Inhibition of Protein Synthesis Alters Protein Degradation through Activation of Protein Kinase B (AKT)

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Key words: Protein metabolism; cycloheximide; AKT; protein turnover; protein degradation

Background: Whether a change of protein biosynthesis modulates protein degradation is unknown.

Results: Inhibition of protein biosynthesis induces activation of AKT (protein kinase B), leading to activation of E3 ubiquitin ligase and thus degradation of its substrate proteins.

Conclusion: Protein degradation is regulated by protein biosynthesis.

Significance: These studies reveal a novel regulation of protein degradation.

SUMMARY

The homeostasis of protein metabolism is maintained and regulated by the rates of its biosynthesis and degradation in living system. Alterations of protein degradation may regulate protein biosynthesis through a feedback mechanism. Whether a change of protein biosynthesis modulates protein degradation has not been reported. In the present study, we found that inhibition of protein biosynthesis induced phosphorylation/activation of AKT and led to phosphorylation of AKT target substrates including FoxO1, GSK-3α/β, p70S6 kinase, AS160 and the E3 ubiquitin ligase MDM2. The phosphorylation of ribosomal protein S6 was also modulated by inhibition of protein biosynthesis. The AKT phosphorylation/activation was mainly mediated through the phophoinositide-3-kinase (PI3K) pathway because it was blocked by the PI3K inhibitor LY294002. The activated AKT phosphorylated MDM2 at Ser166 and promoted degradation of the tumor suppressor p53. These findings suggest that inhibition of protein biosynthesis can alter degradation of some proteins through activation of AKT. These studies reveal a novel regulation of protein degradation and call for a caution for studies of protein half-life by blocking protein biosynthesis.

The most common approach to determine turnover or half-life of a protein in cultured cells is to measure the degradation of the protein after blocking its biosynthesis. Cycloheximide, a protein synthesis inhibitor that acts specifically on the 60S subunit of eukaryotic ribosome (1), is widely used for this purpose. However, inhibition of protein synthesis also confers cellular stress. Cells respond to stress in variety of ways ranging from activation of survival pathways to the initiation of programmed cell death that eventually eliminates damaged cells. Whether cells provide a protective or destructive stress response depends...
on the nature and duration of the stress as well as cell type (2). Treatment of rat hepatocytes or astrocytes, human leukocytes and Burkitt lymphoma cells with cycloheximide can induce apoptosis (3-5). Whether responses of cells to protein synthesis inhibitors alter protein turnover is not known.

The serine/threonine kinase AKT (transforming murine retrovirus AKT8 related oncogene), also known as protein kinase B (PKB), is a downstream effector of phosphatidylinositol 3-kinase (PI3K)/3-phosphoinositide-dependent protein kinase 1 (PDK1) and plays key roles in the regulation of cell survival, cell cycle, cell growth and metabolism (6). AKT is activated through its phosphorylation at Thr\(^{308}\) and Ser\(^{473}\) (7,8). There are three isoforms of AKT in mammals, termed AKT1/PKB\(_{\alpha}\), AKT2/PKB\(_{\beta}\), and AKT3/PKB\(_{\gamma}\). These isoforms are products of distinct genes but are highly related, exhibiting >80% protein sequence homology, and share the same structural organization (9). However, the AKT isoforms distribute differently and have different functions despite the high level of homology. AKT1 is expressed in most tissues, and AKT2 is highly expressed in insulin-responsive tissues including adipose tissue, skeletal muscle and liver, whereas AKT3 expression is prominent in the brain and testes (10). Animal models deficient of AKT1, AKT2 or AKT3 have indicated that three AKT isoforms differ in physiological functions. Mice lacking AKT1 demonstrate increased perinatal mortality and reductions in body weight (11,12). In contrast, AKT2-deficient mice exhibit a diabetes-like syndrome with an elevated fasting plasma glucose level, elevated hepatic glucose output, and peripheral insulin resistance (10,13). Although the functions of AKT3 have not been well known, AKT3-deficient mice exhibit a reduction in brain weight resulting from decreases in both cell size and cell number but maintain normal glucose homeostasis and body weight (14,15).

During investigation of regulation and turnover of AKT, we observed that inhibition of protein biosynthesis by cycloheximide induced phosphorylation/activation of AKT and altered its degradation. Furthermore, we found that the degradation of several other proteins was also altered when protein biosynthesis was inhibited and that this phenomenon involved AKT activation.

**MATERIALS AND METHODS**

*Reagents and antibodies*—LY294002, rapamycin, cycloheximide and p53 antibody were products of Sigma-Aldrich Corp. (St. Louis, MO, USA). NSC119889 was purchased from EMD Chemicals, Inc. (Gibbstown, NJ, USA). Antibodies against Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), MDM2 (SMP14) and p-MDM2 (Ser\(^{468}\)) were products of Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). Antibodies against AKT, p-AKT (Ser\(^{473}\)), p-AKT (Thr\(^{308}\)), FoxO1, p-FoxO1 (Ser\(^{256}\)), GSK3\(_{\alpha/\beta}\), p-GSK3\(_{\alpha/\beta}\) (Ser\(^{21/9}\)), AS160, p-AS160 (Thr\(^{42}\)), S6, p-S6 (Ser\(^{235/236}\)), p70S6K and p-p70S6K (Thr\(^{389}\)) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

*Cell culture*—Human embryonic kidney cell line (HEK-293FT) and mouse neuroblastoma cell line (N2a) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin 100 U/ml, streptomycin 100 U/ml and incubated in a humidified atmosphere containing 5% CO\(_2\) at 37°C.

*Plasmid Construction and DNA Mutagenesis*—The expression construct for human AKT1 was generated by reverse-transcription PCR from RNA isolated from normal human neuronal progenitor cells and confirmed by DNA sequence analysis and was obtained from SignalChem (Richmond, BC, Canada). AKT tagged with hemagglutinin (HA) was cloned into pCI-Neo vector via XhoI and Sal I sites. Mutation of Thr-308 or Ser-473 to Ala of AKT was achieved by using the Quik Change II site-directed mutagenesis kit (Stratagene, La Jolla, CA) with primers (5’-ggtgccacatgaagGccttttgccacacctg-3’, and reversed, 5’-caggtgtgccgcaaaaggCcttcatggtggcacc-3’ for Thr-308 to Ala and 5’-ccacttcccccagttcGcctactcggccagcggc-3’, and reversed, 5’-ccagcttggccgaaagggCcttcgttgtggeacc-3’ for Thr-308 to Ala and 5’-ccacttccccagttgCctactcggcagccgcg-3’, and reversed, 5’-gcegctgtggcagtagCgaactgggggatgg-3’ for Ser473 to Ala). The mutation at Thr-308 and Ser-473 to Ala (AKT2A) were produced by using PCR from vector pCI-Neo-HA AKT T308A with the same forward primers used for Ser-473 to Ala. The mutations were confirmed by DNA sequencing analysis.
Transfection—All transfections of HEK-293FT cells were performed with FuGENE 6 (Roche) in 12-well plates. HEK-293FT cells were transfected with 0.5 µg of plasmid DNA and 1.5 µL of FuGENE 6 in 50 µL Opti-MEM (Invitrogen) for 16 hr, and then the cells were treated with protein synthesis inhibitors cycloheximide or NSC119889. Lipofectamine™2000 reagent (Invitrogen) was used for transfection in N2a cells according to the manufacturer’s instructions.

Cycloheximide chase analysis—For cycloheximide chase analysis, after transfection with HA tagged wild-type (WT)-AKT or mutant AKT for 16 hr, HEK-293FT cells were incubated with 100 µM of cycloheximide (diluted from a 355.4 mM stock solution in DMSO) for various times. Cell lysates were prepared and the expression of HA-AKT was analyzed by Western blots.

Cell lysis and Western blots—Whole-cell extracts were prepared using a buffer consisting of 50 mM Tris-HCl, pH 7.4, 150 mM sodium chloride, 1% NP40, 0.25% sodium deoxycholate, 1.0 mM PMSF and the Roche complete mini protease inhibitor cocktail. Protein concentrations of cell lysates were determined by using the Pierce 660 nm Protein Assay Reagent (Thermo Fisher Scientific Inc., Rockford, IL). Western blots were carried out after 10% SDS-PAGE. After incubation with HRP-conjugated secondary antibodies, the protein-antibody complex were visualized by the Pierce ECL Western Blotting Substrate (Thermo Scientific Inc.) and exposed to Kodak medical X-ray film (Kodak, USA). Specific immunostaining was quantified by using the Multi Gauge software V3.0 from Fuji Film.

Statistical analysis—All experiments were repeated at least three times. To determine differences between groups, ANOVA or Student’s t-test was performed, and a p-value of <0.05 was defined to be statistically significant.

RESULTS

Inhibition of protein synthesis promotes phosphorylation and alters degradation of AKT—When we tried to determine the half-life of HA-tagged WT-AKT and mutant AKT, including T308A/S473A (2A), T308A and S473A, by measuring the level of HA-AKT with anti-HA at various time points after treatment with the protein synthesis inhibitor cycloheximide at the concentration (100 µM) that is used in many studies (16,17), we found that AKT with a single mutation (T308A or S473A) was more stable than WT-AKT, whereas the turnover of 2A mutant was more rapid than WT-AKT (Fig. 1a-e). To investigate what made such apparent differences in their turnover, we determined AKT phosphorylation after cycloheximide treatment because some proteins' turnover is regulated by phosphorylation. We found that cycloheximide dramatically induced AKT phosphorylation at Ser473 in HEK-293FT cells transfected with HA-WT-AKT (Fig. 1a). Phosphorylation of AKT at Thr308 was also detected 36 hr after the cycloheximide treatment. The same phenomenon was seen for endogenous AKT when HA-AKT-2A, of which no phosphorylation could occur on residue 308 or 473 of the exogenous AKT, was expressed in HEK-293FT cells (Fig. 1b). Phosphorylation of AKT at Ser473 and Thr308 was also seen in cells expressing AKT single mutants T308A or S473A (Fig. 1c, d). Treatment of the cells with various concentrations of cycloheximide ranging from 0.1 to 300 µM, which covered almost all the concentrations used in published studies, indicated a dose-dependent induction of AKT phosphorylation (Fig. 1f). Dose-dependent inhibition of protein biosynthesis was also evident as the total amounts of cellular proteins were decreased along with increasing concentrations of cycloheximide (Fig. 1f, lower left graph). Correlation analysis indicated a strong negative correlation (r=-0.99) between AKT phosphorylation at Ser473 and the total amounts of cellular proteins (Fig. 1f, lower right graph), suggesting that AKT phosphorylation correlates positively to inhibition of protein biosynthesis.

To investigate whether AKT phosphorylation we observed above was cells’ response to the stress induced by transfection reagent FuGENE-6, we studied cycloheximide-induced AKT phosphorylation in HEK-293FT cells both with and without FuGENE-6 pretreatment for 16 hr. We found that cycloheximide induced an increase in AKT phosphorylation at Ser473 under both conditions (Fig. 1g). No phosphorylation of AKT at Thr308 was detected within 12 hr after cycloheximide treatment. These results suggest that the AKT phosphorylation we observed in HEK-293FT cells...
was induced by cycloheximide, rather than by FuGENE-6.

To learn whether the cycloheximide-induced AKT phosphorylation results from inhibition of protein synthesis, we treated HEK-293FT cells with NSC119889, another protein synthesis inhibitor that acts by preventing mRNA-ribosome interaction and inhibiting 5'-mediated/cap-dependent initiation of protein synthesis (18). Marked increase in AKT phosphorylation at Ser 473 was also observed (Fig. 1h). Because cycloheximide and NSC119889 are two structurally different compounds and inhibit protein synthesis through different mechanisms, our results suggest that inhibition of protein synthesis induces AKT phosphorylation.

We also investigated the effect of protein synthesis inhibition on AKT phosphorylation in mouse neuroblastoma N2a cells. We found that, as in HEK-293FT cells, inhibition of protein synthesis in N2a cells by either cycloheximide or NSC119889 promoted Ser 473 phosphorylation of both endogenous (Fig. 2a) and transfected AKT (Fig. 2b). AKT phosphorylation at Thr 308 was also seen as early as 3 hr after the treatments with the inhibitors in N2a cells. Taken together, these data suggest that inhibition of protein synthesis induces AKT phosphorylation, which does not appear to be a cell type–specific phenomenon.

AKT phosphorylation mediated by inhibition of protein synthesis leads to phosphorylation of multiple AKT substrates—AKT is activated through its phosphorylation at Thr 308 and Ser 473 in response to various stimuli or stresses(19). To study whether protein synthesis inhibition–induced phosphorylation of AKT indeed lead to the activation of its kinase activity, we measured phosphorylation of several well-known AKT substrates, including AKT1 downstream substrates FoxO1, GSK3α/β, p70S6K and ribosomal protein S6 and AKT2 downstream substrate AS160. We found that the treatment of the WT-AKT-expressing HEK-293FT cells with cycloheximide resulted in marked increase in phosphorylation of both the AKT1 and AKT2 substrates (Fig. 3a). To learn whether these substrates are also phosphorylated by endogenous AKT under these conditions, we treated the untransfected HEK-293FT cells with cycloheximide and found similar increase in phosphorylation of all the AKT substrates studied (Fig. 3b). When the cells were treated with NSC119889, marked increase in phosphorylation of FoxO1, p70S6K and AS160, but not of GSK3α/β, was observed (Fig. 1e). In contrast, phosphorylation of ribosomal protein S6 was significantly decreased with NSC119889 treatment. Taken together, these results indicate that inhibition of protein synthesis induces both phosphorylation and activation of AKT and that this phenomenon is not AKT isoform-dependent.

Inhibition of protein synthesis induces AKT phosphorylation/activation mainly through the PI3K/PDK1 pathway—AKT can be activated through phosphorylation at Thr 308 and/or Ser 473 by its upstream kinases, i.e., PI3K/PDK1 or mammalian target of rapamycin (mTOR) complex 2 (mTORC2) (7,20). To investigate through which upstream pathway the protein synthesis inhibitors induced AKT phosphorylation/activation, we treated cultured cells with cycloheximide in the presence of LY294002, a selective inhibitor of PI3K, or rapamycin, an inhibitor of mTOR. We observed that 10 µM LY294002, which is commonly used for selective inhibition of PI3K (21,22) not only blocked the cycloheximide-induced AKT Ser 473 phosphorylation, but also blocked its basal phosphorylation (Fig. 4a,b), suggesting that cycloheximide-induced AKT phosphorylation/activation in HEK-293FT cells is mainly mediated through the PI3K/PDK1 pathway.

Treatment of HEK-293FT cells with 100 nM rapamycin for 24 hr inhibited AKT Ser 473 phosphorylation, but the treatment for 3 hr instead increased AKT Ser 473 phosphorylation (Fig. 4a,b). The latter might have resulted from the known rapamycin-induced feedback activation of PI3K/PDK1 (8,23,24). Cycloheximide-induced AKT Ser 473 phosphorylation was also inhibited by rapamycin at 24 hr post treatment. Additive effects were seen when both LY294002 and rapamycin were used. Similar results were seen in HEK-293FT cells transfected with WT-AKT (Fig. 4c). As cycloheximide did not induce detectable phosphorylation of AKT at Thr 308 during 24 hr treatment (Fig. 1a), phosphorylation at this site could not be studied here. Taken together, these results suggest that inhibition of protein synthesis induces AKT phosphorylation/activation mainly through the PI3K/PDK1 pathway, but the mTOR pathway could also play a minor role.
Inhibition of protein synthesis induces activation of the E3 ubiquitin ligase MDM2 and degradation of its substrate proteins—Most cellular proteins are degraded through the ubiquitin-proteasome system, in which the E3 ubiquitin ligases are critical. MDM2 is an E3 ligase and is known to be regulated by AKT (25,26). Phosphorylation of MDM2 by activated AKT at Ser^{166} and Ser^{186} activates MDM2 and promotes its nuclear entry (25,26). To determine whether cycloheximide treatment increases MDM2 phosphorylation via AKT activation, we treated HEK-293FT cells transfected with HA-WT-AKT with cycloheximide and observed time-dependent increases in phosphorylation of both AKT at Ser^{473} and MDM2 at Ser^{166} (Fig. 5a-c). The cycloheximide-induced phosphorylation of both proteins could be blocked by PI3K inhibitor LY294002 (20 µM).

The tumor suppressor p53 is a known substrate of MDM2, and MDM2 plays a central role in p53 ubiquitination and rapid degradation by the 26S proteasome (27,28). To study whether cycloheximide-induced phosphorylation/activation of MDM2 leads to p53 degradation, we determined p53 level and found rapid decrease in the p53 level (Fig. 5d) along with phosphorylation/activation of AKT and MDM2 (Fig. 5a-c). This decrease was not the consequence of merely protein synthesis inhibition by cycloheximide because inhibition of AKT and MDM2 phosphorylation with LY294002 in the presence of cycloheximide completely blocked the p53 reduction (Fig. 5d). Because the level of AKT and MDM2 was also decreased after the cycloheximide treatment for 60-120 min, which is consistent with self ubiquitination and degradation upon their activation (29), the total phosphorylated MDM2 and AKT decreased slightly after those time points (Fig. 5a,b). However, after normalization with the corresponding protein level, clear time-dependent increases in phosphorylation of these two proteins were seen with the cycloheximide treatments (Fig. 5a,c). These data indicate that inhibition of protein synthesis can alter the degradation of some proteins via activation of AKT and its downstream E3 ligase.

DISCUSSION

The homeostasis of protein metabolism is maintained and regulated by the rates of its biosynthesis and degradation in living system. In some cases, alterations of protein degradation regulate protein biosynthesis through a feedback mechanism. Whether a change of protein biosynthesis modulates protein degradation has not been reported. In the present study, we found that inhibition of protein synthesis by cycloheximide or NSC119889 induced AKT phosphorylation/activation mainly through the PI3K pathway. AKT signaling regulates many aspects of biological functions including cell survival, proliferation, metabolism, cell migration and metastasis. Activated AKT can phosphorylate the E3 ubiquitin ligase MDM2 and promote MDM2 nuclear entry, and promote p53 degradation via ubiquitin-proteasome pathway (25,26). We thus postulate that cycloheximide alters protein turnover via activation of AKT and its downstream substrates including MDM2. The present study showed increased degradation of two protein examples (MDM2 and p53) when protein biosynthesis was inhibited with cycloheximide or NSC119889.

Accumulating evidence has shown that AKT can be activated by various cellular stresses such as heat, oxidative stress, hyperosmotic stress and sodium arsenite (30). AKT contains a pleckstrin homology (PH) domain that binds to PIP3 [phosphatidylinositol (3,4,5)-triphosphate] with high affinity. Once at correct position in the plasma membrane, AKT can be phosphorylated by PDK1 mainly atThr^{308} (7) and by mTORC2 mainly at Ser^{473} (8). In the present study, we found that the cellular stress of protein biosynthesis inhibition activated AKT. As the phosphorylation/activation of AKT was completely blocked by PI3K inhibitor LY294002 and was also partially affected by the mTORC inhibitor rapamycin, we concluded that the protein biosynthesis inhibition–induced AKT phosphorylation/activation is mainly mediated through the PI3K pathway. Consistent with our observations, Hemi et al. reported that translational inhibitors activate the trans-activation of ErbB2/ErbB2 receptors, which lead to the activation of PI3K regulated pathway (19). AKT activation is also induced by oxidative stress via the epidermal growth factor receptor/PI3K pathway (30).
Inhibition of protein synthesis has been shown to be protective in various apoptosis models (31,32). We speculate that AKT phosphorylation/activation under this condition might underlie this protective role, because AKT signaling is anti-apoptotic (33). Activated AKT can phosphorylate and inactivate the pro-apoptotic proteins Bad and caspase-9 (34-36), leading to inhibition of apoptosis and promotion of cell survival.

The AKT kinase family includes three highly homologous isoforms: AKT1, AKT2 and AKT3. The development of AKT isoform-specific null mice has proven a functional diversity of AKT isoforms in physiology and in disease, although they have overlapping functions. In the present study, we found increased phosphorylation of both AKT1 and AKT2 substrates when protein synthesis was inhibited. These results suggest that protein synthesis inhibition–induced phosphorylation/activation of AKT may not be AKT isoform-dependent.

Rapamycin is a potent inhibitor of mTORC1 (37). We observed that the treatment of HEK-293FT cells with rapamycin for 24 hr partially inhibited protein synthesis inhibition–induced phosphorylation/activation of AKT, which is consistent with previous studies showing that prolonged rapamycin treatment also inhibits mTORC2 and AKT/PKB (38). Interestingly, we observed AKT activation after the treatment of cells with rapamycin for 3 hr. This short-term phenomenon could be mediated by a feedback activation of AKT signaling through an insulin-like growth factor-1 receptor (IGF-1R) mechanism (8,23,24).

Ubiquitination of proteins occurs through three steps that require ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3) (39,40). Ubiquitin itself contains seven lysine residues, and each of these can be further conjugated with the carboxyl terminus of another ubiquitin to form a polyubiquitin chain (41). As the three-steps process advances, specificity increases: E1 interacts with all E2s, which then interact with a more limited subset of E3s. Each E3 in turn targets only a limited array of protein substrates based on shared recognition motif within the proteins to be labeled. This enables the ubiquitination-proteasome pathway to be specific in the selection of the proteins to be labeled (42). The human proteome contains two ubiquitin E1 enzymes, approximately 50 E2s and 600 E3s (43). It is the specificity of the E3 enzyme that determines which proteins in the cell are to be marked for destruction in the proteasome (43).

Although MDM2 originally functions as the E3 ligase to degrade p53 (44-46), MDM2 also targets other proteins, such as androgen receptor (47) and Numb (a protein that plays an important role in specifying cell fate during development) (48,49), for ubiquitination and degradation. Because we observed an increase in the phosphorylation of MDM2 despite of its elevated degradation after treatment of cells with cycloheximide, we speculate altered degradation or half-lives of the proteins that are substrates of MDM2, as a consequence of MDM2 activation, when protein biosynthesis is inhibited.

Activation of AKT in response to inhibition of protein synthesis could alter degradation of other cellular proteins. It has been reported that activated AKT phosphorylates a deubiquitinating enzyme called USP8 at Thr907, resulting in its activation and stabilization, which in turn stabilizes the ubiquitin ligase Nrdp1 and promotes degradation of growth factor receptor ErbB3 (50). Activated AKT1, but not AKT2, phosphorylates the ubiquitin ligase Skp2 at Ser72 and protects it from Cdh1-mediated degradation through disruption of the interaction between Skp2 and its E3 ligase Cdh1 (51). Importantly, the E3 ubiquitin ligase Skp2 targets a growing number of proteins, including the transcription factors E2F1 (52) and c-myc (53,54), cdk inhibitors (cdkis) p21 Cip1 (55) and p27Kip1 (56), and Forkhead transcription factors FoxO1 (57). Collectively, inhibition of protein synthesis may alter turnover of many proteins via activation of AKT and its downstream ubiquitin ligases.

Our observations of increased p53 degradation with cycloheximide treatment are consistent with a previous study showing that the chemo preventive agent apigenin inhibits p53 ubiquitination and increases its stability (58). Apigenin can actually lead to a dose- and time-dependent decrease in AKT phosphorylation at Ser473 (59). In light of our observations, apigenin-induced AKT inhibition may underlie the apigenin-mediated p53 stabilization. AKT activation also promotes proteasome-dependent...
degradation of AKT substrates tuberin, FoxO1 and FoxO3a (60).

It is worth noting that inhibition of protein biosynthesis by either cycloheximide or NSC119889 increased phosphorylation of AKT, and its substrates FoxO1, p70S6K and AS160. However, cycloheximide but not NSC119889 led to increased phosphorylation of GSK-3α/β and S6. The exact mechanism leading to these differential consequences with these two inhibitors remains to be investigated. These observations suggest that besides inhibition of protein biosynthesis, NSC119889 might have an additional activity that would have stimulated other signaling pathways and in turn counteracted the AKT-induced phosphorylation of GSK-3α/β and S6 when protein biosynthesis is inhibited. This additional activity might also affect turnover of some proteins.

A common approach to determine protein half-life is to measure the remaining amount of a protein throughout a period of time after new protein biosynthesis is completely blocked. Cycloheximide is most commonly used for this purpose in biological research. It blocks protein synthesis through interfering with the translocation step (movement of two tRNA molecules and mRNA in relation to the ribosome) and thus blocking translation elongation (61). The present study indicates that inhibition of protein synthesis, such as with cycloheximide, alters turnover of some proteins via activation of AKT. Therefore, for determination of the half-lives of these affected proteins, the approach using protein synthesis inhibitors should be avoided. Pulse-chase radio-labeling analysis, which does not require inhibition of protein biosynthesis, should be used instead.

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REFERENCES


**FIGURE LEGENDS**

**Fig. 1.** Inhibition of protein synthesis induces AKT phosphorylation in HEK-293FT cells. (a-d) HEK-293FT cells were transfected with plasmids expressing HA-WT-AKT (a), HA-AKT-2A (b), HA-AKT-T308A (c) or HA-AKT-S473A (d) for 16 hr, followed by the treatment with 100 µM cycloheximide for the indicated periods of time. The levels and site-specific phosphorylation of AKT in the cell lysates were determined by Western blots. (e) Quantification of AKT level (HA blots) after normalization with GAPDH. (f) After transfection with plasmids expressing HA-WT-AKT for 16 hr, equal amounts of HEK-293FT cells were treated with the indicated concentrations of cycloheximide for 3 hr. The levels of AKT and its phosphorylation in the cell lysates were determined by Western blots. The quantification of the AKT-pS473 blots after normalization with the HA blots are presented at the right side of the blots. The total amounts of cellular proteins were also determined and are presented in relative to that of untreated cells (lower left graph). Linear correlation between AKT-pS473 and the total protein amounts is shown in the lower right graph. (g) HEK-293FT cells with (right panel) or without (left panel) FuGENE 6 pretreatment for 16 hr were treated with 100 µM cycloheximide and harvested at the indicated time points. (h) HEK-293FT cells were treated with 50 µM NSC119889 for 3 hr, and the cell lysates were analyzed by Western blots. The experiments were repeated 3-4 times, and the graphs in panels (e) to (h) are quantification of the blots and are presented as mean ± SEM. *p<0.05 and **p<0.01 vs. 0 hr or 0 µM controls.

**Fig. 2.** Inhibition of protein synthesis induces AKT phosphorylation in N2a cells. N2a cells untransfected (a) or transfected with HA-tagged WT-AKT (b) for 16 hr were treated with 100 µM cycloheximide or 50 µM NSC 119889, and then the cells were harvested at indicated time points. The levels and site-specific phosphorylation of AKT in the cell lysates were determined by Western blots. Quantifications of the blots are shown in the graphs under the blots.

**Fig. 3.** Inhibition of protein synthesis induces phosphorylation of AKT substrates. (a) HEK-293FT cells were transfected with HA-WT-AKT for 16 hr, followed by the treatment with 100 µM cycloheximide for 3 hr. Then the levels and phosphorylation of AKT and its substrates were analyzed by Western blots. (b,c) HEK-293FT cells were treated with 100 µM cycloheximide (b) or 50 µM NSC 119889 (c) for the indicated periods of time, and the cell lysates were analyzed. Lower graphs show the densitometrical quantifications (mean ± SD) of the phosphorylation of individual AKT substrates, which were calculated after being normalized by the levels of the corresponding proteins.

**Fig. 4.** LY294002 and rapamycin inhibit AKT phosphorylation induced by cycloheximide. (a) HEK-293FT cells were treated with 100 µM cycloheximide, 10 µM LY294002 (LY) or 100 nM rapamycin (Rap), or in combination, for 3 or 24 hr, followed by analysis of the level and phosphorylation of AKT using Western blots. GAPDH blot was included as a loading control. (b) Quantification of the blots shown in (a). Relative AKT phosphorylation (mean ± SEM) after being normalized by the total AKT levels is shown. (c) HEK-293FT cells transfected with WT-AKT for 16 hr were treated with cycloheximide, LY294002 or rapamycin for 3 or 24 hr, followed by analysis of the levels and phosphorylation of AKT using Western blots.

**Fig. 5.** Cycloheximide induces p53 turnover through activation of AKT. (a) HEK-293FT cells were pretreated with or without 20 µM LY294002 for 1 hr, followed by the treatment with 100 µM cycloheximide for the indicated periods of time. The cell lysates were then analyzed by Western blots. (b,c) Quantifications of the blots shown on the left side (in the absence of LY294002) of panel (a). (d) Quantifications of p53 levels. The data are representative of three independent experiments with similar results.
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Inhibition of protein synthesis alters protein degradation through activation of protein kinase B (AKT)

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