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Suppression of PI3K/mTOR pathway rescues LLC cells from cell death induced by hypoxia

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Abstract

Cancer cells in solid tumors are challenged by various microenvironmental stresses, including hypoxia, and cancer cells in hypoxic regions are resistant to current cancer therapies. To investigate the mechanism of resistance to hypoxia in cancer cells, we examined mouse Lewis lung carcinoma (LLC) cells, which died due to necrosis at high density under hypoxic but not under normoxic conditions. Levels of mammalian target of rapamycin (mTOR), a central regulator of cellular energy, are reported to be suppressed in hypoxia. We found that phosphorylation of two molecules downstream to it, ribosomal p70 S6 kinase (S6K) and ribosomal protein S6, was markedly suppressed by hypoxia. Overexpression of the active form of S6K increased the sensitivity of LLC cells to hypoxia. On the other hand, inhibition of PI3K or mTOR dramatically reduced hypoxia-induced cell death under hypoxic conditions. Under hypoxic conditions, blockade of the PI3K or mTOR pathway increased levels of intracellular ATP and delayed decreases in pH and glucose level in culture medium, without affecting the cell cycle.

Keywords: Hypoxia resistance; Cancer; PI3K; mTOR; S6K; S6; ATP; Necrosis; Cell cycle

The involvement of hypoxia in tumor progression has recently become a prominent area of research. Cancer cells are exposed to various degrees of microenvironmental deterioration, such as low oxygen tension, low pH, and low nutrient concentrations. It is known that cancer cells surviving in hypoxic regions are refractory to ionizing radiation and also to some chemotherapeutic agents [1,2]. Since the degree of tumor hypoxia is inversely correlated with overall survival of cancer patients, targeting of hypoxia has long been a goal in cancer therapy [1,3].

Under conditions in which energy sources, such as oxygen, glucose, and amino acids, are deficient, cells die of necrosis due to depletion of intracellular ATP. To avoid this, mammalian cells have the ability to reduce oxygen consumption, a phenomenon termed oxygen conformance [4]. Hypoxia-tolerant glioblastoma cells decrease their oxygen consumption rate in response to low oxygen tension [5]. Growth factor signaling is also inhibited in oxygen conformance in cancer cells [6]. However, the molecular mechanism of hypoxia resistance in cancer cells remains unclear.

Overactivation of the PI3K pathway is observed in a wide spectrum of human cancers, and this pathway plays critical roles in transformation and proliferation [7]. The mammalian target of rapamycin (mTOR), one of the downstream molecules of the PI3K pathway, is a key regulator of translation, integrating multiple environmental and nutritional cues, such as availability of

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glucose and amino acids, intracellular ATP, and oxygen tension. mTOR regulates the phosphorylation of p70 S6 kinase (S6K) and eIF4E-binding protein 1 (4E-BP1), which promote translation of certain mRNA transcripts [8]. In the presence of sufficient supply of mitogens and nutrients, mTOR relays a signal to these translational regulators, enhancing the translation of mRNAs encoding proteins essential for cell growth and survival.

On the other hand, under nutritionally starved conditions, which often occur in solid tumors, down-regulation of PI3K or mTOR leads to retardation of growth and accumulation of cells in G1 phase. Recently, Arsham et al. [9] reported that mTOR and its downstream molecules are down-regulated when cancer cells are exposed to hypoxia, suggesting that down-regulation of mTOR activity by hypoxia is an energy conservation strategy to decrease ATP consumption prior to oxygen becoming metabolically limiting. Hypoxia might thus also induce cellular energy conservation strategies in cancer cells through inhibition of mTOR signaling. We examined whether suppression of PI3K and mTOR signaling contributes to tolerance to hypoxia, using hypoxia-sensitive mouse Lewis lung carcinoma cells.

Materials and methods

Cells and culture conditions. Mouse Lewis lung carcinoma (LLC) cells were maintained in Dulbecco's modified Eagle's medium containing a high level of glucose supplemented with 10% fetal calf serum (FCS) and 1% penicillin–streptomycin. The mouse LLC cell line was a gift from Dr. Shibuya, Tokyo University [10]. Hypoxia was achieved by incubating cells with 1% O_2 and 5% CO_2 in a Multigas Incubator (Astec, Fukuoka, Japan). The number of cells was counted in triplicate at each time point, under a microscope using a hemocytometer. Dead cells were excluded by Trypan blue staining. In the text, 'high density' of cells indicates a cell density of more than 130,000 cells per cm^2. LY294002 and rapamycin were purchased from Wako (Osaka, Japan).

Cell death assays. Release of lactate dehydrogenase (LDH) from dead cells into medium was measured with a CytoTox 96 Non-Radioactive Cytotoxicity assay (Promega, Madison, WI), according to the manufacturer's instruction. Propidium iodide (PI) and Hoechst 33342 staining was performed as described previously [11]. Western blotting of caspase-3 and its cleaved active form was performed as described below.

Cell-cycle analysis. Cells were harvested by centrifugation, washed with PBS, resuspended in ice-cold 70% ethanol in PBS, and stored at −20 °C for more than 30 min. After washing in PBS, cells were filtered through a 40-μm nylon mesh, and the solution was mixed with an equal volume of 2× fluorochrome solution (100 μg/ml PI in PBS with sodium citrate 0.2%, Triton X-100 0.2%, and RNase 0.5%) for 20 min at 4 °C. DNA content was analyzed by flow cytometry (FACS-Calibur) using CELLQuest software (Becton–Dickinson).

Measurement of intracellular ATP, medium pH, and glucose. Intracellular ATP levels were measured using a CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI), according to the manufacturer's instructions. The number of viable cells was counted with a hemocytometer. Total amount of ATP was divided by cell number to obtain the intracellular ATP level per cell. The pH and glucose concentration of the culture medium were measured with an ABL 700 (Radiometer, Copenhagen, Denmark).

Immunoblotting. Cells were plated in a 6-cm dish at a density of 1×10^6 cells in 3 ml of culture medium, and cultured under normoxic or hypoxic conditions for 24 h, at which time the cells reached 'high density.' Immunoblotting was performed as previously described [12]. Antibodies to mTOR, p-mTOR (Ser2448, Ser2481), AMPK, p-AMPK (Thr172), S6K, p-S6K (Thr389, Thr421/Ser424), S6, p-S6 (Ser235/236), 4E-BP1, p-4E-BP1 (Thr37/Thr46), caspase-3, and active caspase-3 were purchased from Cell Signaling Technologies (Beverly, MA), flag and β-actin were from Sigma (Saint Louis, MO), and mouse and rabbit secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). ECL reagents were obtained from Amersham Biosciences. Levels of protein expression were quantitatively estimated by densitometric scanning using a 1200 dpi flat-bed scanner with NIH Image 1.55f. Each protein concentration was normalized using β-actin as an internal control for loading.

Plasmid construction. Flag-tagged p70ΔCT104, which lacks the autoinhibitory c-terminus domain of S6K [13], was subcloned into the *Pst*I–*Xba*I site of pBSII(SK−). To substitute for glutamate threonine^{389}, which is known to be a site of autophosphorylation and to be critical for S6K activity [14], a two base-pair mutation was introduced with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), with the oligonucleotides 5′-gtctttctgggttttGAatatgtggctc catc-3′ and 5′-gatggagccacatatTCaaaacccagaaagac-3′, to generate pBSII/flag-p70ΔCT104-E389. The entire sequence was verified by sequencing the double-stranded DNA. The *Sal*I–*Not*I fragment was subcloned into the *Xho*I–*Not*I site of a retroviral expression vector, pMXsPuro. This construct, pMXsPuro/flag-p70ΔCT104-E389, is designated S6K-CA in the text. Transfection of the retroviral vector was performed as described previously [15].

Statistical analysis. Statistical analysis was performed with GraphPad Prism 4 (GraphPad Software, San Diego, CA). The statistical significance of results was tested with an unpaired t test or one-way ANOVA, depending on the hypothesis. Findings of $p < 0.01$ were considered significant.

Results

LLC cells died as a result of necrosis under conditions of prolonged hypoxia

The proliferation and death of LLC cells were examined under normoxic and hypoxic conditions (Fig. 1A). Although hypoxia is known to induce cell-cycle arrest for a wide range of types of cells and culture conditions, proliferation of LLC cells under hypoxic conditions (1% atmosphere) was the same as under normoxic conditions until the cells reached high density. After the cells reached 'high density' (see Materials and methods), their number plateaued under normoxic conditions, while the number of viable cells abruptly decreased with massive cell death under hypoxic conditions. Hypoxia-induced cell death was confirmed by lactate dehydrogenase (LDH) release (Fig. 1B). On day 5, significant LDH release was observed under hypoxic conditions, but remained low under normoxic conditions. To examine the characteristics of the cell death induced by hypoxia, we stained the non-fixed cells with propidium iodide (PI) and Hoechst 33342 dye (Fig. 1C). On day 5 under hypoxic conditions, about 70% of the LLC cells exhibited the staining pattern typically associated with necrotic cell death: PI-positive with intact and rounded nuclei.

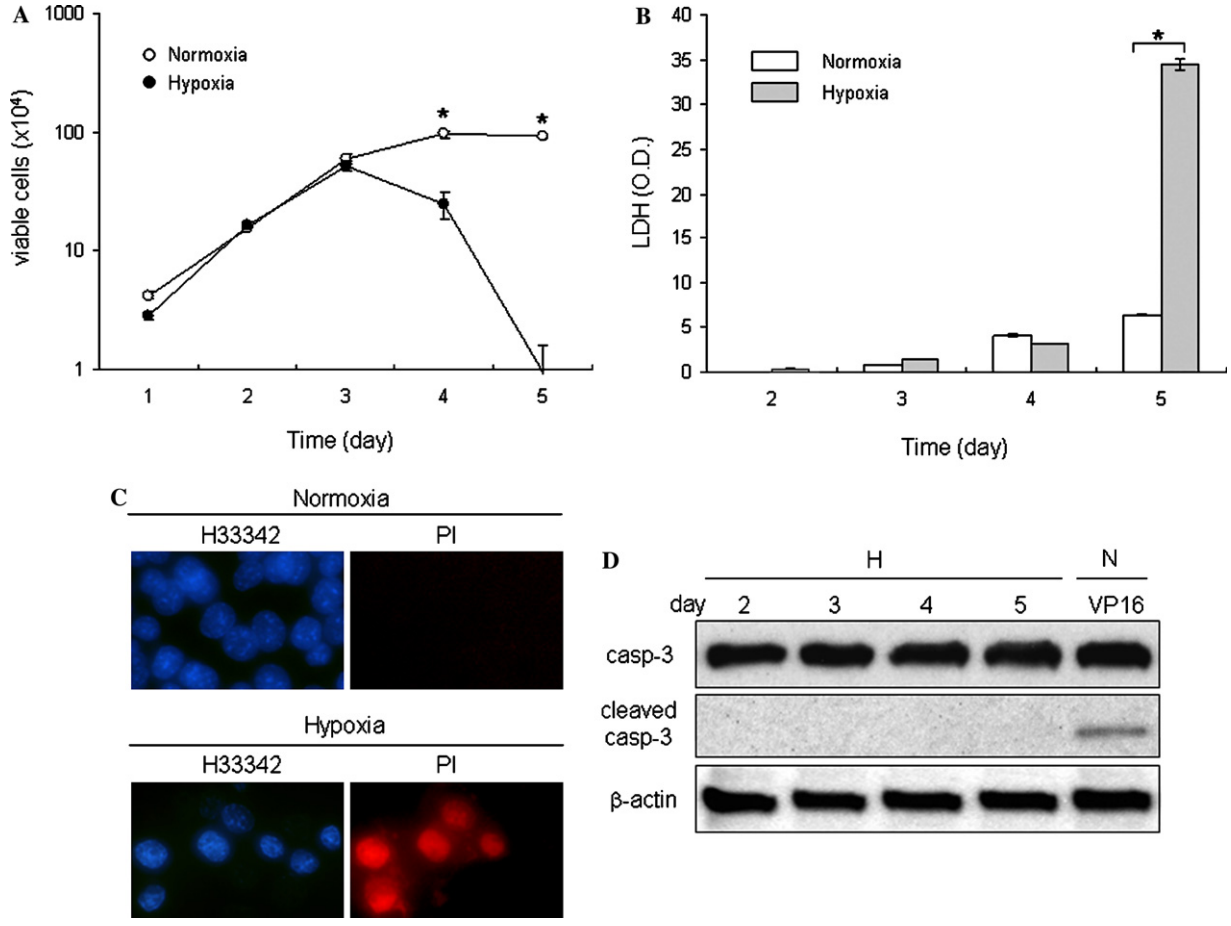

Fig. 1. LLC cells died due to necrosis under conditions of prolonged hypoxia. (A) Time-course changes in cell count. Cells were plated on 12-well plates in triplicate at a density of 1.5×10^4 cells per well in 1 ml of culture medium, and then cultured under normoxic or hypoxic conditions, as indicated in the figure. The number of viable cells was counted: normoxia (open circles); hypoxia (closed circles). (B) The supernatant in (A) was subjected to lactate dehydrogenase (LDH) measurement. OD values are shown: normoxia (open bars); hypoxia (gray bars). The experiment was repeated three times, and mean values (±SD) are shown. Asterisks indicate statistical significance ($p < 0.05$). (C) Morphological changes of LLC cells at day 5 were assessed by fluorescence microscopy with propidium iodide (PI) and Hoechst 33342 staining. Notably, in the panel showing Hoechst 33342 staining of LLC cells under hypoxia, nuclear morphology is the same in PI-positive and -negative cells. (D) Lysates of the cells in (A) were subjected to Western blotting analysis to detect the cleaved (active) form of caspase-3 (casp-3), an indicator of apoptosis. Lysate of LLC cells treated with etoposide (VP-16) is shown as a positive control for apoptosis. N, normoxia; H, hypoxia.

Typical apoptotic cells with condensed and irregularly shaped nuclei were not observed. Consistent with these findings, the cleaved form of caspase-3, an indicator of caspase-cascade activation in apoptosis, did not appear in LLC cells during hypoxic culture (Fig. 1D). Apoptotic cells, which should be Annexin V-positive but negative for PI staining, were rarely observed on flow cytometry analysis (data not shown). These results indicate that prolonged hypoxia-induced death of LLC cells occurred as a result of necrosis and not through typical apoptosis.

Phosphorylation of S6K and S6 was suppressed under conditions of hypoxia

Since PI3K and mTOR have been suggested to be involved in the cellular response to hypoxic stress [16,9], we examined the status of the PI3K/mTOR pathway in LLC cells (Fig. 2A). No difference was observed in AKT/PKB, a downstream molecule of PI3K pathway, in either protein level or extent of phosphorylation. Phosphorylation of mTOR at Ser2448 was marginally lower under hypoxic conditions than under normoxic conditions, but no difference in phosphorylation was observed at Ser2481. Two downstream molecules in the mTOR pathway were examined: ribosomal p70 S6 kinase (S6K) and eukaryotic initiation factor 4E-binding protein (4E-BP1). Under hypoxic conditions, levels of S6K phosphorylation were decreased at Thr421/Ser424 as well as Thr389 (Figs. 2A and B). Phosphorylation of ribosomal protein S6, a substrate of S6K, was suppressed under hypoxic conditions. No significant difference was observed in either protein level or extent of phosphorylation of 4E-BP1. Taken together, these find-

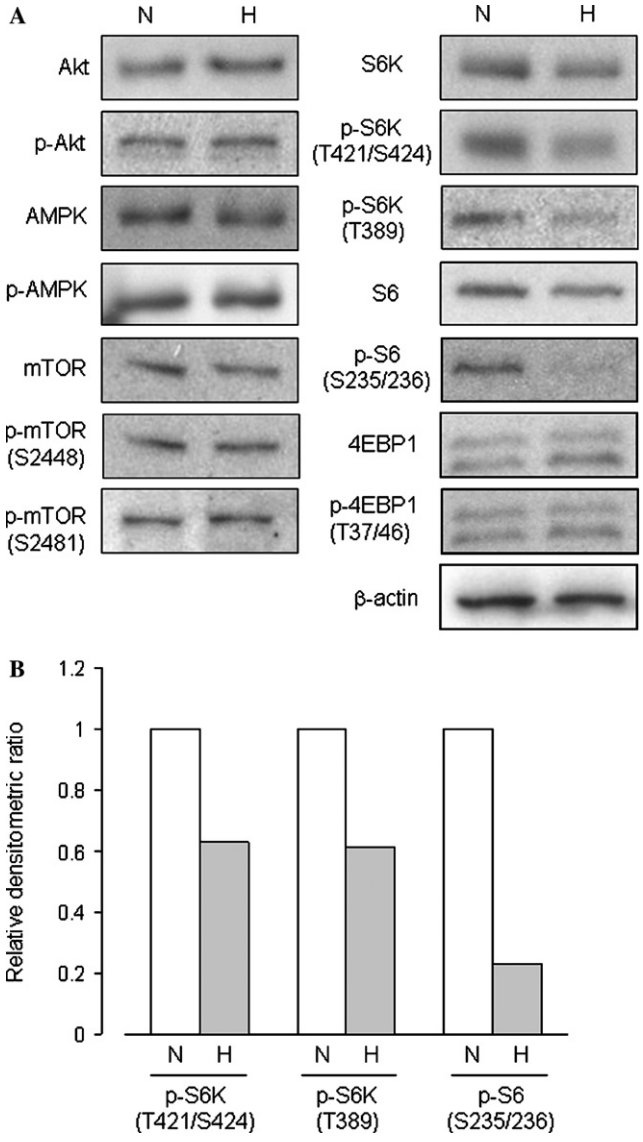

Fig. 2. Phosphorylation of S6K and S6 was suppressed under conditions of hypoxia. LLC cells at 'high density' were subjected to immunoblotting analysis. (A) Western blotting with the antibodies indicated was performed with cell lysates. (B) Quantification of immunoblots. The relative intensities of the indicated phosphoproteins to those of normoxic cells are shown. The experiment was repeated three times, and representative results are shown.

ings suggest that although levels of upstream molecules of the PI3K/mTOR pathway, such as AKT/PKB and mTOR, were not remarkably suppressed under hypoxic conditions, phosphorylation of downstream molecules such as S6K and its substrate S6 was clearly suppressed in LLC cells.

Overexpression of S6K active form sensitizes LLC cells to prolonged hypoxia

To assess the role of suppression of phosphorylation of S6K and S6 in determination of cell survival or death under hypoxic conditions, we introduced an active mutant of S6K, S6K-CA, into LLC cells by retroviral vector, as confirmed by flag tag (Fig. 3A). To avoid the differences in cell number after a few days of culture under different conditions, cells were prepared at 'high density.' The effect of transfection was assessed by increase in phosphorylation of ribosomal protein S6. Phosphorylation of S6 was suppressed under hypoxic conditions, whereas overexpression of S6K-CA maintained S6 phosphorylation at higher levels in hypoxia. We then examined the death of these cells under hypoxic conditions. Intriguingly, under hypoxic conditions, the cells with high levels of S6K activation died to a significantly greater extent than did control vector-transfected cells at day 5 (Fig. 3B), while S6K overexpression did not accelerate cell death under normoxic conditions.

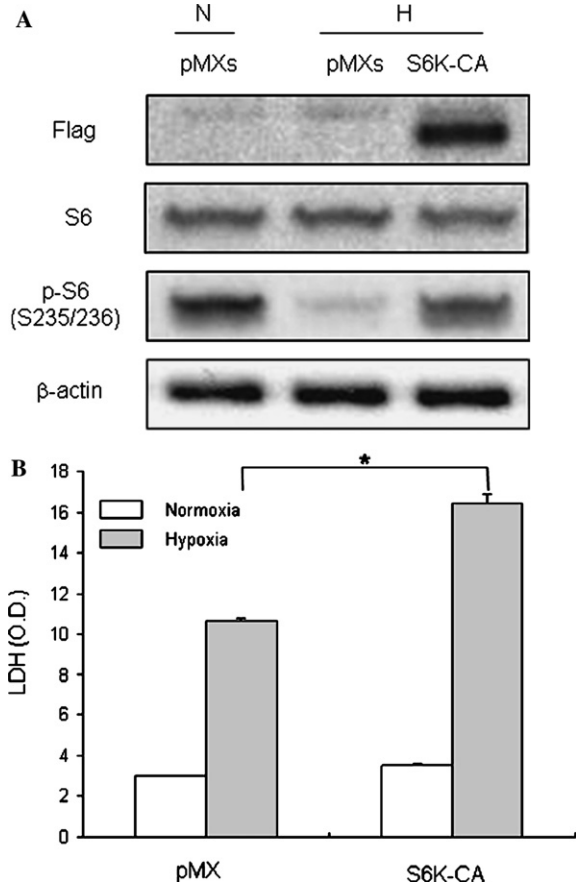

Fig. 3. Overexpression of S6K active form sensitized LLC cells to hypoxia. The cells were plated on 12-well plates at a density of 1.5×10^4 in triplicate and incubated under normoxic conditions for 24 h at 37 °C. The cells were then cultured under normoxic or hypoxic conditions until day 5. (A) Western blots were performed with the antibodies indicated. (B) The supernatant of the culture medium in (A) was subjected to LDH measurement. N, normoxia; H, hypoxia. Each experiment was repeated three times, and mean values (±SD) are shown. Asterisks indicate statistical significance ($p < 0.05$).

Inhibition of the PI3K or mTOR pathway rescued LLC cells from death induced by prolonged hypoxia

To examine whether suppression of S6K activity contributes to cell survival under hypoxic conditions, PI3K and mTOR, which regulate phosphorylation of S6K and consequently S6, were inhibited by specific inhibitors. LLC cells were treated with LY294002 or rapamycin, specific inhibitors of PI3K and mTOR, respectively. LY294002 suppressed phosphorylation of AKT, S6K, and S6 (Fig. 4A), and rapamycin suppressed phosphorylation of S6K and S6. We then examined the effects of LY294002 and rapamycin on cell survival under hypoxia. As assessed morphologically (Fig. 4B) and by LDH release (Fig. 4C), LLC cells treated with LY294002 or rapamycin were intact after 48 h of culture under hypoxic conditions, while most of the LLC cells without such treatment had died by this time point.

Inhibition of PI3K or mTOR increased levels of intracellular ATP and slowed decreases in pH and glucose level in culture medium

Necrosis occurs when intracellular energy is depleted [17]. Since the death of LLC cells under hypoxic conditions was due to necrosis, we examined the change in intracellular ATP levels induced by inhibition of PI3K or mTOR (Fig. 5A). Under hypoxic conditions, intracellular ATP levels were significantly lower than under normoxic conditions. With treatment with LY294002 or rapamycin, intracellular ATP levels of the cells under hypoxic conditions remained at the levels present under normoxic conditions.

Schmaltz et al. [18] reported that marked acidosis is required for hypoxia-induced cell death, and we therefore examined the effects of inhibition of PI3K or mTOR on pH levels in culture medium (Fig. 5B). Under hypoxic conditions, the decrease in pH level was similar to that under

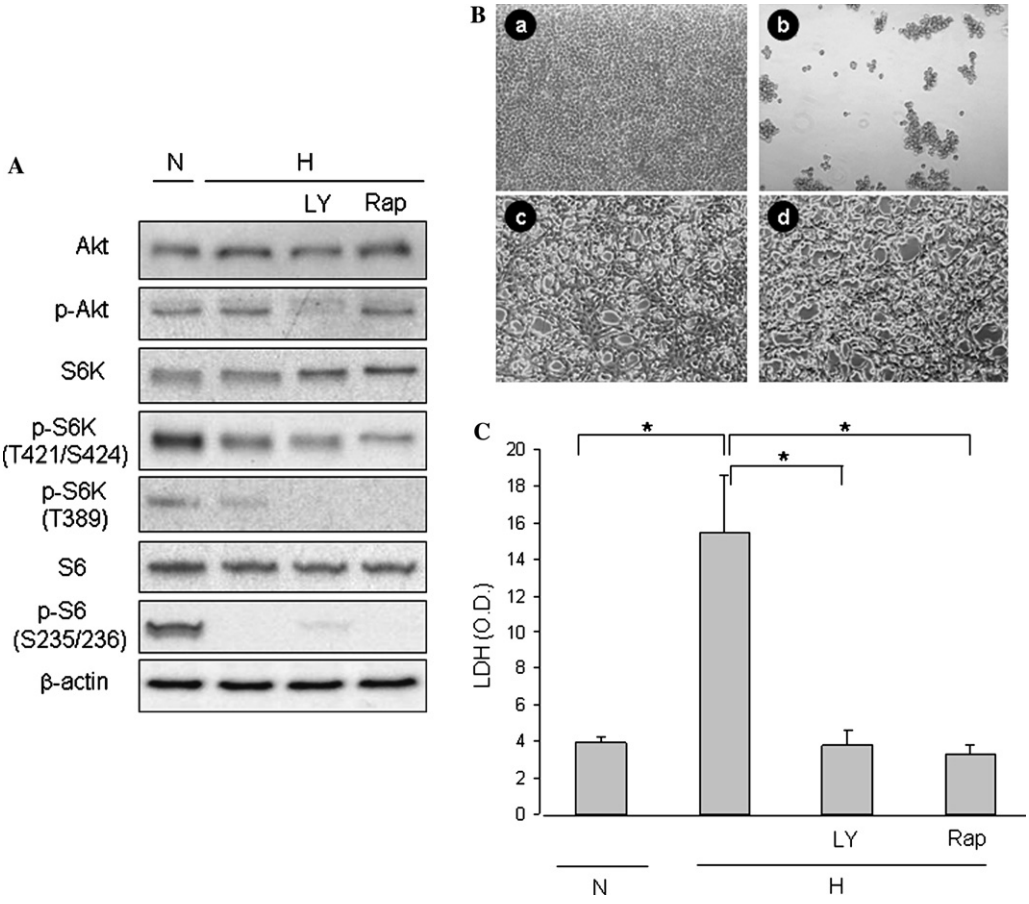

Fig. 4. Inhibition of PI3K or mTOR pathway rescues LLC cells from death induced by prolonged hypoxia. Cells were plated on 12-well plates at a density of 3×10^5 in triplicate and incubated under normoxic conditions for 24 h at 37 °C. The cells were then cultured with or without LY294002 (10 μM) or rapamycin (20 nM) under normoxic or hypoxic conditions for an additional 48 h. (A) Western blotting of the cells was performed with the antibodies indicated. (B) Phase-contrast pictures of the cells: B-a, under normoxic conditions; B-b, under hypoxic conditions; B-c, treated with LY294002 under hypoxic conditions; and B-d, treated with rapamycin under hypoxic conditions. (C) The supernatant of the culture medium in (A) was subjected to LDH measurement. N, normoxia; H, hypoxia; LY, LY294002; and Rap, rapamycin. Each experiment was repeated three times, and mean values (±SD) are shown. Asterisks indicate statistical significance ($p < 0.05$).

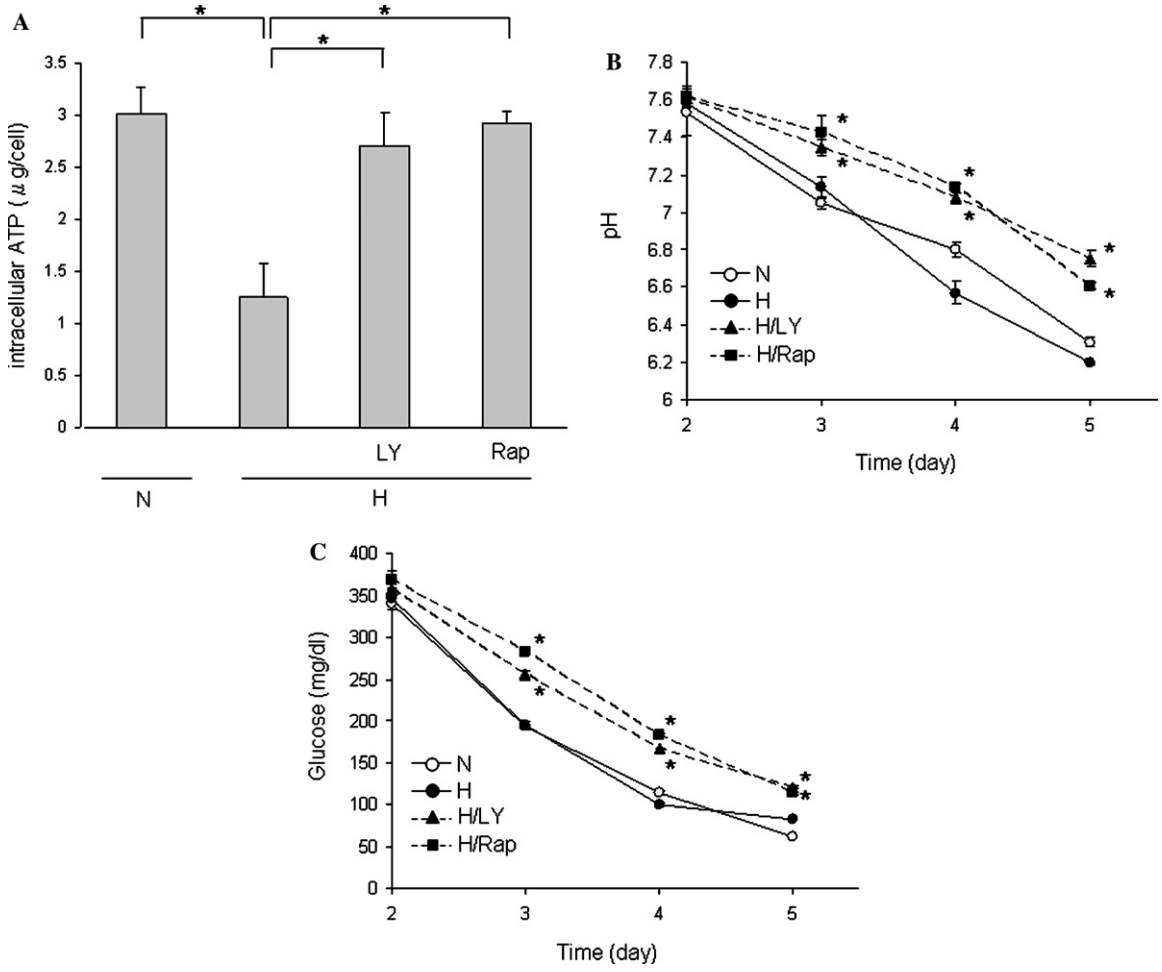

Fig. 5. Inhibition of PI3K or mTOR increased levels of intracellular ATP and delayed decreases in pH and glucose level in culture medium. Cells were plated on 12-well plates at a density of 3×10^5 in triplicate and incubated under normoxic conditions for 24 h at 37 °C. The cells were then cultured with or without LY: LY294002 (10 μM) or Rap: rapamycin (20 nM) under normoxic (N) or hypoxic (H) conditions for the indicated periods. Intracellular ATP levels (μg/cell) on day 5 (A); kinetics of pH (B) and of glucose levels (mg/dl) (C) of the culture medium under normoxic or hypoxic conditions, with or without treatment with LY294002 or rapamycin. Each experiment was repeated three times, and mean values (±SD) are shown. Asterisks indicate statistical significance when compared with the value from the cells incubated under hypoxic conditions ($p < 0.05$).

normoxic conditions. In contrast, the decrease in pH levels of cells treated with PI3K or mTOR inhibitors was significantly delayed. Notably, the pH levels of the treated cells on day 5, when such cells were intact, were as low as those of untreated cells at day 4, when untreated cells started to die. Inhibition of PI3K or mTOR also significantly delayed the decrease in glucose levels in culture medium under normoxic conditions (Fig. 5C). Taken together, these results indicate that LLC cells treated with PI3K or mTOR inhibitor survived under hypoxic conditions with conservation of intracellular ATP, less consumption of glucose, and delay of decrease in extracellular pH, probably due to lesser amounts of lactate production.

Inhibition of neither PI3K nor mTOR affected the cell cycle at high cell density under conditions of hypoxia

As shown in Fig. 1A, LLC cells proliferated under hypoxic conditions at the same rate as under normoxic conditions in the logarithmic growth phase. We examined the cell-cycle pattern at high cell density under normoxic and hypoxic conditions (Table 1). The popu-

Table 1
Inhibition of neither PI3K nor mTOR affected the cell cycle under hypoxia

Normoxia	Hypoxia		
None	None	LY294002	Rapamycin
56.55 ± 1.97	60.62 ± 0.18*	60.00 ± 1.21	61.86 ± 1.80 (%)

Cells were plated on 6-cm dishes in triplicate at a density of 3×10^5 cells per well in 3 ml of culture medium, and analyzed after 48 h incubation under normoxic or hypoxic conditions, with or without indicated inhibitor treatment. LY, LY294002; Rap, rapamycin. Ratio of G0/G1 populations from DNA content analysis is shown. The ratio of number of cells in G0/G1 region to total number of cells is shown as a percentage. Each experiment was repeated three times and mean values (±SD) are shown. Asterisks indicate statistical significance when compared with the value from the cells incubated under normoxic conditions ($p < 0.05$).

lation of cells in G0/G1 phase was slightly larger under hypoxic than under normoxic conditions. Intriguingly, cells treated with neither PI3K nor mTOR exhibited additive cell-cycle arrest under hypoxic conditions. These results indicate that the conservation of intracellular energy levels by PI3K and mTOR inhibitors is not due to suppression of cell-cycle progression.

Discussion

In this study, we found, using hypoxia-sensitive mouse Lewis lung carcinoma (LLC) cells, that suppression of the PI3K and mTOR pathway rescued cancer cells from hypoxia-induced necrotic cell death.

Hypoxia can induce either necrosis or apoptosis in cancer cells, depending on the situation [19,17]. In our study, the morphological characteristics of hypoxia-sensitive LLC cells following hypoxia-induced cell death were mostly compatible with necrosis. Low levels of intracellular ATP might be a determinant of type of death, and thus when tumor cells maintain oxygen consumption under hypoxic conditions, necrosis is likely to be the major cause of cell death, rather than apoptosis. Whether human cancer cells acquire the ability to evade necrosis as well as apoptosis needs to be determined.

The finding of dramatic suppression of sensitivity to hypoxia in LLC cells by LY294002 and rapamycin in this study indicates that PI3K and mTOR signaling is involved in resistance to hypoxia. Neither LY294002 nor rapamycin was toxic to LLC cells under hypoxic conditions, as assessed by clonogenic survival assay (data not shown). Since mTOR is a molecule downstream of PI3K, suppression of mTOR signaling might be a common pathway for resistance to hypoxia. The reason why LLC cells are hypoxia-sensitive might be that they are not able to effectively down-regulate mTOR signaling in response to hypoxic conditions. In contrast to the apparent inhibition of S6K phosphorylation in LLC cells under hypoxic conditions, we observed no change in mTOR phosphorylation at Ser2481, which is an autophosphorylation site and is down-regulated in HEK293 cells under hypoxic conditions [9]. Moreover, hypoxia did not alter phosphorylation levels of 4E-BP1, another molecule downstream of mTOR, in LLC cells. Thus, S6K down-regulation alone might not be sufficient for acquisition of resistance to hypoxia by LLC cells. It remains unclear whether suppressing the mTOR pathway is the only strategy for inducing resistance to hypoxia. Since S6K and eIF4E are regulated through multiple pathways [20], upstream molecules other than mTOR might also play roles in the resistance to hypoxia due to PI3K inhibition.

Since mTOR is situated in the center of a pathway controlling protein synthesis, and is suppressed by hypoxia [9], as well as by depletion of glucose and amino acids [21,22], it is possible that suppression of mTOR activity by low oxygen tension is a survival signal for normal cells to switch to a low-energy-consumption state. Inoki et al. [23] showed that uncontrolled high mTOR activity in fibroblasts due to lack of the TSC2 gene, which is activated by AMPK and suppresses mTOR signaling, is responsible for cell death under conditions of starvation. In addition, upstream signaling molecules in the mTOR pathway, such as the IGF receptor and PI3K, have been shown to be associated with sensitivity to starvation or hypoxia in other species: *C. elegans* with a hypomorphic mutant IGF receptor (daf-2) exhibits a high level of resistance to hypoxia [16], and *Drosophila* with an ectopic insulin receptor or PI3K is starvation-sensitive [24].

Unlike normal cells, cancer cells are thought to proliferate without responding to inhibitory cues related to nutrient or oxygen levels. Since it seems likely that the mTOR pathway is essential in maintaining this transformed phenotype, specific rapamycin-derivative inhibitors of mTOR are under clinical evaluation [25,26]. It is also true that the majority of the surviving cells in severely hypoxic regions in a tumor are not actively dividing [27]. It is possible that cancer cells survive in highly challenging microenvironments by transient suppression of mTOR signaling and its associated conservation of energy. If this hypothesis were correct, inhibition of mTOR might favor the survival of cells in hypoxic regions, as shown in this study. Hence, contrary to attempts to suppress mTOR in cancer therapy, forced activation of mTOR signaling might be used to target hypoxic cancer cells, as suggested by this study. Indeed, inhibition of AMP-activated protein kinase (AMPK) by antisense RNA expression vectors in pancreatic cancer cell lines has been shown to significantly inhibit tumor growth in nude mice [28]. Since AMPK negatively regulates mTOR signaling, the effect of AMPK inhibition should be mediated via activation of mTOR signaling. To avoid accelerating cancer cell growth in the relatively favorable microenvironment present in a tumor, it may be advantageous to use a drug delivery system, as we have recently reported [15,29], to confine mTOR activation to the hypoxic fraction.

The role of cell-cycle arrest in the mechanism of tolerance to hypoxia is controversial. Since cell-cycle progression is an energy-consuming process, suppression of cell-cycle progression is likely to be essential for development of resistance to hypoxia. Gardner et al. [30] reported that a cyclin-dependent kinase inhibitor, p27/KIP1, is induced by hypoxia, and that reduction or absence of p27 abrogates the hypoxia-induced G1 checkpoint. Hence, p27/KIPI may be a key molecule in the suppression of cell-cycle progression under hypoxic conditions. The stability of p27 is also enhanced by suppression of TOR signaling [31], which suggests a relationship between suppression of mTOR signaling

and cell-cycle arrest under hypoxic conditions. Masuda et al. [32] showed that survival of a small cell lung cancer cell line under unfavorable culture conditions, such as lack of nutrients and hypoxia, is enhanced by induction of p27 and suppressed by treatment with antisense oligonucleotides against p27. On the other hand, we show here that suppression of the PI3K or mTOR pathway rendered LLC cells hypoxia-resistant without remarkably affecting the cell cycle. Our result is consistent with a recent report showing that the cell-protective effect of EGFR inhibition under hypoxic conditions is not accompanied by alteration of the cell-cycle distribution [6]. Taken together, these findings suggest that cell-cycle arrest might be necessary but not enough for tolerance to hypoxia. To acquire hypoxia resistance, other mechanisms such as suppression of anabolism that are regulated by PI3K/mTOR pathway might be required.

In conclusion, our results indicate that mTOR signaling plays an important role in resistance to hypoxic stress. The present study used a murine tumor cell line in vitro, and further investigation will be necessary to extend our results to tumor cells in vivo, especially those of human cancers.

Acknowledgments

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