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Advances in Brief

Inhibition of Epidermal Growth Factor Receptor Signaling Protects Human Malignant Glioma Cells from Hypoxia-Induced Cell Death

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Abstract

Epidermal growth factor receptor (EGFR) signaling has become an important target for drug development because EGFR signaling enhances tumor cell proliferation, migration, and invasion and inhibits apoptosis. However, the results of clinical trials using EGFR inhibitors in patients with solid tumors have been disappointing. Here, we report a protective effect of the EGFR inhibitors AG1478 and PD153035 against cell death induced by acute hypoxia, which contrasts with their proapoptotic effects under normoxia. Under hypoxic conditions, both agents reduced glucose consumption, delayed ATP depletion, and preserved the mitochondrial membrane potential. Exposure either to hypoxia or the EGFR inhibitors under normoxic conditions resulted in the dephosphorylation of ribosomal protein S6, a player in the energy and nutrient-sensing pathway governed by mammalian target-of-rapamycin (mTOR). Combined inhibition of phosphatidylinositol 3′-kinase (PI3K) and extracellular signal-regulated kinase-1/2 (ERK1/2) mimicked the protective effects of EGFR inhibition on hypoxia-induced cell death and protein S6 dephosphorylation. These results caution that therapies targeting EGFR signaling pathways can protect tumor cells from acute hypoxia.

Introduction

Epidermal growth factor receptor (EGFR) amplification and overexpression are observed in the majority of *de novo* (primary) glioblastomas (1, 2) and associated with a worse prognosis especially among younger patients (3, 4). Under normoxic conditions, EGFR signaling may enhance proliferation, invasion, and migration of glioma cells (5). Furthermore, EGFR signaling has antiapoptotic properties (6–8), *e.g.,* the pharmacological inhibition of EGFR sensitizes human malignant glioma cells to death ligand-induced apoptosis (9). Effects of EGFR on proliferation and survival are thought to be mediated by the mitogen-activated protein kinase (MAPK) and PI3K/Akt pathways, respectively (7, 8, 10). Several small molecule inhibitors of EGFR and humanized monoclonal antibodies to EGFR have been developed for clinical use (11). However, the results from clinical trials with EGFR inhibitors have been disappointing thus far. In particular, the combination of EGFR inhibitors with cytostatic drugs has failed to produce additive or synergistic results, which had been expected from preclinical studies, in several trials (12). However, these preclinical studies had been limited by a lack of attention to the special conditions of the tumor microenvironment. In particular, the role of tumor hypoxia has been disregarded (13). Large fields of necrosis are much more frequent in the subgroup of primary glioblastomas (2), the majority of which overexpress EGFR, and the presence of necrosis itself is also associated with a worse outcome (14). We have previously characterized a paradigm of cell death induced by acute hypoxia in human malignant glioma cells and demonstrated that cell death is mainly nonapoptotic because mitochondrial injury is decoupled from caspase activation under circumstances of energy deprivation (15). Here, we investigated the effects of EGFR inhibition on hypoxia-induced cell death in human malignant glioma cells *in vitro*.

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Materials and Methods

Reagents and Cell Lines. AG1478, U0126, LY294002, and EGF were purchased from Calbiochem (San Diego, CA). PD153035 was from Tocris Cookson (Bristol, United Kingdom). LN-T229 and U87MG human malignant glioma cell lines and LN-T229 cells transduced with wild-type (pLWERNL), constitutively active (pLERNL), and kinase-deficient (pLDKRNL) EGFR have been described previously (9). All chemicals not specified below were purchased from Sigma (Deisenhofen, Germany).

Induction of Hypoxia. Hypoxia was induced as described previously (15) by incubating cells in Gas Pak pouches for anaerobic culture (Becton-Dickinson, Heidelberg, Germany). In brief, cells were plated on 96-well plates (Falcon; Becton-Dickinson) at 2×10^4/well or Petri dishes of various sizes and allowed to attach in medium containing 10% FCS for 24 h. Then the medium was removed, and the cells were incubated in serum-free DMEM without glucose (Life Technologies, Inc., Basel, Switzerland) adjusted to 2 mM glucose under normoxia or hypoxia for the indicated lengths of time. The protective effect of EGFR inhibition was verified in a different system for induction of hypoxia, using sealed chambers flooded with a mixture of 200 parts/million O_2, 5% CO_2, and 95% argon. No decrease in pH <7.2 occurred under hypoxia. lactate dehydrogenase release assays and electron microscopy after treatment as indicated were performed as described previously (15).

ATP Assay. Immediately after hypoxia, the plates were placed on ice, and the cells were pelleted by centrifugation and lysed in ATP releasing agent (Sigma). ATP was determined by luciferase assay with the CLS II kit (Boehringer Mannheim, Mannheim, Germany) as described previously (15).

JC-1 Assay. After treatment as indicated, cells were washed in PBS and incubated for 15 min in JC-1 staining reagent (Biocarta, Hamburg, Germany) at 37°C. The cells were then washed once in assay buffer, resuspended in PBS, and analyzed by flow cytometry or immunofluorescence microscopy.

Glucose Assay. Immediately after hypoxia, the cells were pelleted by centrifugation, and the supernatant was assayed for glucose employing the Gluco-quant kit (Roche, Mannheim, Germany).

Cell Cycle Analysis. The cells were treated with vehicle or AG1478 and exposed to hypoxia or normoxia as indicated, washed in PBS, permeabilized in 75% ethanol overnight, and stained with propidium iodide (50 μg/ml) in the presence of RNase A (100 μg/ml; Roth, Karlsruhe, Germany) for 20 min at 4°C. DNA content was analyzed by flow cytometry on a fluorescence-activated cell sorter (FACS-calibur) employing Cell-Quest software (Becton-Dickinson).

Bromodeoxyuridine (BrdUrd) Assay. The cells were grown in 96-well plates. Immediately before the onset of hypoxia, the medium was removed, and the cells were incubated in medium with 2 mM glucose, vehicle or AG1478, and BrdUrd (10 μM). After the end of hypoxia, the cells were fixed, and BrdUrd incorporation was detected with the colorimetric cell proliferation ELISA (Roche).

Immunoblot Analysis. Immediately after hypoxia or normoxia, cells were washed with cold PBS and harvested into ice-cold PBS containing protease

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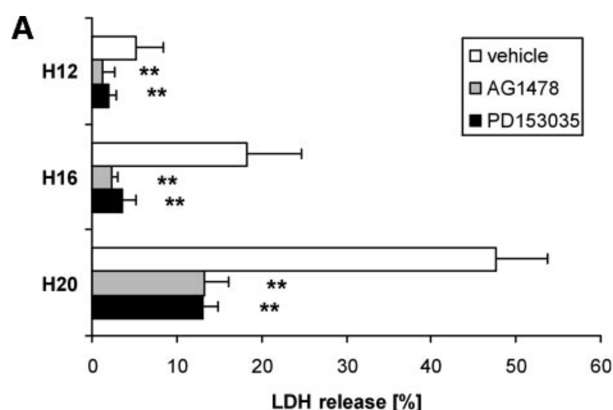
inhibitors. Cellular lysates were prepared as described previously (15) and subjected to SDS-PAGE analysis. Membranes were probed with an antibody to phospho-EGFR (Tyr1173; Biomol, Hamburg, Germany), stripped, and reprobed with anti-EGFR antibody (Santa Cruz Biotechnology, San Diego, CA). The secondary antibodies, protein A and antimouse IgG, were purchased from Amersham Biosciences (Braunschweig, Germany), and antigoat antibody was from Santa Cruz Biotechnology. Enhanced chemiluminescence (ECL+; Amersham Biosciences) was used for detection. Immunoblot for phospho-Akt (Ser473), phospho-p42/44 mitogen-activated protein kinase (Thr202/Tyr204), phospho-S6 (Ser235/236), and eIF4E (used here as internal standard for equal protein loading) was performed using the Pathscan Multiplex Western Cocktail I (Cell Signaling, Beverly, MA).

Results

EGFR Inhibition Protects Malignant Glioma Cells from Hypoxia-Induced Cell Death. To investigate a possible modulation by EGFR signaling of hypoxia-induced cell death, LN-T229 cells were incubated with tyrphostine AG1478 or the chemically unrelated EGFR inhibitor, PD153035 and subjected to hypoxia and partial glucose deprivation. Hypoxia-induced cell death as determined by lactate dehydrogenase release was significantly reduced by EGFR inhibition (Fig. 1*A*). Ultrastructural analysis corroborated the protective effect of EGFR inhibition. AG1478 alone did not alter the morphological features of LN-229 cells under normoxic conditions. After hypoxia for up to 12 h, AG1478 protected LN-229 cells from the morphological changes of hypoxic cell death while vehicle-treated cells underwent a mixed type of cell death with some features of apoptosis (nuclear chromatin condensation and margination and cytoplasmatic blebbing) and secondary necrosis with cytoplasmic vesiculation and vacuolization, organelle disruption, and cell lysis (Ref. 15; Fig. 1*B*). A protection from hypoxia-induced cell death by AG1478 was also evident in LN-T229 cells overexpressing wild-type EGFR and EGFR vIII, a constitutive active deletion mutant, and in U87MG malignant glioma cells (data not shown). LN-T229 cells were used to further characterize the hypoxia-resistant phenotype conferred by EGFR inhibition.

Glucose and ATP Depletion and Loss of the Mitochondrial Membrane Potential during Hypoxia Are Attenuated by EGFR Inhibition. Depletion of glucose and ATP and mitochondrial injury appear to be critical for hypoxic glioma cell death (15). Because EGFR signaling increases glucose consumption in cancer cells (16), EGFR inhibition was hypothesized to alter these processes: The depletion of glucose and ATP was significantly delayed by AG1478 and PD153035 (Fig. 2*A*). Furthermore, the hypoxia-induced decrease in the mitochondrial membrane potential was determined by staining with the potential-sensitive dye JC-1. Flow cytometric analysis demonstrated a decrease in red fluorescence (FL-2H) in mitochondria at H16. This corresponds to a shift of the color of mitochondria from red



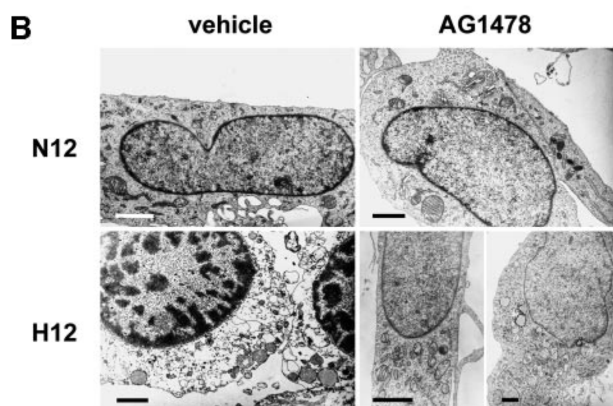


Fig. 1. EGFR inhibition protects malignant glioma cells from hypoxia-induced cell death. *A*, LN-T229 cells were exposed to vehicle, AG1478 (10 μM), or PD153035 (10 μM) and incubated under hypoxic conditions with 2 mM glucose for the indicated lengths of time. Cytotoxicity was assessed by lactate dehydrogenase (LDH) release assay ($n = 4$; **, $P < 0.001$; Student's *t* test). *B*, LN-T229 cells were exposed to vehicle or AG1478 (10 μM), incubated under normoxia or hypoxia with 2 mM glucose for 12 h, and processed for electron microscopy (*N12*, normoxia 12 h; *H12*, hypoxia 12 h; *scale bar* = 1 μM).

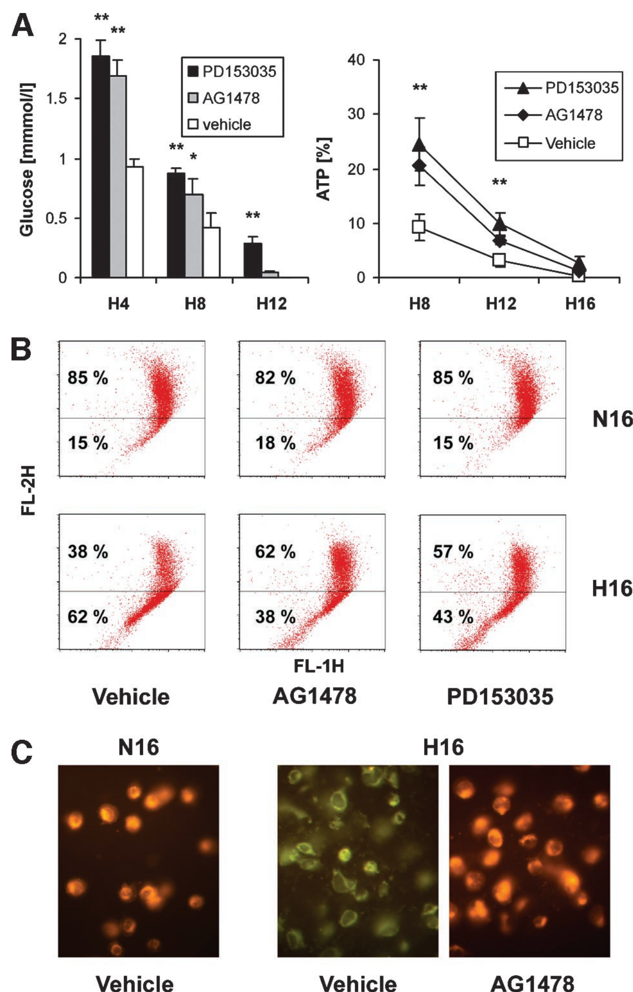


Fig. 2. Glucose and ATP depletion and mitochondrial membrane potential decline during hypoxia are attenuated by EGFR inhibition. *A*, LN-T229 cells were exposed to vehicle, AG1478 (10 μM), or PD153035 (10 μM) and incubated under hypoxia with 2 mM glucose. *Left*, glucose concentration in the supernatant was determined by enzymatic assay ($n = 3$; *, $P < 0.05$; **, $P < 0.001$; Student's *t* test). *Right*, cellular ATP was determined by luciferase assay ($n = 5$; **, $P < 0.001$; Student's *t* test). *B* and *C*, LN-229 cells were exposed to vehicle, AG1478 (10 μM), or PD153035 (10 μM) and incubated under hypoxia with 2 mM glucose for 16 h. Mitochondrial membrane potential was determined by JC-1 staining and analyzed by flow cytometry (*B*) or immunofluorescence microscopy (*C; N16*, normoxia 16 h; *H16*, hypoxia 16 h).

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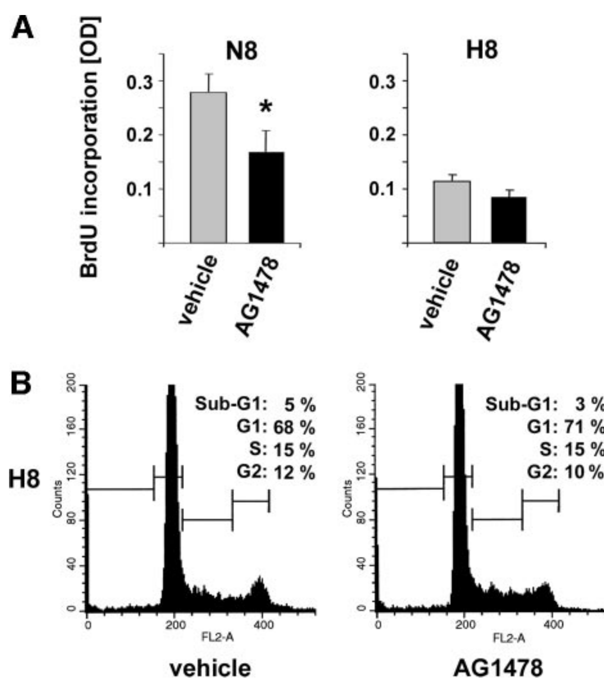

Fig. 3. EGFR inhibition does not alter DNA synthesis under hypoxia. *A,* LN-T229 cells were exposed to vehicle or AG1478 (10 μM) and incubated under normoxia or hypoxia with 2 mM glucose for 8 h in the presence of BrdUrd. BrdUrd incorporation was analyzed by ELISA (n = 5; *, P < 0.05, for the comparison of vehicle *versus* AG1478 under normoxia; Student's t test). *B,* LN-T229 cells were exposed to vehicle or AG1478 (10 μM), incubated under hypoxia with 2 mM glucose for 8 h, and prepared for cell cycle analysis by flow cytometry (*N8,* normoxia 8 h; *H8,* hypoxia 8 h).

to green as observed by fluorescence microscopy using a wide band-pass filter suitable for both fluorescein and rhodamine spectra and implies loss of mitochondrial membrane potential. EGFR inhibition attenuated the loss of the mitochondrial membrane potential for up to 16 h (Fig. 2, *B* and *C*)

EGFR Inhibition Does Not Alter DNA Synthesis under Hypoxia. Processes involved in DNA replication and mitosis are energy consuming, and cycling cells may be more vulnerable to cell death induction. We therefore analyzed the effect of EGFR inhibition on cell cycle distribution and DNA incorporation. Under normoxic conditions, a reduction in the rate of BrdUrd incorporation by EGFR inhibition was already apparent after 8 h. Hypoxia suppressed DNA synthesis as determined by BrdUrd incorporation, but there was no significant additional reduction by EGFR inhibition (Fig. 3*A*). Cell cycle analysis indicated a slight reduction of cells in G_2 phase and a slight increase in cells in $G_{0/1}$ phase at H8 in the presence of EGFR inhibitors (Fig. 3*B*), but the cell cycle distribution was not altered by hypoxia itself for 8 h (data not shown). Because many cell cycle-associated energy consuming processes, in particular protein synthesis, are initiated before DNA replication (*e.g.,* in G_1), these may be sensitive to EGFR inhibition in the absence of an effect on DNA synthesis.

Inhibition of PI3K and ERK1/2 Mimics the Effect of EGFR Inhibition on Hypoxia-Induced Cell Death. To identify downstream steps in the signaling cascade initiated by EGFR that may regulate the sensitivity to hypoxic cell death, we assessed the effects of the PI3K inhibitor, LY294002, and the ERK1/2 inhibitor, U0126, on glioma cell death induced by acute hypoxia. Both agents inhibited hypoxia-induced cell death substantially. When LY294002 and U0126 were combined, they afforded a degree of protection similar to EGFR inhibition (Fig. 4*A*). However, only the inhibition of PI3K with LY294002 reduced glucose consumption to a similar degree as AG1478, whereas ERK1/2 inhibition with U0126 had no effect on glucose levels (Fig. 4*B*). We then proceeded to investigate the patterns of protein kinase phosphorylation during hypoxia (Fig. 4*C*). First, we verified the presence of EGFR phosphorylation in the absence of stimulation with serum or exogenous ligands (Fig. 4*C, top panels*), which was only slightly decreased compared with normoxia. We then

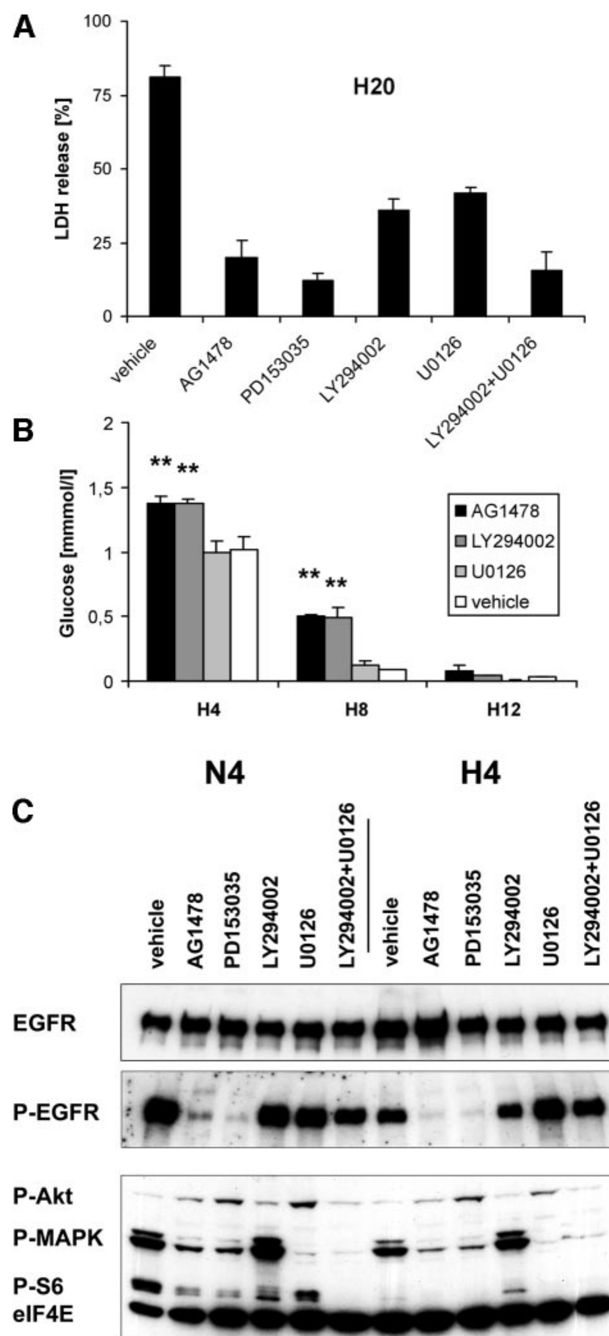

Fig. 4. PI3K and ERK1/2 inhibition mimic the effect of EGFR inhibition on hypoxia-induced cell death. *A,* LN-229 cells were exposed to vehicle, AG1478 (10 μM), PD153035 (10 μM), LY294002 (10 μM), U0126 (20 μM), or LY294002 and U0126 and incubated under hypoxia with 2 mM glucose for 20 h. Cytotoxicity was assessed by lactate dehydrogenase (LDH) release assay (n = 4, P < 0.001, for all treatments *versus* vehicle, Student's t test). *B,* LN-229 cells were exposed to vehicle, AG1478 (10 μM), LY294002 (10 μM), or U0126 (20 μM) and incubated under hypoxia with 2 mM glucose for the indicated lengths of time. Glucose concentration in the supernatant was determined by enzymatic assay (n = 3; **, P < 0.001; Student's t test). *C,* LN-229 cells were exposed to vehicle, AG1478 (10 μM), PD153035 (10 μM), LY294002 (10 μM), U0126 (20 μM), or LY294002 and U0126 and incubated under normoxia or hypoxia with 2 mM glucose for 4 h. Cellular lysates were analyzed by immunoblot with antibodies to *EGFR*, phospho-EGFR (*P-EGFR*), phospho-Akt (*P-Akt*), phospho-p42/p44 mitogen-activated protein kinase (*P-MAPK*), phospho-S6 (*P-S6*), and *eIF4E* (*N4,* normoxia 4 h; *H4,* hypoxia 4 h).

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investigated the pattern of the downstream signaling components phospho-Akt, phospho-p42/44 MAPK, and phospho-S6 (Fig. 4*C, bottom panel*). After 4 h, the most prominent effect of hypoxia was the dephosphorylation of protein S6. Inhibition of EGFR or PI3K at normoxic conditions also resulted in decreased S6 phosphorylation, whereas ERK1/2 inhibition alone had only a slight effect on S6 phosphorylation. Combined inhibition of PI3K and ERK1/2 abolished S6 phosphorylation. Of note, Akt phosphorylation appeared to be up-regulated at 4 h after treatment with EGFR inhibitors or the ERK1/2 inhibitor both under normoxic and hypoxic culture conditions. This finding may reflect compensatory signaling (*e.g.,* via insulin-like growth factor receptor) because Akt phosphorylation is initially abolished by EGFR inhibitors (not shown).

Discussion

The present study demonstrates that the inhibition of EGFR signaling protects human malignant glioma cells from cell death induced by acute hypoxia. This effect contrasts with antiapoptotic properties of the EGFR in other paradigms (7, 9, 17, 18). We hypothesize that the maintenance of energy-consuming cellular processes by EGFR-dependent signal transduction accelerates the depletion of energy resources under conditions of acute hypoxia and reduced nutrient supply, thereby sensitizing glioma cells toward cell death.

How does inhibition of EGFR signaling affect the consumption of oxygen and nutrients under hypoxic conditions? Besides detecting hypoxia *per se, e.g.,* by stabilization of HIF-1α, cells also sense starvation-like signals such as deprivation of glucose, amino acids, or ATP and generate adaptive responses such as reduced synthesis of proteins, lipids, and nucleic acids and increased nutrient catabolism. The mTOR protein kinase, a target of growth factor signaling via PI3K and Akt, has been implicated as such an energy and nutrient sensor (19, 20), and the ribosomal protein S6 is one possible effector of altered translation of target genes involved in metabolic control. In support of this model, inhibition of mTOR signaling protects from glucose deprivation-induced cell death (21). EGFR inhibition may mimic such a starvation signal and thereby prepare cells for the forthcoming hypoxic and hypoglycemic conditions. These considerations are well compatible with our data demonstrating S6 dephosphorylation in response to EGFR inhibition (Fig. 4*C*). Converging pathways originating both from the PI3K-Akt-mTOR cascade and ERK1/2-MAPK-ribosomal S6 protein kinase $p90^{RSK}$ signaling may contribute to S6 phosphorylation, thus explaining the necessity of combined inhibition of both pathways to match the efficacy of EGFR inhibition.

Inhibition of ERK1/2 may also have specific protective effects distinct from decreased nutrient consumption, possibly related to cell cycle-associated changes, because the effect on glucose depletion appeared to be specific for PI3K inhibition (Fig. 4*B*). The importance of growth factor receptor-modulated energy depletion for hypoxic cell death as a more general phenomenon is also underscored by the hypoxia-resistant phenotype of *Caenorhabditis elegans* carrying mutations in the insulin/insulin-like growth factor receptor homologue *daf-2* gene that exhibit reduced metabolic rate and lower O_2 consumption (22). Antiapoptotic properties of EGFR signaling may be of lesser importance here because energy deprivation promotes decoupling of mitochondrial cytochrome *c* release from caspase processing, resulting in predominantly necrotic cell death (15).

Limitations of the current study include the use of established tumor cell lines and the focus on an acute type of hypoxic injury. Therefore, caution is necessary when extrapolating these data to tumor cells in human tumors *in vivo*, where both chronic and acute types of hypoxia occur. Nevertheless, dissecting the molecular mechanisms underlying the opposing effects of EGFR inhibition on apoptosis and cell death induced by acute hypoxia may help to enhance the efficiency of treatment strategies targeting signal transduction pathways.

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