Activation of Insulin-like Growth Factor Signaling Induces Apoptotic Cell Death Under Prolonged Hypoxia by Enhancing Endoplasmic Reticulum Stress Response

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Abstract

Malignant cells in solid tumors survive under prolonged hypoxia and can be a source of resistance to current cancer therapies. Mammalian target of rapamycin (mTOR), one of the downstream molecules of the insulin-like growth factor (IGF) pathway, is a key regulator of translation, integrating multiple environmental and nutritional cues. The activity of mTOR is known to be suppressed under hypoxic conditions in cancer cells, whereas the contribution of this suppression to cell survival has not yet been clarified. We show that stimulating IGF signaling provoked caspase-dependent apoptosis under low oxygen tension in two cancer cell lines, COLO 320 and AsPC-1. In concurrence with increased levels of BAD phosphorylation, cell death was not accompanied by cytochrome *c* release from mitochondria. The cells were rescued from apoptosis when phosphatidylinositol 3-kinase (PI3K) or mTOR activity was inhibited, suggesting that these signals are critical in the observed cell death. IGFs and insulin enhanced the endoplasmic reticulum (ER) stress response as monitored by induction of the CCAAT/enhancer binding protein homologous protein (CHOP) proteins and the X box protein-1 splicing under hypoxic conditions, and this response was suppressed by inhibiting PI3K and mTOR activity. IGF-induced cell death under hypoxic conditions was prevented by treatment with cycloheximide, suggesting that *de novo* protein synthesis is required. Indeed, suppression of CHOP protein levels with small hairpin RNA reduced cell death. Taken together, the data suggest that stimulating IGF signaling under hypoxic conditions provokes apoptosis by enhancing the ER stress response. [Cancer Res 2007;67(17):8095–103]

Introduction

Regions with low oxygen tension are commonly observed in experimental as well as solid tumors in humans. The tumor cells in hypoxic regions are resistant to current cancer therapies (1). Besides resistance to therapy, there is substantial evidence that hypoxia is a selective force toward a more aggressive tumor phenotype. Thus, the poorer the tumor oxygenation, the poorer prognosis for the patient (2). Much progress has been made in understanding the molecular mechanisms of the hypoxic response in tumor cells (3). However, these studies have focused on short periods of hypoxia (hours) so that the molecular mechanism of cancer cells to respond to the prolonged hypoxia (days) that commonly exists in solid tumors remains to be further elucidated.

The insulin-like growth factor (IGF) family, which consists of two ligands, IGF-I and IGF-II, interact with the receptor IGF-I receptor (IGF-IR), a transmembrane tyrosine kinase that is structurally and functionally related to the insulin receptor (4). The role of IGF-IR in malignant transformation is well documented (4, 5). IGF-IR signaling can induce many effects including mitogenesis, transformation, and cell survival. However, the role of IGF signaling under hypoxic conditions is not yet clarified. Mammalian target of rapamycin (mTOR) is one of the downstream molecules in the IGF pathway and a key regulator of translation, integrating environmental and nutritional cues. Although many studies report that mTOR activity is up-regulated in various types of cancers (6), it is also true that mTOR activity is suppressed under hypoxic conditions in cancer cells (7–9). Because hypoxia is an apoptosis-inducing stress (10) and the IGF/mTOR signal is considered to be an important factor for the survival of cancer cells, suppression of mTOR signaling under hypoxic conditions seems to be paradoxical.

Recently, hypoxia was shown to generate an endoplasmic reticulum (ER) stress (11, 12). Proteins must be folded into proper conformation to carry out their cellular functions. In eukaryotic cells, the ER serves as a processing station for protein folding and the posttranslational modification of secreted and transmembrane proteins (13, 14). Unfolded or misfolded proteins, which occur under various physiologic and pathologic conditions, are harmful to cells. To overcome this stress, cells use the ER stress response signaling pathway, which has three functional components: (*a*) transcriptional up-regulation of a set of genes to process the misfolded proteins, called the unfolded protein response (UPR); (*b*) repression of translation to decrease the load of client proteins; and (*c*) programmed cell death, which occurs when ER functions are severely impaired (15). Although the precise mechanism of how protein misfolding is provoked under low oxygen tension remains to be elucidated, it is now clear that a stress response is critical for cell survival during hypoxia (11, 12).

Given that hypoxia is an ER stress, promoting protein synthesis via IGF signaling under hypoxic conditions could induce excessive protein levels that might lead to cell death rather than protect cells from apoptosis, as is widely observed under normoxic conditions. Here, we showed that IGF signaling and the consequent activation of mTOR signaling under prolonged hypoxic conditions induce apoptotic cell death through enhancement of the ER stress response in human colon and pancreatic cancer cell lines.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Materials and Methods

Cells and cell culture. The colon cancer cell line COLO 320 and the pancreatic cancer cell line AsPC-1 were obtained from the American Type Culture Collection. The cells were cultured in RPMI 1640 with or without 10% fetal bovine serum. Hypoxia was achieved by incubating cells with 1% O_2 and 5% CO_2 in a Multigas Incubator (ASTEC, Fukuoka, Japan). Anoxia (0% O_2) was achieved by the AnaeroPack system (Mitsubishi Gas Chemical, Tokyo, Japan). Cells were seeded at a density of 3×10^5 or 1×10^5 per well of 12-well tissue culture plates for counting viable cells, and at 1.5×10^6 or 1×10^6 per plate in 60-mm tissue culture plates for Western blotting and reverse transcription-PCR (RT-PCR) for the COLO 320 or AsPC-1 cells, respectively. The COLO 320 cells were preincubated in control media for 6 h under normoxic conditions, then treated with or without reagents and immediately transferred to the hypoxic chamber. The AsPC-1 cells were preincubated in culture media for 17 h under normoxic conditions, then the medium was changed to serum-free medium and the cells were treated with or without reagents and immediately transferred to the Multigas Incubator or the AnaeroPack system. The concentration of IGF-I, IGF-II, and insulin was 100 ng/mL, 100 ng/mL, and 100 nmol/L, respectively, when not otherwise indicated. After the indicated periods, cells were subjected to cell counting. Viable cell number was assessed by the trypan blue dye exclusion assay.

Reagents. Cycloheximide, tunicamycin, and insulin were purchased from Sigma Chemical Co.; zVAD-fmk was from Peptide Institute; IGF-I and IGF-II were from R&D Systems; and LY 294002, rapamycin, and etoposide (VP-16) were from Wako Pure Chemical Industries.

Immunoblot analysis. Western blotting analysis was done as previously described (16). The primary antibodies against AKT, eukaryotic initiation factor 4E–binding protein 1 (4EBP1), S6 kinase (S6K), S6, and BAD were purchased from Cell Signaling Technology. The antibodies raised against the phosphorylated proteins AKT (Ser^{473}), 4EBP1 ($Thr^{37/46}$), S6K (Thr^{389}), S6 ($Ser^{235/236}$), and BAD (Ser^{112}) were also from Cell Signaling Technology. Other antibodies used included growth arrest– and DNA damage–inducible gene 153 (GADD153)/CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP), GRP78/BiP, and cyclic AMP–responsive element binding protein 2/activating transcription factor 4 (ATF4) from Santa Cruz Biotechnology; anti–β-actin from Sigma; anti–cytochrome *c* from Becton Dickinson; and anti-porin from Invitrogen. The secondary anti-rabbit immunoglobulin G (IgG) and antimouse IgG antibodies, both horseradish peroxidase conjugated, were purchased from Santa Cruz Biotechnology.

Cytosol fractionation. Fractionation of the cytosol was done using the Mitochondria/Cytosol Fractionation Kit (BioVision) according to the manufacturer's instructions. Briefly, 7.5×10^7 COLO 320 cells were detached by treatment with trypsin and collected by centrifugation. After washing with PBS, the cell pellet was resuspended with Cytosol Extraction Buffer Mix containing DTT and protease inhibitors and mixed by pipetting. After centrifugation at $700 \times g$, the supernatant was recovered and further centrifuged at $10{,}000 \times g$. The supernatant from the second spin was used as the cytosolic fraction.

Semiquantitative RT-PCR. Total RNA was isolated from the cells using RNeasy Mini (Qiagen). One microgram of total RNA was reverse transcribed to obtain cDNA using Superscript III (Invitrogen) according to the manufacturer's protocol. The PCR reactions were done in the iCycler (Bio-Rad). One microliter of reverse transcription reaction solution was used in 20 μL of total PCR reaction buffer as a template. For CHOP and β-actin mRNA amplification, the cycling program was an initial melting step of 5 min at 95°C followed by 25 cycles at 94°C for 60 s, annealing at 58°C for 60 s, and elongation at 72°C of 30 s. The same program was used for X box protein-1 (XBP1) amplification, except for annealing temperature (56°C) and number of cycles (30 cycles). The primer sequences were described in Supplementary Table S1. Amplified PCR products were visualized on a 1% agarose gel (2% gel for XBP1) stained with ethidium bromide.

RNA interference. Gene silencing was done using the pSuperRetro plasmid purchased from Oligoengine as previously described (17). The corresponding sequence of the CHOP oligonucleotide was 5′-gtcctgtcttcagatgaaa-3′. The small hairpin RNA (shRNA)/CHOP vector was introduced into cells by retroviral vector transfection (18). After selection with puromycin for 1 week, the cells were subjected to further experiments.

Statistical analysis. The experiments were repeated at least thrice with representative results shown. Statistical analysis was done with GraphPad Prism 4 (GraphPad Software). The statistical significance of the results was tested with the unpaired *t* test. $P < 0.05$ was considered to be statistically significant.

Results

IGF-I, IGF-II, or insulin induced cell death in COLO 320 cells under hypoxic conditions. To explore the mechanism of cell death under hypoxic conditions, we used the human colon cancer cell line COLO 320. When these cells were cultured without serum under hypoxic conditions, the COLO 320 cells showed minimal death with a slight increase in cell number until day 4 (Fig. 1*A*). No further increase in cell number was observed at day 1 after treatment with IGF-I or insulin. The second day after IGF-I or insulin treatment under hypoxic conditions, COLO 320 cells showed massive cell death along with a prominent decrease in viable cells. The cell death induced by IGF-I or insulin under hypoxic conditions was not remarkable in 24 h of culture, whereas after 24 h, cell death became prominent and increased with time. Cell death was similarly induced by IGF-II, and the degree of cell death with IGF-I, IGF-II, and insulin was dose dependent (Fig. 1*B*). The IGFs and insulin bind the receptors IGF-IR and insulin receptor, respectively, which share a high degree of identity in their primary and tertiary structures. Both receptors activate highly similar sets of downstream intracellular events including the Ras/Raf/mitogen-activated protein kinase and phosphatidylinositol 3-kinase (PI3K) pathways. As the degree of cell death was observed equally between the IGFs and insulin, the cell death would be expected to occur through a pathway common to both the IGF-IR and insulin receptor pathways.

The cell death induced by IGFs or insulin under hypoxic conditions is caspase-dependent apoptosis. Morphologic analysis under microscopy showed that the type of death induced by IGFs or insulin under hypoxic conditions was typical apoptosis with nuclear condensation and fragmentation (data not shown). Although COLO 320 cells exhibited low caspase-3 activity under both normoxic and hypoxic conditions without serum, treatment with IGF-I, IGF-II, or insulin under hypoxic conditions clearly enhanced the cleavage of procaspase-3 to the active form, but not under normoxic conditions (Fig. 2*A*). At the same time, the cleavage of poly(ADP-ribose) polymerase (PARP), which is a substrate of caspase-3, was also remarkably enhanced by treatment of the cells with the IGFs or insulin under hypoxic conditions. Caspase-9, a molecule upstream of caspase-3 in the caspase-activation cascade, was also cleaved into an active form under hypoxic conditions in cells treated with the IGFs or insulin (Supplementary Fig. S1). The contribution of caspases to the cell death induced by IGFs and insulin under hypoxic conditions was confirmed by the result that the death was circumvented by the broad-specificity caspase inhibitor zVAD-fmk (Fig. 2*B*). Taken together, the cell death induced by IGFs or insulin under hypoxic conditions was caspase-dependent apoptosis.

IGF- or insulin-induced apoptosis under hypoxic conditions was not accompanied by cytochrome *c* release from mitochondria. The finding that IGFs or insulin induced typical apoptosis under hypoxic conditions was surprising because IGF is a well-known antiapoptotic factor in various settings (5). To explore the apoptotic pathway in IGF-treated COLO 320 cells under

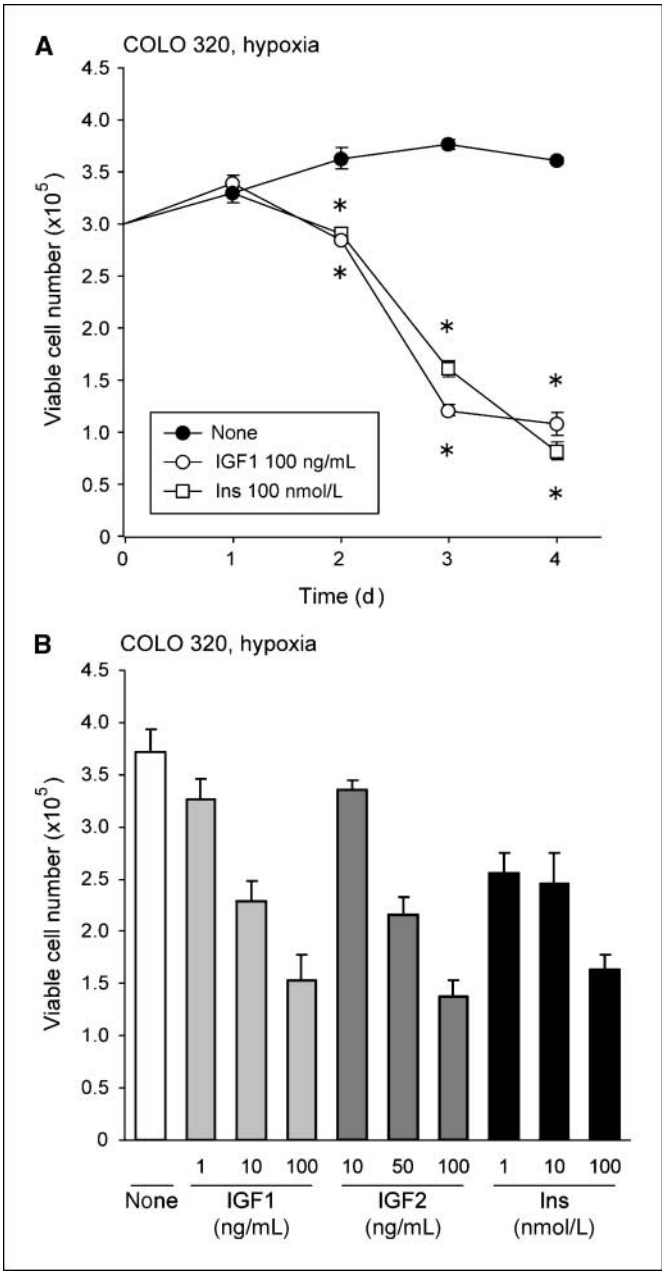


Figure 1. IGF-I, IGF-II, or insulin induced cell death of COLO 320 cells under hypoxic conditions. *A,* viable number of COLO 320 cells at the indicated time points under hypoxic conditions without treatment (●), with 100 ng/mL IGF-I (*IGF1*; ○), or with 100 nmol/L insulin (*Ins*; □). *B,* viable cell number at day 3 under hypoxic conditions. The indicated doses of IGF-I (*light gray column*), IGF-II (*IGF2*; *dark gray column*), or insulin (*black column*) were added to the medium at day 0. The experiment was done in triplicate and repeated thrice. *Bars,* SD. * $P < 0.05$.

hypoxic conditions, the phosphorylation of BAD, a proapoptotic member of the Bcl-2 family, was examined. BAD is a BH3-only protein that induces apoptosis by forming a dimer with and inactivating the antiapoptotic proteins Bcl-2 and Bcl-xL (19). Once phosphorylated by IGF treatment, BAD binds to a 14-3-3 protein and is thus unavailable to interact with members of the Bcl-2 family (20). The BAD protein was clearly phosphorylated after treatment of the cells with the IGFs or insulin under hypoxic conditions (Fig. 2*C*). Because IGF treatment caused BAD phosphorylation, mitochondria might be protected from apoptotic stimuli. Hence, we examined cytochrome *c* release from mitochondria into the cytosol to determine whether the mitochondrial apoptotic machinery was involved in the IGF- or insulin-induced cell death in COLO 320 cells under hypoxic conditions. Cytochrome *c*

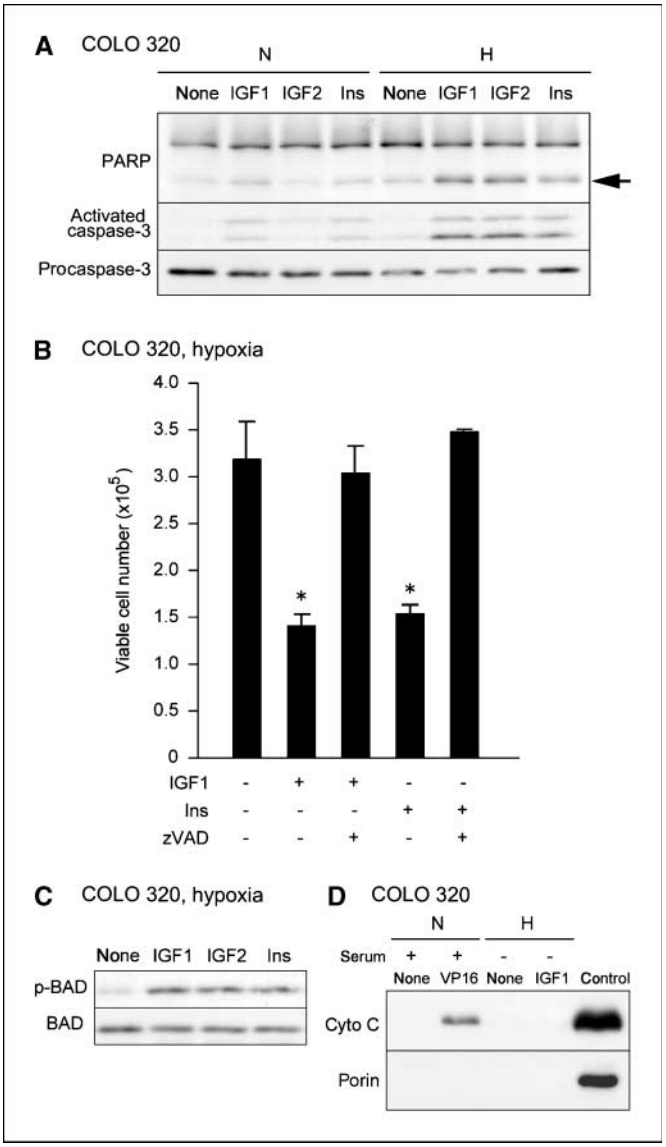


Figure 2. The cell death induced by IGF or insulin treatment under hypoxic conditions in COLO 320 cells was apoptosis, not accompanied by cytochrome *c* release from mitochondria. *A,* Western blot analysis of the cleavage of procaspase-3 and PARP. Cells were cultured under the indicated conditions for 17 h. *N,* normoxia; *H,* hypoxia. *Arrow,* cleaved form of PARP. *B,* viable cell number at day 3 of COLO 320 cells with or without IGF-I, insulin, or zVAD-fmk (*zVAD*), 10 μmol/L treatment as indicated. The experiment was done in triplicate and repeated thrice. *Bars,* SD. *, $P < 0.05$, versus control (nontreated). *C,* phosphorylation of the proapoptotic protein BAD after IGF or insulin treatment was examined by Western blotting. The cells were cultured under hypoxic conditions with the indicated treatments and subjected to Western blotting. Membranes were probed with anti–phosphorylated BAD (*p-BAD*) or anti-BAD (*BAD*) antibody. *D,* Western blot of the cytosolic fractions from cells cultured under normoxic or hypoxic conditions with the indicated factors. The concentration of VP-16 was 10 μmol/L. The membrane was probed with anti–cytochrome *c* antibody. The membrane was also probed with anti-porin antibody to exclude the possibility that the cytosolic fraction was contaminated with the mitochondrial fraction. The equality of protein loading was confirmed by staining the transferred proteins with Ponceau S (Sigma; data not shown). Whole-cell lysate was used as a positive control for both antibodies.

was not detected in the cytosolic fraction after IGF-I treatment (Fig. 2*D*), which was confirmed by immunocytochemistry showing that cytochrome *c* was confined to mitochondria in IGF-I–treated cells (Supplementary Fig. S3). Meanwhile, VP-16 treatment clearly induced cytochrome *c* release from mitochondria (Fig. 2*D*; Supplementary Fig. S2). Preferential distribution of mitochondria to the perinuclear region was confirmed by MitoTracker Red staining (data not shown). Taken together, both IGFs and insulin caused prominent apoptosis under hypoxic conditions in COLO 320 cells that was not accompanied with mitochondrial cytochrome *c* release.

PI3K and mTOR signals are involved in IGF- or insulin-induced apoptosis under low levels of oxygen tension. To explore the intracellular signaling that leads to the IGF- or insulin-induced apoptosis under hypoxic conditions, the PI3K and mTOR signaling pathways were examined in COLO 320 cells because these molecules are known to be activated by IGF or insulin stimulation. Under normoxic conditions, phosphorylation of AKT, a downstream molecule of PI3K, as well as that of S6K, S6, and 4EBP1, downstream molecules of mTOR, was increased by IGF or insulin treatment (Fig. 3*A*). Meanwhile, under hypoxic conditions, phosphorylation of these proteins in IGF- or insulin-treated cells was increased but to a lesser extent than was observed under normoxic conditions (Fig. 3*A*). In contrast, treatment with IGFs or insulin restored phosphorylation of AKT completely even under hypoxic conditions. Thus, the IGFs or insulin was able to fully activate the PI3K pathways and partially the mTOR pathways under hypoxic conditions.

To generalize the findings, the human pancreas cancer cell line AsPC-1 was used in similar experiments. AsPC-1 cells treated with IGF-I also showed remarkable cell death under anoxic conditions (Fig. 3*D*) but not under hypoxic conditions (data not shown). The phosphorylation of AKT, as well as of S6K, S6, and 4EBP1, was increased by IGF-I treatment under normoxic conditions (Fig. 3*B*). Meanwhile, as was observed in COLO 320 cells, S6K, S6, and 4EBP1 were partially phosphorylated in IGF-I–treated cells under hypoxic conditions (Fig. 3*B*). Further suppression of the phosphorylation of 4EBP1, S6K, and S6 was not observed under anoxic conditions. Treatment with IGF-I restored phosphorylation of AKT under hypoxic and anoxic conditions as well.

Next, to examine the effect of these signals on cell death, LY 294002 (a PI3K inhibitor) or rapamycin (an mTOR inhibitor) was added into the culture medium with IGFs or insulin. When the PI3K/mTOR pathways were blocked by these inhibitors, the IGF- or insulin-induced apoptosis was suppressed in both cell lines (Fig. 3*C* and *D*). These results indicate that activation of the PI3K/mTOR pathways played critical roles in the cell death induced by IGF or insulin treatment under hypoxic or anoxic conditions, although the oxygen tension at which IGFs or insulin induced cell death was cell type specific. Hypoxia (1% O_2) was sufficient for IGF- or insulin-induced death in COLO 320 cells, whereas anoxia was necessary for AsPC-1 cells.

IGFs and insulin enhance the ER stress response under low levels of oxygen tension. Protein synthesis is suppressed under hypoxic conditions (9, 21, 22), at least partially due to ER stress (11, 12), in which suppression of protein synthesis is one of the essential components. Because activation of PI3K and mTOR signaling promotes protein synthesis (23, 24), we speculated that activation of PI3K and mTOR may enhance ER stress under hypoxic conditions. To investigate the role of IGFs and insulin in ER stress under hypoxic conditions, we assessed the UPR.

Under normoxic conditions in COLO 320 cells, the mRNA transcription of CHOP/GADD153 was undetectable in basal levels and slightly induced by IGF or insulin treatment (Fig. 4*A, top*). Transcripts of the UPR gene *XBP1* are spliced into short, transcriptionally active forms under ER stress, indicating the presence of IRE1 endoribonuclease activity, one of the principal arms of the ER stress response (25). Under normoxic conditions, the spliced short form of XBP1 was undetectable in basal levels and slightly induced by IGF or insulin treatment. Under hypoxic conditions alone, mRNA of CHOP and the spliced short form of XBP1 were slightly induced when compared with those under normoxic conditions. In contrast, when the cells were treated with IGFs or insulin under hypoxic conditions, the levels of CHOP and splicing of XBP1 were remarkably induced to levels observed in the cells treated with tunicamycin, a known inducer of ER stress. The induction of CHOP mRNA and XBP1 splicing was increased by IGF-I and insulin in a dose-dependent manner (Supplementary Fig. S3). Other UPR genes, *BiP* and *ATF4*, were examined to confirm the induction of ER stress by IGF or insulin treatment under hypoxic conditions (Fig. 4*A, bottom*). IGFs or insulin increased the protein levels of BiP and ATF4 under hypoxic conditions but not under normoxic conditions.

Next, we examined the downstream signal activated by IGFs or insulin that enhanced ER stress. When the activation of PI3K or mTOR was inhibited by LY 294002 or rapamycin, respectively, IGF-I- or insulin-induced UPR was suppressed (Fig. 4*B, top*). These results were confirmed by the protein levels of CHOP (Fig. 4*B, bottom*). Thus, IGFs and insulin provoked the UPR under hypoxic conditions in COLO 320 cells through the PI3K/mTOR pathway. In AsPC-1 cells, anoxia itself induced UPR. Under anoxic conditions, CHOP transcription was induced and XBP1 was extensively spliced (Fig. 4*C, top*). IGF-I treatment further enhanced the mRNA transcript levels of both CHOP and XBP1. Inhibition of PI3K or mTOR suppressed the elevated levels of CHOP protein induced by IGF-I treatment and attenuated the cleavage of PARP at the same time (Fig. 4*C, bottom*). Thus, in AsPC-1 cells, anoxia itself induced ER stress, and IGF-I enhanced the UPR through the PI3K/mTOR pathway.

Cell death induced by IGFs or insulin under low levels of oxygen tension is attenuated by suppression of *de novo* protein synthesis. One of the well-characterized roles of mTOR is the regulation of mRNA translation. Because IGFs and insulin activated mTOR signaling under hypoxic conditions, it is possible that IGFs or insulin reactivates protein synthesis under hypoxic conditions. To explore the contribution of protein synthesis in IGF-induced cell death, we treated COLO 320 cells with cycloheximide, which inhibits protein synthesis by inhibiting translational elongation. At the 125 ng/mL dose, cycloheximide had no cytotoxic effect on the cells under normoxic conditions (data not shown). Strikingly, IGF-I– and insulin-induced cell death under hypoxia was negated by treatment of the cells with cycloheximide (Fig. 5*A*). The activation status of the caspases observed in the IGF- or insulin-induced apoptosis under hypoxic conditions was examined (Fig. 5*B, top*). The cleavage of caspase-3 induced by IGF-I or insulin in untreated cells under hypoxic conditions was inhibited by cycloheximide treatment. To determine whether cycloheximide treatment affected ER stress induced by the IGFs or insulin, the mRNA transcripts of UPR genes were measured. Treatment with cycloheximide strongly suppressed CHOP mRNA levels and XBP1 splicing in both IGF-I– and insulin-treated cells under hypoxic conditions (Fig. 5*B, bottom*).

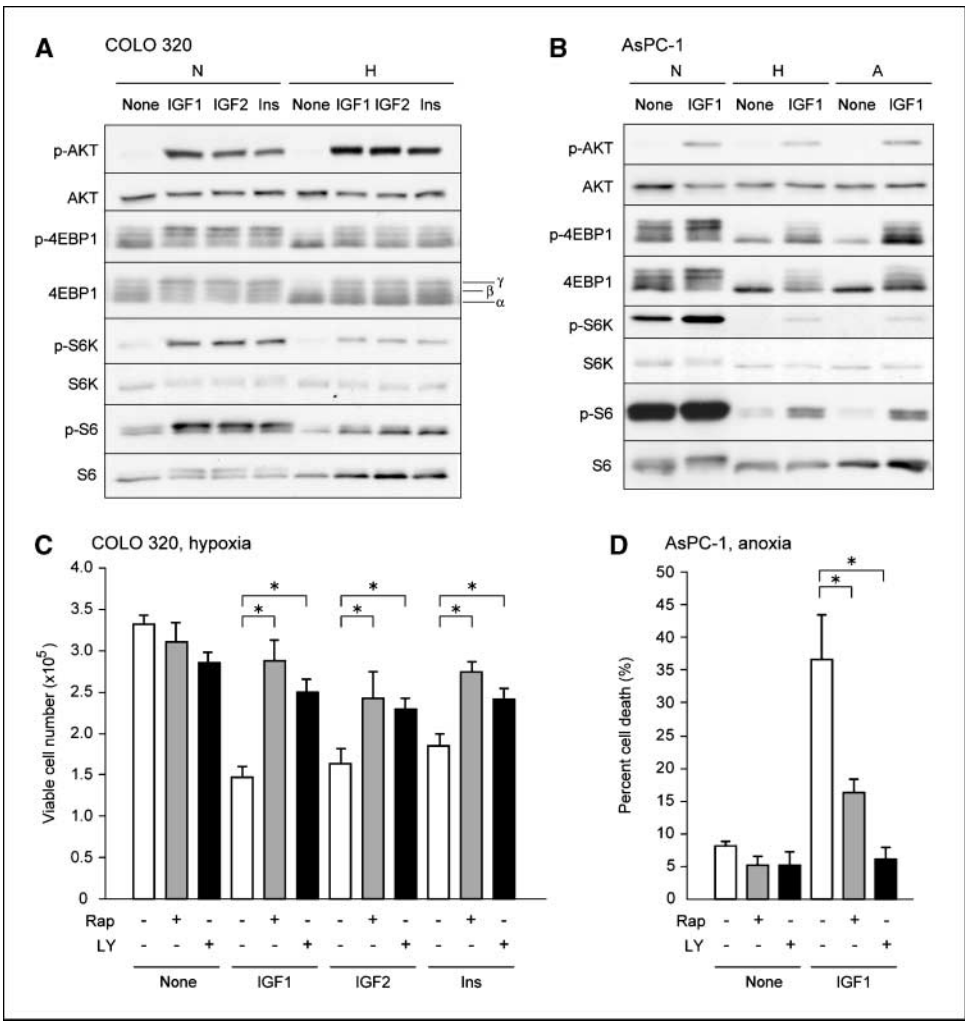


Figure 3. The PI3K and mTOR signals are involved in IGF- or insulin-induced apoptosis under low levels of oxygen tension. *A,* Western blot probed with antibodies against the downstream molecules of PI3K and mTOR. COLO 320 cells were cultured under normoxic or hypoxic conditions with the indicated factors for 17 h and the lysate was subjected to the experiments. α, β, and γ, hypophosphorylated, intermediately phosphorylated, and hyperphosphorylated bands of 4EBP1, respectively. Anti–phosphorylated protein antibodies are indicated with a "p" prefix. *B,* AsPC-1 cells were cultured under normoxic, hypoxic, or anoxic (*A*) conditions for 24 h with the indicated treatments and the lysate was subjected to Western blotting with the indicated antibodies. *C,* viable cell number at day 3 of COLO 320 cells cultured under hypoxic conditions with the indicated reagents. *Rap,* rapamycin (100 nmol/L; *gray column*); *LY,* LY 294002 (10 μmol/L; *black column*). LY 294002 and rapamycin were added 30 min before the addition of either IGFs or insulin. *D,* percent death at day 3 of AsPC-1 cells cultured under anoxic conditions with the indicated treatments as in (*C*). The experiment was done in triplicate and repeated thrice. *Bars,* SD. * $P < 0.05$.

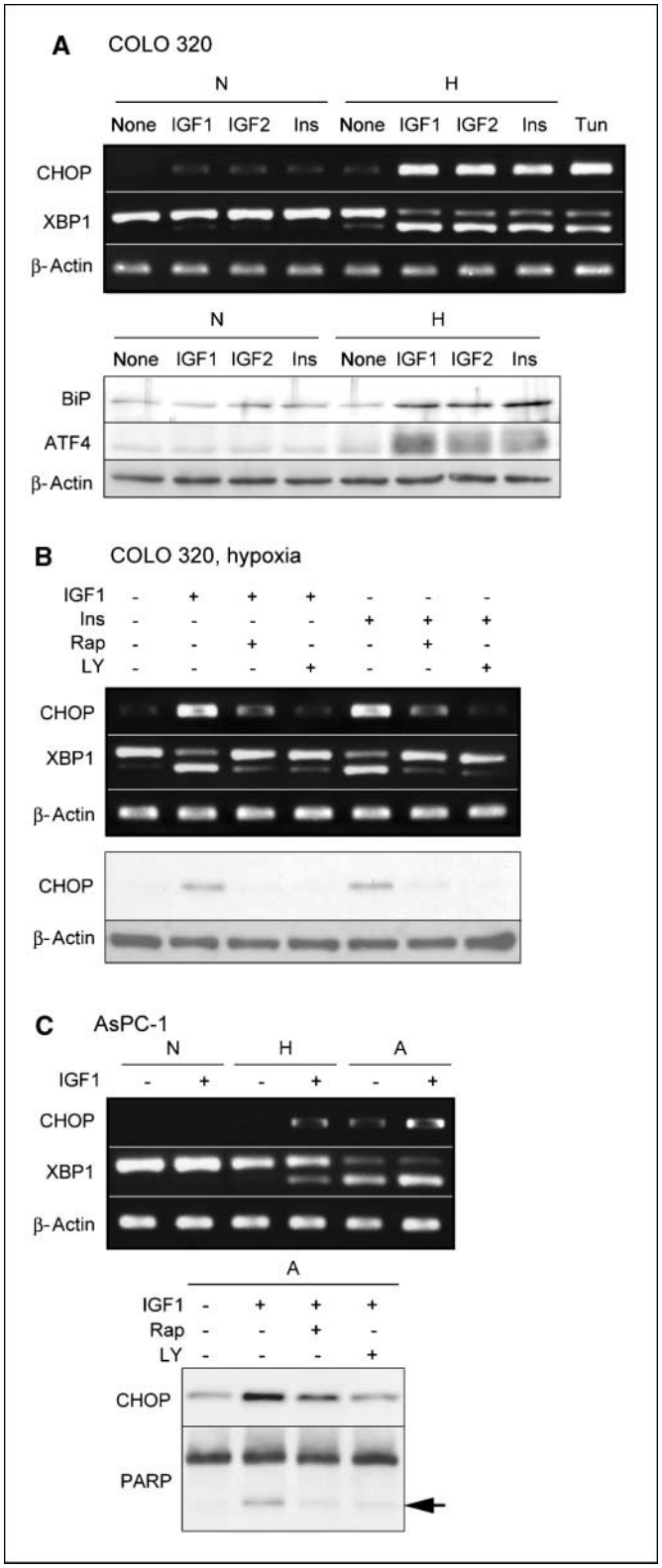
In AsPC-1 cells, suppression of protein synthesis by cycloheximide also inhibited the cell death induced by IGF-I under anoxic conditions (Fig. 5*C, left*). The enhanced CHOP mRNA levels and splicing of XBP1 transcripts were inhibited by cycloheximide (Fig. 5*C, right*). Thus, suppression of *de novo* protein synthesis attenuated the UPR and rescued cells from apoptosis induced by the IGFs or insulin under hypoxic or anoxic conditions in two different cell lines.

These results indicate two possible effects of cycloheximide in suppressing IGF- or insulin-induced cell death under hypoxic conditions. First, cycloheximide might repress global protein synthesis stimulated by IGF or insulin to levels that induced cell death because increasing the influx of nascent peptides enhances the protein load on the ER processing and folding machineries. Second, cycloheximide might suppress cell death by repressing the *de novo* synthesis of specific protein(s). To examine the first possibility, we assessed global protein synthesis using a [^{35}S]methionine and cysteine incorporation assay in COLO 320 cells (Supplementary Fig. S4). Global protein synthesis was suppressed after 18.5 h of hypoxia to approximately half of that found under normoxic conditions. In cells cultured with IGF-I, protein synthesis was slightly but significantly increased compared with the nontreated cells. Meanwhile, cycloheximide prominently suppressed protein synthesis in both IGF-I–treated and nontreated cells. Taken together, IGF-I marginally enhanced the global protein synthesis under hypoxic conditions.

IGF- or insulin-induced apoptosis under low levels of oxygen tension is attenuated by suppression of CHOP protein levels. We next evaluated whether the *de novo* synthesis of a specific protein or proteins is critical for the IGF- or insulin-induced cell death under hypoxic conditions. We focused on CHOP/GADD153 because it is a candidate in the cell death pathway due to excessive ER stress, although the mechanism is still poorly understood (26). A retroviral vector containing small hairpin CHOP RNA (shRNA/CHOP) was introduced into the cells. The level of CHOP protein in the cells transfected with shRNA/CHOP was remarkably reduced compared with the cells transfected with empty vector, and the protein levels remained low after IGF-I stimulation (Fig. 6*A* and *B, bottom*). When the levels of CHOP were reduced both in COLO 320 and AsPC-1 cells, the IGF-I–induced death was dramatically suppressed (Fig. 6*A* and *B, top*), and caspase-3 activation and PARP cleavage were suppressed in parallel (Fig. 6*A* and *B, bottom*). Thus, CHOP is critical for the cell death in two cell lines treated with IGF-I under hypoxic or anoxic conditions.

Based on the collective data, we propose a mechanism of IGF-induced cell death under low levels of oxygen tension (Fig. 6*C*). The IGFs or insulin triggers multiple intracellular pathway including

PI3K, AKT, and mTOR. Activated AKT phosphorylates BAD, which in turn might protect mitochondria and prevent these organelles from releasing cytochrome *c*. Thus, the mitochondrial death pathway is blocked by IGF, as is widely reported in cells under normoxic conditions. On the other hand, activated mTOR enhanced the ER stress response under hypoxic conditions. Although CHOP plays a critical role in the activation of caspase-3 and subsequent apoptosis, it is unclear how the caspase is activated.



Discussion

We show that the IGFs or insulin could induce apoptosis under hypoxic conditions. The IGFs or insulin enhanced the UPR. CHOP, a UPR gene, is necessary for the cell death induced by IGF or insulin under hypoxic conditions. Adaptation to ER stress is critical for the survival of tumor cells during hypoxia (11, 12), whereas cell death is also conspicuous in cells that have encountered insurmountable ER stress (13, 15). We are currently investigating whether these two cell lines are representative for tumor cell lines or tumor cells in general.

The mechanism of IGF or insulin enhanced UPR under hypoxic conditions is obscure. The first possibility is the up-regulation of protein synthesis by IGFs or insulin. It is widely accepted that IGFs or insulin stimulates an anabolic reaction including the promotion of protein synthesis during normoxia. In COLO 320 cells, IGF-I slightly but significantly increased protein synthesis. Inhibition of mRNA translation is a well-characterized and universal cellular response to oxygen deprivation (21). Repression of translation is mediated by three distinct mechanisms during hypoxia: (*a*) phosphorylation and inhibition of eukaryotic initiation factor 2α by the PERK (eukaryotic translation initiation factor 2α kinase3), (*b*) inhibition of the mTOR pathway (27), and (*c*) inhibition of the eukaryotic elongation factor 2 by AMP-activated protein kinase (9). To fully restore protein synthesis under hypoxic conditions, the activation of multiple pathways would be required, although the slight increase in global protein synthesis under hypoxic conditions might be enough to enhance ER stress.

The second possibility is that IGF or insulin causes the up-regulated translation of UPR gene–specific mRNA in spite of global suppression of protein synthesis. The UPR genes have the ability to compete for translation under ER stress conditions that limit initiation of other cellular mRNAs (13). IGFs or insulin might promote translation of individual UPR genes or increase the ligands that activate ER-resident protein kinases to initiate UPR. In these situations, IGFs or insulin is able to enhance the UPR without increasing global protein synthesis. Induction of CHOP by amino acid deprivation, which is also accompanied by suppression of global protein synthesis, requires IGF-I/PI3K/mTOR signaling (28).

The CHOP protein, also known as GADD153, is a member of the C/EBP family (29). It is ubiquitously expressed at very low levels and robustly induced by a wide variety of cell stressors including genotoxic stress, nutrient depletion, and ER stress. Although it is

Figure 4. Addition of IGFs or insulin enhanced ER stress under low levels of oxygen tension. *A, top,* semiquantitative RT-PCR of CHOP and XBP1 transcripts. COLO 320 cells were cultured under normoxic or hypoxic conditions for 17 h with the indicated factors. The cells cultured under normoxic conditions with 2 μg/mL tunicamycin (*Tun*) were used as a positive control for UPR. *A, bottom,* Western blot of BiP and ATF4 from the cells treated as in (*A, top*). *B, top,* semiquantitative RT-PCR of CHOP and XBP1 transcripts. COLO 320 cells were cultured under hypoxic conditions for 17 h and then treated with IGF-I, insulin, rapamycin (100 nmol/L), and/or LY 294002 (10 μmol/L) as indicated. *B, bottom,* Western blot of CHOP from the cells treated as in (*B, top*). *C, top,* semiquantitative RT-PCR of CHOP and XBP1 transcripts. AsPC-1 cells were cultured under normoxia, hypoxia, or anoxia for 24 h with or without IGF-I. *C, bottom,* Western blot of CHOP and PARP. AsPC-1 cells were cultured under anoxic conditions for 48 h with IGF-I, rapamycin (100 nmol/L), and/or LY 294002 (10 μmol/L). *Arrow,* cleaved form of PARP.

now clear that CHOP plays an important role in ER stress-induced apoptosis (26), the mechanisms whereby CHOP induces apoptosis are still unclear. In some cellular contexts, the CHOP-mediated death signal is transmitted to the mitochondria through repression of Bcl-2 proteins (30, 31). Contrarily, we showed that IGF-induced apoptosis under hypoxic conditions was CHOP dependent but independent of mitochondrial cytochrome *c* release in COLO 320 cells.

Recently, Marciniak et al. (32) reported that CHOP is not only induced by ER stress but also that CHOP per se enhances ER stress by increasing the ER client protein load and changing the redox conditions within this organelle. In this case, CHOP does not induce cell death directly but enhances ER stress and the cell death that occurs as a consequence. Various downstream effectors of cell death induced by ER stress other than CHOP have been identified, including activation of the mitochondrial death pathway (33), activation of calpain by Ca^{2+} release from the ER (34), and activation of the ER-resident caspase, caspase-12 in mice (35) and caspase-4 in humans (36). Mitochondria were unlikely to be involved in our experimental system. Calpain was unlikely to be involved in caspase activation because calpain inhibitor I (*N*-acetyl-leucinyl-leucinyl-norleucinal) did not reduce the cell death (data not shown). Activation of caspase-4, the human counterpart of mouse caspase-12, is also a possible explanation of the mitochondria-independent activation of caspase-9. Morishima et al. reported that, on activation, caspase-12 translocates from the ER to the cytosol where it directly cleaves procaspase-9 independent of mitochondria (37). When caspase-4 levels were repressed by shRNA, we observed a partial rescue from cell death in COLO 320 cells but not in AsPC-1 cells (data not shown). These data suggest that the downstream effectors of IGF- or insulin-induced cell death, which activate executional caspases, may be cell type specific.

It is widely accepted that IGF/PI3K/mTOR signaling plays an important role in mitogenesis, transformation, and protection from apoptosis (5) and may accordingly contribute to carcinogenesis. Indeed, germ-line mutations of the *LKB1* and *TSC1/2* genes, which suppress the mTOR signal, predispose individuals to a variety of human tumors. Phosphatase and tensin homologue, which suppresses PI3K activity, is frequently mutated in various tumors. Overexpression of IGF-II and IGF-IR is often observed in cancer specimens, and their contribution to tumorigenesis was clearly shown in experimental animals (38, 39). Nonetheless, induction of cell death by IGF/PI3K/mTOR signaling has also been documented. IGF-I induces apoptosis in osteosarcoma cells (40) and glioma cells (19). Overexpression of fragments of the IGF-IR intracellular domain in cancer cells was shown to induce cell death and inhibit tumorigenesis in nude mice (41–43). Activation of the mTOR signal under hypoxic conditions accelerated cell death in Lewis lung carcinoma cells (8). Furthermore, although early-stage tumors express abundant levels of the IGF-IR, the expression is rather reduced in advanced prostate cancer and breast cancer (4).

These reports and our current findings suggest that IGF signaling would not invariably work in favor of cancer cell survival

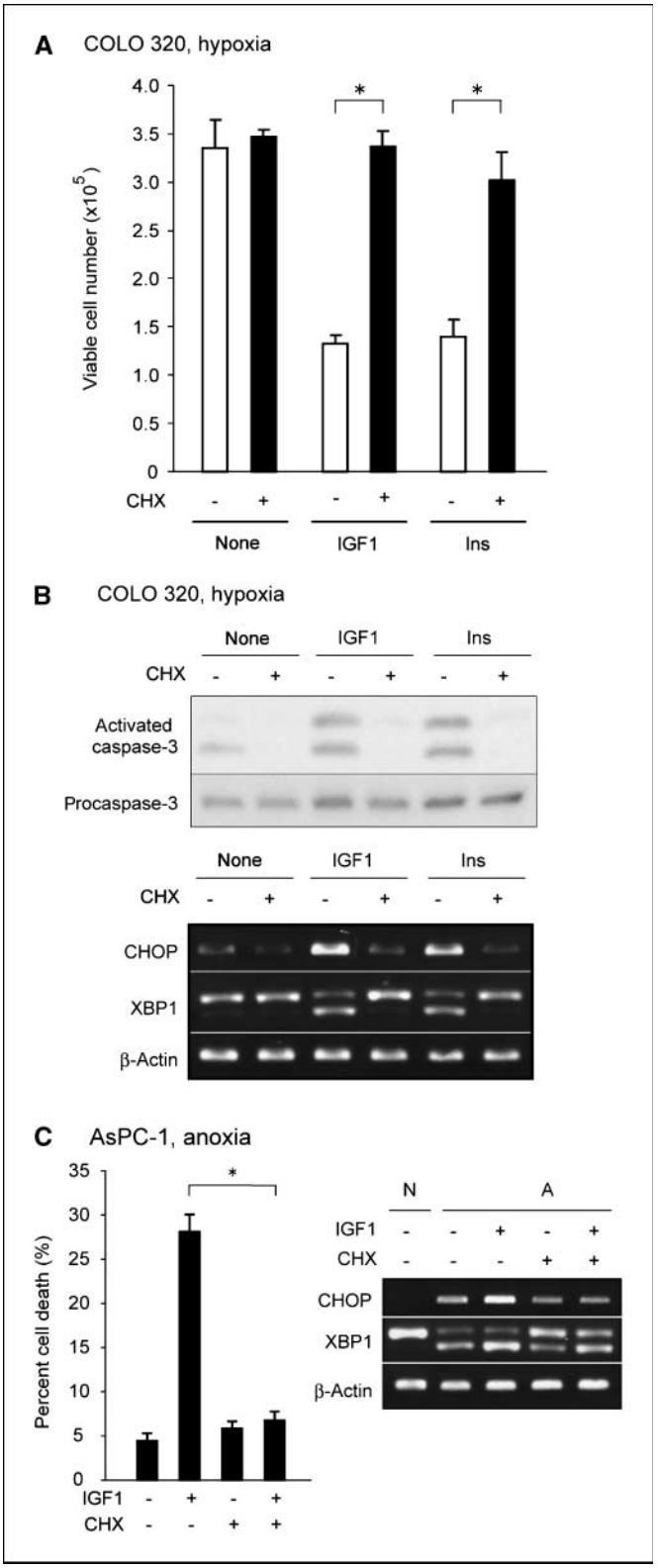


Figure 5. IGF-I– or insulin-induced cell death under low levels of oxygen tension was attenuated by suppression of protein synthesis. *A,* viable number at day 3 of COLO 320 cells cultured under hypoxic conditions with the indicated factors. *CHX,* cycloheximide (125 ng/mL). The experiment was done in triplicate and repeated thrice. *Bars,* SD. *, $P < 0.05$. *B, top,* Western blot analysis of caspase-3. Cells were cultured under hypoxic conditions for 17 h with the indicated factors. Procaspase-3 and activated caspase-3 were detected with the corresponding antibody. *B, bottom,* semiquantitative RT-PCR of CHOP and XBP1 transcripts. COLO 320 cells were cultured under hypoxic conditions for 17 h with the indicated factors. *C, left,* percent death at day 3 of AsPC-1 cells cultured under anoxic conditions with IGF-I and/or cycloheximide, 12.5 μg/mL. *C, right,* semiquantitative RT-PCR of CHOP and XBP1 transcripts. AsPC-1 cells were cultured for 24 h with the indicated factors as in (*C, left*).

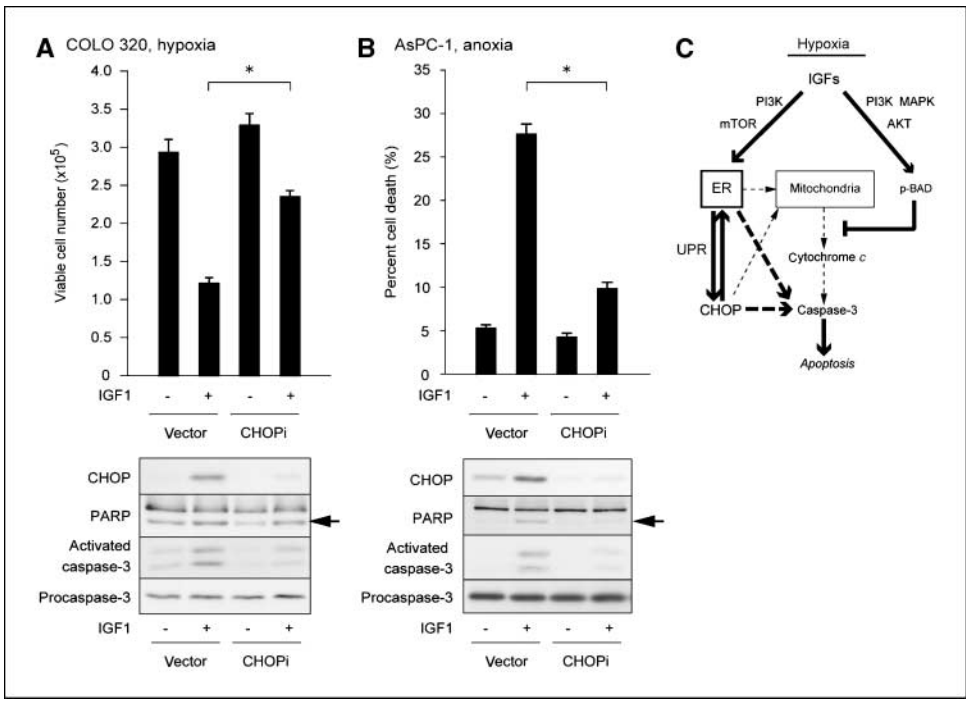


Figure 6. IGF-I–induced cell death under low levels of oxygen tension was attenuated by repression of CHOP/GADD153 expression. *A, top,* viable number at day 3 of COLO 320 cells transfected with shRNA/CHOP (*CHOPi*) or empty vector (*vector*). Cells were cultured under hypoxic conditions with or without IGF-I. The experiment was done in triplicate and repeated thrice. *Bars,* SD. *, $P < 0.05$. *A, bottom,* Western blot analysis of indicated proteins from COLO 320 cells cultured under hypoxic conditions for 17 h with the indicated treatments. *Arrow,* cleaved form of PARP. *B, top,* percent death at day 3 of AsPC-1 cells transfected with shRNA/CHOP or empty vector. Cells were cultured under anoxic conditions with or without IGF-I. *B, bottom,* Western blot analysis of indicated proteins from AsPC-1 cells cultured under anoxic conditions for 48 h with the indicated treatments. *Arrow,* cleaved form of PARP. *C,* a model of IGF-induced cell death under low levels of oxygen tension. *MAPK,* mitogen-activated protein kinase.

throughout cancer development. Suppression of IGF signaling could be also a strategy for the cancer cells to counteract the potentially harmful effects of signaling through this pathway. A number of gene expression studies have reported that hypoxia induces some of the IGF binding proteins (IGFBP) such as IGFBP-1 and IGFBP-3, which are transcriptionally regulated by hypoxia-inducible factor (44). ER stress also induces IGFBP-1 through ATF4 (28). Although the physiologic role of IGFBPs under hypoxic conditions is not yet clarified, local up-regulation of IGFBPs in hypoxia might support cell survival by binding IGFs and inhibiting IGF signaling.

The experiments in this study were mostly done under serum-free conditions to focus on the effects of IGF signaling. Under the existence of serum, COLO 320 cells showed massive necrosis with no signs of apoptosis under hypoxia, probably because of overgrowth, and the death was also reduced by inhibitors of PI3K and mTOR.[4] Thus, suppressing PI3K/mTOR signaling might contribute in multiple ways to survival of cancer cells in tumor hypoxic regions. Because many cancer cells acquire the ability to secrete IGFs in autocrine fashion (4) and IGF-II is reportedly induced by hypoxia (45), the experimental settings in this study might recapitulate the hypoxic tumor microenvironment, which is isolated from the blood perfusion. Further studies are required to clarify the growth factor milieu of hypoxic regions *in vivo*.

[4] H. Endo and M. Inoue, unpublished observation.

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