydrogen peroxide treatment was a more potent activator of PI 3-K (Fig. 1B). PI 3-K activity induced by hypoxia and hydrogen peroxide was also inhibited by wortmannin, a specific PI 3-K inhibitor, further supporting the specificity of this lipid kinase activity to PI 3-K (Fig. 1B).

**Hypoxia Treatment of Cells Resulted in the Activation of Akt.** To investigate whether hypoxia-induced PI 3-K activation resulted in stimulation of downstream target Akt, we incubated cells in a hypoxic tissue culture chamber at 0.01% oxygen for a range of time from 30 min to 4 h. While in the chamber, the cells were washed with PBS buffer and lysed to avoid the confounding effect of reoxygenation. Extracts of untreated and hypoxia-treated cells were subjected to immunoblotting with phospho-specific antibodies to Akt. Hypoxia-treated cells exhibited an increase in the amount of phosphorylated Akt at Ser-473 and hence activated Akt by 60–120 min (Fig. 2A). This induction of Akt activity was sustained through a 4-h hypoxia treatment, with a 2–2.5-fold increase at 240 min (Fig. 2B). Similarly, hydrogen peroxide treatment also caused an increase in Akt phosphorylation, although this occurred more rapidly within 15 min of stimulation. To verify the specificity of wortmannin for PI 3-K and to inhibit the activity induced by hypoxia and that other growth factor receptors still functioned to activate ERK1/2. In contrast to Akt activation, hypoxia and hydrogen peroxide both caused significant increases in ERK1/2 phosphorylation (Fig. 2, A and C). ERK1 seemed to be more responsive to hypoxia treatment than ERK2 in both the ATW and FF740/51 cells, with a 3-fold average induction of ERK1 and a 1.5-fold average induction of ERK2 in each cell line (Fig. 2, A and C). These results suggested that PI 3-K activity signaled from the PDGFR had a minor role in modulating ERK1/2 activity induced by hypoxia and that other growth factor receptors still functioned to activate ERK1/2.

**Hypoxia Activated Akt in a PI 3-K-dependent Manner.** Previous studies have demonstrated both PI 3-K-dependent and independent pathways in the activation of Akt. We used the PI 3-K specific inhibitor wortmannin to determine whether PI 3-K activity was necessary for hypoxic induction of Akt phosphorylation in ATW+ cells (Fig. 3A). In contrast to the nearly complete inhibition of hypoxia-induced Akt activation by wortmannin treatment, we found only partial inhibition of Akt activation by hydrogen peroxide at 100 nm wortmannin, suggesting that hydrogen peroxide used both PI 3-K-dependent and -independent pathways to activate Akt. In ATW+ cells, the activation of ERK1/2 by either hypoxia or hydrogen peroxide was unchanged by wortmannin, further supporting its specificity of action on PI 3-K at the 100 nm concentration.

**Activation of Akt by Hypoxia Did Not Depend upon Extracellular Ligand-induced Phosphorylation of Growth Factor Receptors.** To investigate the potential activation of growth factor receptors by extracellular ligands, we used the growth factor receptor poison, suramin. Although the mechanism by which suramin blocks growth factor receptor function is probably multifactorial, studies suggest that suramin binds directly to the growth factor and thus prevents growth factor binding and activation of the receptor. With a 2-h pretreatment of suramin in serum-free medium, suramin was able to block the PDGF BB activation of Akt in the ATW+ cells (Fig. 3B); however, suramin failed to block hypoxia or hydrogen peroxide induction of
Akt phosphorylation (Fig. 3A). The lack of inhibition by suramin and the relatively short period of time for receptor activation made a hypoxia-induced paracrine or autocrine release of growth factor ligand to activate the receptor highly unlikely. From these results, hypoxia appeared to activate growth factor receptors and downstream effectors in a ligand-independent manner. Furthermore, both the failure to activate Akt by PDGF BB and the diminished activation of Akt by hypoxia in the FF740/51 cells confirmed that mutation of the PI 3-K association domain is specific for PDGFRβ. These results also indicated that other growth factor receptors were capable of activating Akt by hypoxia in addition to the PDGFR.

**Hypoxia Induced an Inactivating Phosphorylation of GSK-3, a Downstream Target of Akt.** Because the cell lines used in this study exhibited little hypoxia or hydrogen peroxide-induced apoptosis, we investigated the role of Akt in metabolism with GSK-3 as a relevant downstream target. With similar kinetics to Akt activation, hypoxia treatment resulted in the phosphorylation of GSK-3α and GSK-3β at Ser-9 and Ser-21, respectively, in HT1080 cells (Fig. 4A). The phosphorlyations at these two sites have been shown to be Akt-dependent and to inactivate the kinase. Using an *in vitro* kinase assay with a glycogen synthase peptide as the substrate, we found that hypoxia decreased GSK-3 activity 50% within 60 min in HT1080 cells and a human hepatoma cell line HepG2 (Fig. 4B). Although hydrogen peroxide treatment activated Akt to a greater degree than hypoxia, it resulted in a minimal increase in GSK-3 phosphorylation and a concomitant slight decrease in GSK-3 activity. Thus, hypoxia and hydrogen peroxide diverged in their signaling pathways downstream of Akt. Previous studies have shown that inactivating GSK-3 activates glycogen synthase in some types of cells, increasing the synthesis of glycogen. Whether inactivation of GSK-3 by hypoxia results in activation of glycogen synthase *in vivo* has yet to be determined.

**Discussion**

In this study, we propose that hypoxia activates a PI 3-K/Akt pathway by inducing the tyrosine phosphorylation of growth factor receptors. Similar to what has been reported for other stresses, the activation of PDGFR by hypoxia appears to be ligand independent.

Some studies suggest that stresses such as UV irradiation and osmotic stress can cause a change in membrane dynamics and result in the clustering and presumably dimerization of growth factor receptors. In fact, dimerization alone is sufficient to cause receptor autophosphorylation. Therefore, it is possible that hypoxia by altering the fluidity of the membrane may lead to receptor dimerization and activation. We are currently investigating this hypothesis.

Another mechanism of growth factor receptor activation involves ROS. Recent publications have cited the role for ROS as signaling molecules in growth factor receptor signal transduction pathways (Refs. 36, 44, and 45; reviewed in Ref. 46). Huang et al. (36) showed that UV treatment caused an accumulation and extracellular release of ROS, which then activated several growth factor receptors, most notably the EGFR. In this study, UV activation of EGFR was inhibited by suramin. However, because hypoxia-induced activation of PDGFRβ was not inhibited by suramin, this mechanism of receptor activation by ROS is unlikely, and hypoxia activation may be mechanistically different.

Although UV, ionizing radiation, and reoxygenation after ischemia have been documented to cause the accumulation of ROS, which then play a role as intracellular signaling molecules, the involvement of ROS in hypoxia signaling is highly controversial. Recent studies (37–39) have suggested that hypoxia blocks cytochrome c in the electron transport chain, causing a release of ROS from the mitochondria. However, as HIF-1 complex formation, DNA binding, and HIF-1 protein accumulation have been shown to be inhibited by hydrogen peroxide and activated by antioxidants (13, 47), perhaps it is unlikely that hypoxia would cause the accumulation of ROS like hydrogen peroxide that would result in the inhibition of HIF-1. Because ROS formation is dependent on oxygen availability, it also seems logical that hypoxia would result in decreased ROS. We have some preliminary data that show that mitochondrial function is necessary for hypoxia signaling to Akt, but the role of ROS in this pathway is unknown at present.

The activation of the PI 3-K/Akt/GSK-3 pathway by hypoxia has several potential physiologically relevant consequences. Although the most studied role of GSK-3 in mammalian biology is its role in regulation of glycogen synthesis in response to insulin, several recent studies have shown that it may also be important in cell survival, proliferation, and differentiation. Pap and Cooper (41) report that GSK-3 is an important downstream target of the PI 3-K/Akt survival pathway. Ectopic overexpression of a dominant-negative GSK-3 resulted in cellular protection from cell apoptosis, whereas catalytically active GSK-3 promotes apoptosis. In addition, GSK-3β has been linked to the phosphorylation, turnover, and subcellular localization of cyclin D1 and hence plays a potential role in cell cycle regulation (48).
Diehl et al. (41) suggest that a mitogen-activated Ras/PI 3-K/Akt pathway modulates cyclin D1 turnover, decreasing its proteolysis, increasing its stability, and allowing the cells to progress to the synthesis phase of the cell cycle. Although Akt activity appears necessary for hypoxia-induced vascular endothelial growth factor expression via HIF-1 in some cell types, HIF-1 is not a good substrate for Akt or GSK-3, suggesting that GSK-3 inactivation and HIF-1 activation represent divergent downstream pathways of hypoxia-induced Akt. Activation of Akt and subsequent inactivation of GSK-3 may be critical events in hypoxia-induced signal transduction pathways modulating several cellular processes, including metabolism, proliferation, differentiation, and survival.

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References

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