Activation of RhoB by Hypoxia Controls Hypoxia-Inducible Factor-1α Stabilization through Glycogen Synthase Kinase-3 in U87 Glioblastoma Cells

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Abstract

Hypoxia is a crucial factor in tumor aggressiveness and resistance to treatment, particularly in glioma. Our previous results have shown that inhibiting the small GTPase RhoB decreased oxygenation of U87 human glioblastoma xenografts, in part, by regulating angiogenesis. We investigated here whether RhoB might also control a signaling pathway that would permit glioma cells to adapt to hypoxia. We first showed that silencing RhoB with siRNA induced degradation and inhibition of the transcriptional activity of the hypoxia-inducible factor by the proteasome in U87 hypoxic cells. This RhoB-dependent degradation of hypoxia-inducible factor-1α in hypoxic conditions was mediated by the Akt/glycogen synthase kinase-3β pathway. While investigating how hypoxia could activate this signaling pathway, using the GST-Rhotekin RBD pulldown assay, we showed the early activation of RhoB by reactive oxygen species under hypoxic conditions and, subsequently, its participation in the ensuing cellular adaptation to hypoxia. Overall, therefore, our results have not only highlighted a new signaling pathway for hypoxia controlled by the small GTPase RhoB, but they also strongly implicate RhoB as a potentially important therapeutic target for decreasing tumor hypoxia. (Cancer Res 2006; 66(1): 482-9)

Introduction

Decreased cellular oxygen levels, termed hypoxia, have been identified during heart disease, acute and chronic vascular disease, pulmonary disease, and cancer. Mammalian cells are able to sense decreased oxygen levels through a conserved hypoxic response pathway that is involved in the execution of many physiologic events such as vessel formation during embryogenesis and in pathologic processes such as tumorigenesis. In particular, hypoxia is an important selective force both in the clonal evolution of tumors and in their resistance to radiotherapy. The transcriptional hypoxia-inducible factor 1 (HIF-1) is an essential regulator of oxygen homeostasis by controlling a battery of target genes involved in angiogenesis, glycolysis, proliferation, and pH regulation. HIF-1 is a heterodimer composed of HIF-1α and HIF-1β subunits. Whereas HIF-1β is constitutively expressed, the intracellular level of HIF-1α is dependent on oxygen conditions. At normal oxygen levels, HIF-1α is prolyl-hydroxylated by oxygen-dependent specific prolyl hydroxylases, which require oxygen, ferrous iron and 2-oxoglutarate for activity. Three prolyl hydroxylases have been identified, although recent work has shown that prolyl-hydroxylase 2 is the critical oxygen sensor setting the low steady-state levels of HIF-1α in normoxia (1). This prolyl-hydroxylase promotes the interaction of HIF-1α with the von Hippel Lindau (VHL) ubiquitin-ligase complex initiating rapid ubiquitination and subsequent HIF-1α protein destruction via the proteasome. During hypoxia, the prolyl-hydroxylases cease to function and HIF escapes from degradation. In addition to this regulation, kinase/phosphatase activity is required for hypoxia signal transduction. It has been shown that treatment of cells with the tyrosine kinase inhibitor, genestein, or with the serine/threonine phosphatase inhibitor, sodium fluoride, blocks hypoxia-induced HIF-1α expression (2). Moreover, the phosphoinositide-3-kinase (PI3K)/Akt pathway modulates hypoxia-induced HIF-1α activation (3). For example, treating human prostate cancer cells with pharmacologic agents that target PI3K or Akt inhibits HIF-1α expression (4). Active PI3K activates Akt, which in turn, inhibits glycogen synthase kinase (GSK-3) via phosphorylation of its Ser21 and Ser9 residues. On the other hand, GSK-3 activity is up-regulated by Tyr279/Tyr216 phosphorylation, which according to recent results, may be an intramolecular autophosphorylation event (5). GSK-3 which had first been shown to regulate glycogen synthase activity and had been identified as a negative regulator of glycogen synthesis, was also shown to regulate a number of transcription factors through phosphorylation. Recent work has shown that prolonged hypoxia activates GSK-3β and decreases HIF-1α protein suggesting that GSK-3β may regulate HIF-1α protein stability (3, 6).

Under hypoxic conditions, HIF-1α stabilization is also controlled by various small GTPases. Overexpression of Ras has been shown to lead to increased expression of HIF-1α via the PI3K/Akt signaling pathway under hypoxic conditions in breast cancer cell lines (7). Recent studies have shown that small GTPases such as RhoA, Rac1, and Cdc42 are induced in vitro during hypoxia by reactive oxygen species (ROS) and inhibition of these GTPases by the C3 exoenzyme prevented HIF-1α accumulation in hypoxia (8). Moreover, we have recently shown the involvement of another member of this family, RhoB, in the control both of radioresistance (9) and of hypoxia. We showed that inhibition of RhoB in glioblastoma xenografts leads to a decrease of hypoxia associated with a regulation of the vasculature (10). Similar results have been obtained by targeting RhoB—which is farnesylated and geranylgeranylated in vitro (11, 12)—with a farnesyltransferase inhibitor (13). Because an in vitro decrease in hypoxia can be due to regulation of
the vasculature and of intracellular pathways controlling hypoxia signaling, in the present study, we have investigated the involvement of RhoB in the control of HIF-1α regulation under hypoxic conditions. Our results show the importance of RhoB activation by hypoxia in the control of HIF-1α cellular accumulation and thus elucidate a new signaling pathway controlling hypoxia.

Materials and Methods

Cell culture. Human U87 glioblastoma cells were routinely maintained in DMEM supplemented with 10% calf serum at 37°C in 5% CO₂ humidified incubators and were subcultured weekly. Hypoxic conditions were obtained by incubating U87 cells in a sealed “Bug-Box” anaerobic work station (Ruskinn Technologies, Leeds, United Kingdom/Jouan, St. Herblain, France). The oxygen in the work station was maintained at 1% with the residual gas mixture being 94% nitrogen and 5% carbon dioxide.

Small interfering RNA transfection. U87 cells were transfected with different small interfering RNA (siRNA): an aleatory sequence, SiScramble (Eurogentec), RhoB (SiRhoB1; 5’CCGUCUUCCAGAAGAGAAGdTdT3; Eurogentec), SiRhoB2 (5’CCGUCUUCCAGAAGAGAAGdTdT3; Eurogentec), and a pool of four siRNAs specific for GSK-3β (Smartpool Kit; Dharmacon, Chicago, IL). Cells were transfected with 20 nmol/L of the different siRNA (SiScramble, SiRhoB1, SiRhoB2, and SiGSK-3β) using the OligofectAMINE transfection reagent following the instructions provided by the manufacturer.

Western blot analysis. U87 cells, untransfected or transfected with siRNA, were incubated under normoxia or hypoxia for various times, with or without inhibitors, 20 μmol/L Clasto-lactacystine β-lactone (Calbiochem, Fontenay sou Bois, France), 10 μmol/L MG132 (Calbiochem), 10 μmol/L SB216763 (Sigma, St-Quentin, France). Blots were probed overnight at 4°C with a mouse monoclonal anti-HIF-1α (diluted 1:1,000; BD Transduction Laboratories, LePont de Claix, France), with rabbit polyclonal anti-RhoA, anti-RhoB, anti-RhoC (diluted 1:1,000; Santa Cruz Technology, Santa Cruz, CA), with mouse monoclonal anti-HSP90, anti-HSP70 (diluted 1:1,000; BD Transduction Laboratories), with a rabbit polyclonal anti-phospho-Ser9 GSK-3β (diluted 1:1,000; Cell Signaling, Beverly, MA), with a rabbit polyclonal anti-total Akt (diluted 1:1,000); Cell Signaling), with a rabbit polyclonal anti-phospho-Ser473 Akt (diluted 1:1,000), Cell Signaling), with a rabbit polyclonal anti-phospho-Ser9 GSK-3β (diluted 1:1,000; Cell Signaling), with a rabbit polyclonal anti-total GSK-3 (diluted 1:1,000) Cell Signaling), with a rabbit polyclonal anti-phospho-Tyr279/216 GSK-3β (diluted 1:1,000; Cell Signaling), with a rabbit polyclonal anti-carbonic anhydrase 9 (CA-9; diluted 1:1,000; Santa Cruz Technology), by quantifying the level of CA-9 in U87 cells under various conditions of hypoxia.

RhoB activation assay. The Rho binding domain of Rhotekin (GST-Rhotekin RBD pulldown assay) was expressed as a recombinant fusion with GST in Escherichia coli and purified through binding to GSH-Sepharose beads (Amersham Pharmacia Biotech AB, Les Ulis, France) as previously described (14). After removing ~80% confluence, U87 cells, treated with vehicle or with 15 μmol/L diphenylene iodinum (DPI; Sigma), were incubated under hypoxia and lysed on ice by scraping and vigorous mixing in 1 mL (per 100 mm plate) of lysis buffer [50 mmol/L Tris-Cl (pH 7.5), 500 mmol/L NaCl, 10 μmol/L MgCl₂, 1% Triton X-100, 10 mmol/L DTT, 10 mmol/L PNP, 2 mmol/L NaVO₄, 20 mmol/L NaF, 1% protease inhibitor cocktail (Sigma)]. The lysates were precleared for 5 minutes at 13,200 rpm in an Eppendorf tabletop centrifuge. An aliquot (400 μg) from each lysate was removed as a control for equivalent input into the assay and the remaining lysate was then combined with 100 μL of GST-Rhotekin RBD pulldown assay beads and rotated for 35 minutes at 4°C. Beads were washed twice with 500 μL ice-cold wash buffer [50 mmol/L Tris-Cl (pH 7.5), 500 mmol/L NaCl, 10 mmol/L MgCl₂, 1% Triton X-100]. Bound protein was eluted from the beads with 35 μL SDS-PAGE loading buffer at 95°C. RhoB protein was resolved with SDS-PAGE 12.5% acrylamide and detected by immunoblot analysis.

Statistical analysis. Student’s t test was done to compare the means of values from different experiments. Differences were considered statistically significant at P < 0.05.

Results

RhoB silencing inhibits the accumulation and transcriptional activity of HIF-1 in hypoxic U87 cells. In order to analyze the regulation of hypoxia by RhoB, we chose to inhibit RhoB using siRNA techniques. For this purpose, we designed two different RhoB siRNAs against RhoB. We first checked that these two siRNA, SiRhoB1 and SiRhoB2, induced a significant decrease in RhoB protein level in U87 cells without affecting the two Rho proteins known to present >88% sequence homology with RhoB, i.e., RhoA and RhoC. As shown in Fig. 1A, transfection of U87 cells with either SiRhoB1 or SiRhoB2 at 20 nmol/L decreased the RhoB protein level without affecting levels of RhoA and RhoC. No inhibition was detected after transfection with a scrambled sequence of siRNA (SiScramble). These data showed that SiRhoB1 and SiRhoB2 were able to specifically inhibit RhoB when transfected into U87 cells.

It is now widely accepted that HIF, and more specifically HIF-1α, is a central regulator of the cellular pathways that control hypoxia. To further investigate the role of RhoB in the regulation of these pathways, we next determined whether RhoB was able to regulate HIF-1α in U87 cells under hypoxic conditions. We first quantified the amount of HIF-1α protein in untransfected U87 cells and those transfected either with SiScramble, SiRhoB1, or SiRhoB2 under normoxic and hypoxic conditions (Fig. 1B). Under normoxic conditions, the level of HIF-1α was not affected by transfection with the different siRNAs. However, under hypoxic conditions, the level of HIF-1α was significantly increased both in U87 cells themselves as well as in U87 cells transfected with SiScramble (P < 0.01; Fig. 1C). In contrast, no significant increase of the HIF-1α signal was observed under hypoxic conditions when U87 cells had been transfected with either SiRhoB1 or SiRhoB2, confirming that RhoB inhibition regulates the intracellular level of HIF-1α in hypoxic U87 cells (Fig. 1C).

To determine whether this decrease in HIF-1α was associated with an inhibition of HIF-1α transcriptional activity, we next examined whether RhoB silencing might regulate the expression of HIF-1α-induced genes. Specifically, we chose to analyze the level of one of these genes, CA-9, which is frequently used as a hypoxic marker (15), by quantifying the level of CA-9 in U87 cells under different conditions using Western blotting. As shown in Fig. 1D, the CA-9 level significantly decreased in cells transfected with either SiRhoB1 (0.16 ± 0.03) or SiRhoB2 (0.17 ± 0.03) under hypoxic conditions compared with the SiScramble-transfected cells (0.30 ± 0.02; P < 0.01). This finding confirmed that silencing RhoB inhibited the HIF-1α transcriptional activation of target genes. Overall, these results showed that RhoB is able to regulate the level of HIF-1α and in turn its transcriptional activity in vitro under conditions of hypoxia.

HIF-1α degradation induced by RhoB inhibition is controlled by the proteasome. Because it has been convincingly shown that the intracellular level of HIF-1α is mainly regulated by proteasome-dependent degradation, we next investigated whether the decreased amount of HIF-1α in U87 cells after RhoB silencing might be due to the stimulation of HIF-1α degradation by the proteasome under these conditions. Untransfected U87 cells or those transfected with either of the two siRNAs directed against
RhoB were incubated under hypoxic conditions in the presence of the specific proteasome inhibitor, lactacystin. As expected, an accumulation of HIF-1α was observed under normoxic conditions after lactacystin treatment in both untransfected U87 cells or in those transfected either with SiScramble, SiRhoB1, or SiRhoB2 (data not shown). Under hypoxia, lactacystin treatment did not modify the amount of HIF-1α in either untransfected U87 or in SiScramble-transfected U87 cells. However, in hypoxic cells transfected with either SiRhoB1 or SiRhoB2, treatment with this proteasome inhibitor, significantly increased the level of HIF-1α compared with the vehicle-treated hypoxic transfected cells (P < 0.01; Fig. 2A and B). Moreover, following proteasome inhibitor treatment, the HIF-1α levels of the SiRhoB1- or SiRhoB2-transfected cells under hypoxic conditions were equivalent to those of hypoxic untransfected U87 cells or of those transfected with SiScramble (Fig. 2B). Similar results were obtained by treating cells with another proteasome inhibitor, MG 132 (data not shown).

Overall, therefore, these results showed that under hypoxic conditions, RhoB inhibits the degradation of HIF-1α by the proteasome.

**RhoB controls HIF-1α degradation in hypoxic U87 cells by regulating Akt and GSK-3 activity.** Various intracellular pathways can control HIF-1α proteasome-dependent degradation. Under hypoxic conditions, it has been previously shown that HIF-1α degradation can be regulated by the interaction of HIF-1α with heat shock proteins and, more specifically, with HSP70 and...
HSP90 (16). We therefore examined whether silencing of RhoB might induce regulation of the expression of HSP70 and HSP90. No significant modification of the levels of HSP70 and HSP90 was observed in hypoxic U87 cells transfected by one of the two siRNAs directed against RhoB (data not shown). It is known that HIF-1α recognition by the proteasome can also be controlled by its phosphorylation. Several studies have shown a role for HIF-1α phosphorylation either by mitogen-activated protein kinase (MAPK) under normoxic conditions (1) or by the PI3K/Akt/GSK-3 pathway under hypoxic conditions (3). Because MAPK-dependent HIF-1α phosphorylation does not modify the stability of the protein (1), we focused our attention on a possible regulation of RhoB-dependent HIF-1α degradation via Akt/GSK-3β. Akt is activated by phosphorylation of its Ser473 and Thr308 residues. It has been shown that RhoB may control Akt trafficking (17), hence, we first determined the phosphorylation status on the Ser473 has been shown that RhoB may control Akt trafficking (17), hence, of its Tyr216 residue. Using Western blotting to determine whether RhoB silencing inhibits Ser 473 phosphorylation of Akt, we first analyzed the level of GSK-3β phosphorylated on Ser9 and Tyr216 in hypoxic untransfected cells or those transfected with either SiRhoB1 or SiRhoB2 (Fig. 4A). The amount of phosphorylated Ser9 or Tyr216 GSK-3β was not modified by silencing RhoB under normoxic conditions (Fig. 4). Hypoxia induced a significant increase (P < 0.05) in the phosphorylated Ser9 inactive form of GSK-3β in untransfected as well as SiScr-transfected cells which was, however, significantly decreased (P < 0.05) when cells were transfected with either SiRhoB1 or SiRhoB2 (Fig. 4A and B). Under these same conditions, hypoxia significantly reduced the amount of the phosphorylated Tyr216 active form of GSK-3β (P < 0.01) in either untransfected or SiScr-transfected cells, whereas inhibiting RhoB in hypoxic U87 cells increased the amount of this active form (P < 0.01; Fig. 4A and C). These results strongly suggested that in hypoxic cells, RhoB regulates HIF-1α degradation by modulating GSK-3β activity.

To confirm this observation, we analyzed the effect of GSK-3β inhibition on HIF-1α degradation in RhoB-silenced U87 hypoxic cells. First, we treated U87 cells, transfected with either SiRhoB1 or SiRhoB2, with various concentrations of the specific GSK-3 inhibitor, SB216763 (18). No modification of HIF-1α levels was observed under normoxic conditions. However, treating hypoxic SiRhoB1-transfected U87 cells with this inhibitor reversed the RhoB1-induced degradation of HIF-1α in a dose-dependent manner, so that after treatment with SB216763 from 50 μmol/L, the HIF-1α level of hypoxic SiRhoB1-transfected U87 cells was comparable with that of SiScr-transfected U87 cells (Fig. 5A and B). Treating cells with the siRNA directed against GSK-3β (Fig. 5C) induced the same reversal (Fig. 5D). Overall, therefore, these results showed that the RhoB-dependent HIF-1α regulation under hypoxic conditions is mediated by GSK-3β.

Figure 3. RhoB silencing inhibits Ser473 phosphorylation of Akt in hypoxic U87 cells. A, U87 cells either untransfected or transfected with siRNA, were incubated under conditions of normoxia or hypoxia for varying times. Total cell lysates were resolved on SDS-PAGE and immunoblotted with phospho and total antibody of Akt. Data are representative of at least three different experiments. B, histograms represent the ratio of phospho Akt levels to total Akt levels, as described in Materials and Methods. Columns, mean of three different experiments; bars, ± SD; *, P < 0.001, under hypoxic conditions, transfection of U87 cells with either SiRhoB1 or SiRhoB2 significantly decreased the level of phosphorlated Akt compared with that of untransfected or of SiScr-transfected cells.
RhoB is activated by hypoxia via ROS production. To decipher the upstream mechanisms of HIF-1α stabilization by RhoB under hypoxic conditions, we then investigated whether hypoxia could regulate RhoB expression and activation. We first quantified the intracellular levels of RhoB during hypoxia. The level of RhoB in U87 cells remained unmodified until they were exposed to hypoxic conditions for 16 hours (Fig. 6A). We then quantified the level of activated RhoB in hypoxic or normoxic U87 cells by

**Figure 4.** GSK-3β activation is regulated by RhoB in hypoxic U87 cells. A, U87 cells, either untransfected or transfected with siRNA, were incubated under conditions of normoxia or hypoxia for 16 hours. Total cell lysates were resolved on SDS-PAGE and immunoblotted with the anti-phospho-Ser9, anti-phosphoTyr216 antibodies, and total anti-GSK-3β antibody. Data are representative of at least three different experiments (B and C). Histograms represent the ratio of the phospho GSK-3β levels to total GSK-3β levels, as described in Materials and Methods. Columns, mean of three different experiments; bars, ± SD; *, P < 0.05, under hypoxic conditions, transfection of U87 cells with either SiRhoB1 or SiRhoB2 significantly inhibited GSK-3β Ser9 phosphorylation compared with the untransfected or to SiScr-transfected cells (B); §, P < 0.01, under hypoxic conditions, transfection of U87 cells with either SiRhoB1 or SiRhoB2 significantly increased the phosphorylation of Tyr216 compared with untransfected or to SiScr-transfected cells (C).

**Figure 5.** GSK-3β inhibition reverses the effect of silencing RhoB on HIF-1α accumulation in hypoxic U87 cells. A, U87 cells, either untransfected or transfected with siRNA and treated either with vehicle (data not shown) or with 50 μmol/L of the GSK-3 inhibitor, SB216763, were incubated in normoxia or hypoxia for 16 hours. HIF-1α levels were analyzed by immunoblotting using the anti HIF-1α antibody, as described in Materials and Methods. Data are representative of at least three different experiments (B). The graph represents quantification of HIF-1α levels in SiRhoB1 and SiScr-transfected cells, as described in Materials and Methods. Points, mean of three different experiments; bars, ± SD. C, GSK-3β levels were analyzed in U87 cells, either untransfected or transfected with siRNA against GSK-3β by immunoblotting using the anti GSK-3β antibody, as described in Materials and Methods. Data are representative of at least three different experiments. D, U87 cells, transfected with 20 nmol/L of siRNA directed against either RhoB or GSK-3β as indicated, were incubated under hypoxia for 16 hours. HIF-1α levels were analyzed by immunoblotting using the anti HIF-1α antibody, as described in Materials and Methods. Data are representative of at least three different experiments.
performing pulldown assays using GST-fusion proteins conjugated to the Rho-binding domain of the Rho effector, Rhotekine (14). As shown in Fig. 6B and C, hypoxia induced an early RhoB activation, i.e., within 30 minutes, reaching a maximum at 2 hours, which was maintained for 16 hours at a level higher than that observed under normoxic conditions.

It has been well documented that one of the main mediators of hypoxia in cells is ROS production. Recent work has shown that other members of the Rho family, in particular RhoA and CDC42, could be activated by ROS (8). To determine whether similar regulation might control hypoxia-induced RhoB activation, hypoxic U87 cells were treated with a specific inhibitor of NADPH oxidase and thus of ROS production (DPI; ref. 19), and the levels of activated RhoB were analyzed. As shown in Fig. 6D, treating cells with DPI significantly decreased the level of activated RhoB under hypoxia conditions (0.95 ± 0.10 versus 0.66 ± 0.07; P < 0.05) whereas DPI treatment did not modify the level of activated RhoB under normoxic conditions (0.51 ± 0.09 versus 0.46 ± 0.13) demonstrating that hypoxia regulated RhoB activation via ROS production.

Our results have shown that under hypoxic conditions, RhoB is activated by ROS production, leading to an inhibition of HIF-1α recognition by the proteasome through the inactivation of GSK-3β (Fig. 7).

Discussion

Our previous results have shown that inhibiting RhoB in U87 human glioblastoma xenografts increased their radiosensitivity, at least in part, by increasing the oxygenation of these tumors (10). Similar results have been obtained by targeting RhoB with a farnesyltransferase inhibitor (13). In both approaches, this oxygenation was associated with regulation of vessel morphology and density and, after farnesyltransferase inhibitor treatment, with a decrease in the level of HIF-1α. However, these studies did not investigate whether the regulation of hypoxia observed after RhoB inhibition was due to a direct regulation of angiogenesis by this small GTPase or by an effect of RhoB on the cellular pathways controlling hypoxia and thus on angiogenesis. Here, we have investigated whether silencing RhoB in the U87 human glioblastoma cell line might affect hypoxia-inducible cellular pathways and, in particular, the degradation of the hypoxia-inducible transcription factor, HIF-1α, that has been identified as a central regulator of the hypoxia-induced pathways. Silencing RhoB led to the inhibition of hypoxia-induced HIF-1α accumulation and thus transcriptional activity in hypoxic U87 cells. This regulation of HIF-1α intracellular levels by RhoB was due to an activation of HIF-1α proteolytic degradation, thus demonstrating for the first time the involvement of RhoB in the proteasome-dependent HIF-1α regulation under hypoxic conditions. We next dissected out the biological pathway by which RhoB controls HIF-1α stabilization, focusing our attention on the Akt/GSK-3β pathway. In fact, it has already been shown that activated Akt inhibits GSK-3β preventing recognition of the HIF-1α oxygen-dependent degradation domain as a substrate and thus favoring HIF-1α protein stabilization (6). We have shown that silencing RhoB reduced the amount of phosphorylated, activated Akt, and thus increased the amount of activated GSK-3β in hypoxic U87 cells. Furthermore, inhibiting GSK-3β activity, either by a specific siRNA or by treating cells with various concentrations of the specific inhibitor SB216763, reversed the SiRhoB-induced HIF-1α degradation under hypoxic conditions, demonstrating that GSK-3β controls RhoB-dependent HIF-1α stabilization. Other small GTPases like Rac and RhoA have been involved in HIF-1α regulation. Hirota and Semenza have shown that in hypoxic Hep3B cells, Rac1 is activated in response to hypoxia and is required for the induction of HIF-1α protein expression, and for HIF-1α-dependent gene transcription in response to hypoxia (20). More recently, work from Turcotte’s team has shown that hypoxia induces pVHL expression in renal
cancer cells and that this induction is mediated by RhoA through Rho-kinase (21). Our results, for the first time, implicate the small GTPase RhoB as a regulator of HIF-1α stabilization under hypoxic conditions. These findings, combined with those of the previously described studies, strongly suggest that small GTPases controlling the cytoskeleton might be important factors in terms of mechanisms associated with hypoxia.

We next turned to determining how RhoB pathways could regulate hypoxia. It has already been shown that hypoxia increases expression of Rho proteins like Cdc42, Rac1, and RhoA in renal carcinoma cells (8). RhoB has been described as a stress gene whose mRNA can be activated in <30 minutes by UV or by DNA-damaging agents (22). We have shown here that incubating cells under hypoxic conditions induces an early activation of RhoB, occurring within 30 minutes of hypoxia, without any modification in RhoB levels. This early activation has already been observed for RhoA and for Cdc42 in hypoxic renal cells (8), as well as in hypoxic arterial endothelial cells (23). Our results strongly suggest that RhoB might be a sensor able to undergo activation when intracellular oxygen levels decrease. This activation would then induce hypoxia-inducible pathways, thus permitting tumor cells to adapt themselves to this novel environment.

Increased ROS production by mitochondria has been widely described as mediating the effects of hypoxia (19). In particular, ROS production is necessary for the transcriptional response induced by hypoxia (24). We therefore examined whether RhoB might be activated by ROS production. We were able to show that treating cells with the ROS production inhibitor, DPI, dramatically reduced the amount of activated RhoB in hypoxic U87 cells. These results show that production of ROS by hypoxia activates RhoB. Other small GTPases, such as Cdc42 and RhoA, are also activated by hypoxia via ROS production in renal cell carcinoma (8). Relatively little is known about the activation of the small GTPases by ROS. It has been shown that Ras can also be activated by ROS and that this activation is mediated by the activity of Ras GEF (as CDC 25<sup>Nez265</sup>), GAP (as p120-GAP), and effectors such as Raf-1 (25). RhoB activation by ROS may also be mediated through the regulation of specific GAP or GEF. Further investigations are under way to determine the precise mechanism of RhoB activation.

This study has highlighted the role of RhoB in the regulation of a new pathway controlling hypoxia in U87 human glioblastoma cells. Taking into account our previous results that have identified a role for RhoB in angiogenesis and tumor radiosensitivity in glioma, it clearly becomes of major interest to synthesize inhibitors of the RhoB pathways with the aim of reducing hypoxia in glioma and ultimately increase the overall efficiency of anticancer treatments, including radiotherapy.
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