eEF-2 Kinase Dictates Cross-Talk between Autophagy and Apoptosis Induced by Akt Inhibition, Thereby Modulating Cytotoxicity of Novel Akt Inhibitor MK-2206

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Abstract

Inhibition of the survival kinase Akt can trigger apoptosis, and also has been found to activate autophagy, which may confound tumor attack. In this study, we investigated regulatory mechanisms through which apoptosis and autophagy were modulated in tumor cells subjected to Akt inhibition by MK-2206, the first allosteric small molecule inhibitor of Akt to enter clinical development. In human glioma cells, Akt inhibition by MK-2206 or siRNA-mediated attenuation strongly activated autophagy, whereas silencing of eukaryotic elongation factor-2 (eEF-2) kinase, a protein synthesis regulator, blunted this autophagic response. Suppression of MK-2206–induced autophagy by eEF-2 silencing was accompanied by a promotion of apoptotic cell death. Similarly, siRNA-mediated inhibition of eEF-2 kinase potentiated the efficacy of MK-2206 against glioma cells. Together, these results showed that blunting autophagy and augmenting apoptosis by inhibition of eEF-2 kinase could modulate the sensitivity of glioma cells to Akt inhibition. Our findings suggest that targeting eEF-2 kinase may reinforce the antitumor efficacy of Akt inhibitors such as MK-2206. Cancer Res; 71(7); 2654–63. ©2011 AACR.

Introduction

Akt/protein kinase B, an oncoprotein with serine/threonine kinase activity, plays a central role in cell signaling downstream of growth factors. The Akt family consists of Akt1, Akt2, and Akt3, all of which share a similar domain structure (1). Aberrant activation of Akt promotes cell growth, survival, and proliferation, and is associated with cancer development and progression (2). Thus, Akt represents an attractive target for therapeutic intervention against cancer. The anticancer action of Akt inhibitors is believed to result from induction of apoptosis triggered by Akt inhibition (3). Recently, it has been found that autophagy, a cellular process that degrades cytoplasmic components via the lysosomal machinery, is also activated when Akt is inhibited (4, 5). Although activation of autophagy (also termed type II programmed cell death) can promote apoptotic cell death, this form of intracellular degradative processes has also been appreciated to support cell survival (6, 7). Yet, the exact effects of autophagy on the cytoidal activity of Akt inhibitors, and the functional association of autophagy with apoptosis in response to Akt inhibition, remain largely unknown, but this knowledge should be useful to optimizing the use of Akt inhibitors, which are actively being developed as anticancer agents.

Eukaryotic elongation factor-2 (eEF-2) kinase, also known as calmodulin-dependent protein kinase III, is a unique calcium/calmodulin-dependent enzyme that regulates protein synthesis (8). The only known substrate of this kinase is eEF-2, a 95 kDa protein that promotes ribosomal translocation from the A site to the P site, the reaction that induces movement of mRNA along the ribosome during translation in eukaryotic tissues. eEF-2 kinase phosphorylates eEF-2 on Thr56 and terminates peptide elongation by decreasing the affinity of this kinase for the ribosome (9). The implication of eEF-2 kinase in cancer was suggested by the observation that this kinase is upregulated in various types of neoplasms such as malignant glioma and breast cancer (10–13), and that inhibiting this kinase results in a decreased viability of tumor cells (14). More recently, we have reported that eEF-2 kinase can act as a positive regulator of autophagy under environmental or metabolic stresses, including nutrient deprivation (15), growth factor inhibition (16), and energy stress caused by the glycolytic inhibitor, 2-deoxy-D-glucose (17). As the Akt-mediated signaling participates in the regulation of protein synthesis through mTOR and S6 kinase, a pathway known to control the activity of eEF-2 kinase, in this study we sought to determine whether eEF-2 kinase was involved in the activation of autophagy caused by Akt inhibition, and whether and how autophagy regulated by this kinase affected the cytoidal

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efficacy of MK-2206, a small molecule and allosteric inhibitor of Akt (18, 19). We found that inhibiting Akt by either MK-2206 or by RNA interference induced an eEF-2 kinase–dependent, cytoprotective autophagic response, and suppression of eEF-2 kinase enhanced the effectiveness of MK-2206 against glioma cells by promoting the switch from autophagy to apoptotic cell death.

Materials and Methods

Cell lines and culture
The human glioblastoma cell lines, T98G and LN-229, were purchased from and genetically characterized by American Type Culture Collection. T98G cells were cultured in Ham’s F-10/DMEM (10:1) medium, and LN-229 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) medium. These media were supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO2/95% air. All cultures were monitored routinely and found to be free of contamination by mycoplasma or fungi. All cell lines were discarded after 3 months and new lines propagated from frozen stocks.

Reagents and antibodies
MK-2206 was a gift from Merck & Co. Inc. Bafilomycin A1, acridine orange (AO), MTT, 7,7’-dichlorofluorescin diacetate (DCF-DA), and 3-methyladenine (3-MA) were purchased from Sigma. Anti-microtubule-associated protein 1 light chain 3 (LC3), anti-α-tubulin, anti-p62, anti–phospho-eEF-2 (Thr56), anti–eEF-2, anti–eEF-2 kinase, anti-Akt, anti-Akt1, anti-Akt2, anti-Akt3, anti–phospho-mTOR, anti-mTOR, anti–phospho-S6 kinase (Ser371), anti–S6 kinase, anti-PARP, anti-actinomycin D (7-AAD) and anti–cytochrome c, and anti–COX-IV antibodies were purchased from Cell Signaling Technologies. Anti–hypoxia-inducible factor (HIF)-1α antibody was purchased from BD Biosciences. Anti–tuberous sclerosis complex 2 (TSC2) and anti–BCL2 adenovirus E1B 19kDa protein-interacting protein 3 (BNIP3) antibodies were purchased from Santa Cruz. All cell culture media and other reagents were purchased from Invitrogen. Western blot reagents were obtained from Pierce Biotechnology.

siRNA transfection
siRNA duplexes targeting eEF-2 kinase, Akt1, Akt2 and Akt3 were prepared by Dharmacon Research. Nonsilencing scrambled (nontargeting) siRNA was used as a control. Transfection of siRNA was performed according to the manufacturer’s protocol. Briefly, cells in exponential phase of growth were plated in 6-well tissue culture plates at 1 x 10^5 cells per well, grown for 24 hours, then transfected with siRNA by using Oligofectamine and OPTI-MEM I–reduced serum medium. The concentrations of siRNAs were chosen on the basis of dose–response studies.

Measurement of autophagy
Autophagy was monitored by using the following methods as described previously (15, 16): (i) Western blot analysis of LC3; (ii) microscopic observation of green fluorescent protein (GFP)-LC3 puncta; (iii) flow cytometry analysis of AO staining for acidic organelles; (iv) electron microscopic examination of double- or multimembrane vacuoles in the cytoplasm.

Western blot analysis
Cells were lysed in M-PER mammalian protein extraction reagent (Thermo Scientific) supplemented with a protease inhibitor cocktail (Roche) at room temperature for 5 minutes followed by centrifugation at 14,000 × g for 10 minutes. Protein concentrations of the cell lysates were measured by the Bio-Rad DC assay (Bio-Rad). Proteins (20–40 μg) were resolved on SDS-PAGE and transferred to polyvinylidene difluoride membrane (Bio-Rad). The blots were incubated with indicated antibodies in 3% bovine serum albumin/Tris-buffered saline Tween-20 at 4°C for overnight followed by incubation with secondary antibodies at room temperature for 1 hour. The protein signals were detected by enhanced chemiluminescence method.

Apoptosis assays
Apoptosis was determined by the following procedures.

Flow cytometric analysis of Annexin V and 7-amino-actinomycin D (7-AAD) staining. Briefly, 100 μL Guava Nexin reagent (Millipore) was added to 1 x 10^5 cells (in 100 μL) and the cells were incubated with the reagent for 20 minutes at room temperature in the dark. At the end of incubation, the cells were analyzed by a Guava EasyCyte Plus Flow Cytometry System (Millipore).

Flow cytometric analysis of activated caspases. The Guava multi-caspase assay protocol (Millipore) was followed. Briefly, 10 μL of the Guava caspase detection reagent (Millipore) was added to 1 x 10^5 cells (in 100 μL) and the cells were incubated for 1 hour at 37°C in a humidified atmosphere containing 5% CO2/95% air. At the end of incubation, the cells were washed twice with washing buffer, resuspended in 200 μL of the caspase/7-AAD working solution (Millipore), and then further incubated for 10 minutes at room temperature. Populations of cells with activated caspases were analyzed on an EasyCyte Plus Flow Cytometry System (Millipore).

Measurement of mitochondrial membrane potential
Mitochondrial membrane potential was measured by flow cytometry following JC-1 staining. Briefly, 4 μL of Guava cell staining solution (including JC-1 and MitoPotential 7-AAD) from Millipore was added to 2 x 10^5 cells (in 200 μL) and the cells were incubated with the reagent for 30 minutes at a 37°C in a humidified atmosphere containing 5% CO2/95% air. At the end of incubation, the cells were analyzed by a Guava EasyCyte Plus Flow Cytometry System.

Detection of ROS
Reactive oxygen species (ROS) generation was measured by flow cytometry. Briefly, treated cells were collected and incubated with 10 μmol/L of DCF-DA for 30 minutes at 37°C in a humidified atmosphere containing 5% CO2/95% air. At the end of incubation, the samples were analyzed on an EasyCyte Plus Flow Cytometry System.
Inhibition of Akt activates an eEF-2 kinase–dependent autophagy in glioma cells

Inhibition of Akt can trigger apoptosis, and this is believed to account for the antitumor effect of Akt inhibitors. To better understand how inhibiting Akt produces antitumor actions and the possible influence of autophagy, we examined the effects of the Akt inhibitor, MK-2206 (18), on the level of LC3 II, a hallmark of autophagy. We observed that treatment with MK-2206 caused a robust, concentration-dependent activation of autophagy in human glioma cell lines LN229 and T98G, as evidenced by an increase in the amount of LC3 II (Fig. 1A). LC3 II level was also elevated in the presence of bafilomycin A1, an inhibitor of autophagosome–lysosome fusion and LC3 II degradation, indicating that autophagic flux was enhanced by this Akt inhibitor (Fig. 1B). The stimulatory effect of MK-2206 on autophagy was confirmed by an increase in the amount of LC3 II (Fig. 1A), indicating that autophagic response to MK-2206 treatment was blunted by inactivation of eEF-2 kinase.

To corroborate the involvement of eEF-2 kinase in the Akt inhibition–activated autophagy and to assess whether the effect of inhibiting Akt on autophagy was isozyme-selective, we silenced the expression of Akt1, Akt2, and Akt3 using the siRNAs specific for each of these Akt isozymes. We found that respective silencing of Akt1, Akt2, or Akt3 expression all caused an increase in the amount of LC3 II (Fig. 2C), indicating that inhibition of each of these isozymes contributes to induction of autophagy. Depletion of eEF-2 kinase by siRNA also blunted autophagy induced by knockdown of all these Akt isozymes (Fig. 2C), providing further support for a role of this enzyme in mediating autophagy activated by Akt inhibition.

The TSC2/mTOR/S6 kinase/eEF-2 kinase pathway is involved in activation of autophagy by MK-2206

To explore the pathways that mediate the effect of eEF-2 kinase on Akt inhibition–induced autophagy, we knocked down TSC2, a tumor suppressor protein that is negatively regulated by Akt and suppresses mTOR activity (20). As shown in Figure 3, knockdown of TSC2 led to a reduction in the amount of LC3 II in the cells treated with MK-2206, in comparison with the nontargeting controls (Fig. 3A). Concomitantly, knockdown of TSC2 also resulted in a decreased activity of eEF-2 kinase (decreased phospho-eEF-2), an increased activity of S6 kinase (increased phospho-S6 kinase) and mTOR (increased phospho-mTOR; Fig. 3B). These results suggest that induction of autophagy by Akt inhibition is mediated via the TSC2/mTOR/S6 kinase/eEF-2 kinase pathway.

Silencing of eEF-2 kinase augments apoptosis in glioma cells subjected to Akt inhibition

As the pro-survival or pro-death role of autophagy in stressed cells is often associated with alteration of apoptosis, we next queried whether there was a functional relationship between apoptosis and the eEF-2 kinase–mediated autophagy when Akt was inhibited. We compared the degree of apoptosis in the MK-2206–treated glioma cells with or without silencing of eEF-2 kinase. As shown in Figure 4A and B, MK-2206 caused a concentration-dependent increase in apoptosis in the treated cells, as manifested by increases in Annexin V staining (Fig. 4A) and in activated pan-caspases (Fig. 4B); notably, silencing of eEF-2 kinase strengthened the apoptotic effect of MK-2206 in the glioma cells (Fig. 4A and B).

Hypoxic microenvironments of solid tumors are believed to be linked to the malignant features, including poor response...
to therapies. Thus, we next assessed whether suppression of eEF-2 kinase–mediated autophagy could also sensitize hypoxic glioma cells to the apoptogenic effect of MK-2206. As shown in Figure 4C, under hypoxic condition, MK-2206 induced a stronger autophagic response and higher eEF-2 kinase activity than those under normoxic condition; knock-down of eEF-2 kinase not only blunted autophagy activated by MK-2206 and hypoxia (Fig. 4D), but also further amplified apoptosis in the MK-2206–treated glioma cells subjected to hypoxia (Fig. 4E). To test whether these findings are relevant to in vivo circumstance, we inoculated mice with LN229 cells either with or without knockdown of eEF-2 kinase, and then treated the tumor-bearing mice with MK-2206 (120 mg/kg, p. o.). We observed that following MK-2206 treatment, the tumor xenografts with knockdown of eEF-2 kinase had a lower level of the autophagy marker, LC3 II, but a higher level of the apoptosis marker, cleaved caspase-3, than xenografts without eEF-2 kinase knockdown (Fig. 4F, top). TUNEL assay also
showed an increase of apoptosis in tumor specimens with knockdown of eEF-2 kinase (Fig. 4F, bottom). These in vivo tests were performed by a nonorthotopic xenograft model, and it remains to be shown that these findings are relevant to glioma in an orthotopic setting.

Mitochondrial ROS is a mediator of the MK-2206–stimulated autophagy and apoptosis

To understand the mechanism governing the functional connection between autophagy and apoptosis in the MK-2206–treated tumor cells and the role of eEF-2 kinase in these cellular processes, we determined the effects of MK-2206 and eEF-2 kinase on mitochondrial integrity and ROS production, as mitochondria and ROS play an important role in the regulation of both apoptosis and autophagy (21, 22). As shown in Figure 5A and B, treatment of LN229 or T98G cells with MK-2206 impaired the mitochondrial integrity, as evidenced by a decline in mitochondrial membrane potential (ΔΨm; Fig. 5A) and an increase in release of cytochrome c into cytosol (Fig. 5B). Knockdown of eEF-2 kinase aggravated the MK-2206–induced loss of mitochondrial integrity (Fig. 5A and B). MK-2206 also stimulated the generation of ROS (Fig. 5C and D), and knockdown of eEF-2 kinase further increased the production of ROS (Fig. 5D). The overproduction of ROS appeared to be, at least partially, responsible for activation of both autophagy and apoptosis, as cotreatment with MK-2206 and the ROS scavenger, N-acetyl cystein (NAC), weakened the stimulatory effect of MK-2206 on both autophagy (Fig. 5E) and apoptosis (Fig. 5F). Because mitochondria are a source of ROS production, to further show the importance of the mitochondria in the MK-2206–induced autophagy and apoptosis and the role of the eEF-2 kinase–regulated autophagy in mitigating cellular stress, we compared the level of the mitochondrial marker cytochrome c oxidase subunit IV (COX IV), a specific marker for mitochondria, in the MK-2206–treated cells with or without silencing of eEF-2 kinase. Figure 5G shows that silencing of eEF-2 kinase, which can suppress autophagy (Figs. 2 and 4), rescued the decreases of COX IV in cells treated with MK-2206, suggesting that suppression of autophagy slowed down the degradation of the damaged mitochondria. The results shown in Figure 5H demonstrated an increase of the amount of LC3 II mainly in the mitochondrial fraction of the MK-2206–treated cells, verifying the mitochondrial autophagy that was activated by MK-2206.

Suppression of autophagy via inhibiting eEF-2 kinase enhances the cytotoxic effect of MK-2206 in human glioma cells

To evaluate whether the eEF-2 kinase–mediated autophagy has an impact on the antitumor action of Akt inhibitors, we determined the effects of inhibiting eEF-2 kinase and autophagy on viability of the glioma cells treated with MK-2206. We found that the cytotoxicity of MK-2206 against T98G and LN229 cells was significantly greater when eEF-2 kinase was silenced, as compared with that in the cells transfected with a nontargeting RNA (Fig. 6A). Figure 6B shows that enhanced
sensitivity to MK-2206 was also achieved in glioma cells cotreated with 0.25 μmol/L of a small molecule inhibitor of eEF-2 kinase, NH125 (14). To ascertain that the reinforcing effect of inhibiting eEF-2 kinase on cytocidal activity of MK-2206 was attributed to suppression of autophagy, we tested MK-2206 in the glioma cells, in which autophagy was suppressed by either silencing of beclin 1, a key autophagy regulator, or by use of 3-MA, a chemical inhibitor of autophagy. We observed that the cytocidal activity of MK-2206 was also markedly increased in the cells subjected to silencing of beclin 1 expression (Fig. 6C) or with the treatment of 1 mmol/L of 3-MA (Fig. 6D), suggesting that inhibition of autophagy can indeed sensitize tumor cells to the Akt inhibitor, MK-2206.

### Discussion

What exact roles autophagy plays in response of tumor cells to cancer therapies, what signaling pathways are involved in the regulation of autophagy, and how to exploit autophagy in devising novel strategies for cancer therapy, are the questions being actively and extensively investigated. Inhibiting Akt, a serine/threonine kinase mediating mitogenic and antiapoptotic responses that result from activation of multiple signaling cascades, has been shown to induce autophagy (4, 5) and to promote apoptotic cell death and suppress proliferation and growth of tumor cells (23); yet, whether and how autophagy affects the efficacy of Akt inhibitors, a promising and new class of cancer therapeutic agents, remains poorly understood. The results of this study informs that autophagy induced by Akt inhibition can indeed affect the cytotoxicity of Akt inhibitors, and that manipulating autophagic activity can modulate sensitivity of tumor cells to the cytoidal effect of Akt inhibitors. We show that, like other Akt inhibitors (5), the novel allosteric Akt inhibitor, MK-2206, could induce autophagy in tumor cells (Figs. 1, 2B, 3A, and 4C). Moreover, we show in this study that autophagy induced by Akt inhibition was cytoprotective and could mitigate the cytotoxicity of the Akt inhibitor, as blunting autophagic response by either genetic or pharmacologic approaches could enhance the cytocidal efficacy of MK-2206 (Fig. 6) and the apoptotic cell death induced by the compound (Fig. 4). Furthermore, we revealed that eEF-2 kinase, a unique calcium/calmodulin-dependent enzyme that regulates protein synthesis and is overexpressed in malignant gliomas and other malignancies, played an essential role in regulating autophagy that is activated by Akt inhibition (Fig. 2), and this regulatory role appeared to be mediated through the TSC2/mTOR/S6 kinase pathway (Fig. 3). We have previously reported that eEF-2 kinase serves as a central component of the mammalian macroautophagy pathway that is activated in response to environmental or metabolic stress (15–17). This study further underscores the importance of this kinase as a regulator of
autophagy. However, the precise molecular mechanism by which eEF-2 kinase controls autophagy is yet to be defined.

As reported previously (3–5) and shown in this study (Figs. 1, 2, and 4), inhibition of Akt can induce both autophagy and apoptosis; nevertheless, how apoptosis and autophagy affect cellular sensitivity to Akt inhibitors remains unclear. Our observation indicates that the Akt inhibition–activated autophagy plays a protective role in tumor cells, as suppression of autophagy can enhance the cytocidal activity of the Akt inhibitor, MK-2206. Then, is there a functional association between autophagy and apoptosis in cells subjected to Akt inhibition, and how is the interplay between these 2 cellular processes controlled? To answer these questions, we examined the effects of MK-2206 and eEF-2 kinase activity on level of ROS, a critical regulator of both apoptosis and autophagy, and on integrity of mitochondria, which are both source and target of ROS. We found that MK-2206 increased ROS level in a concentration-dependent manner (Fig. 5C, top) and enhanced mitochondrial permeability (Fig. 5A and B) in the treated tumor cells. Thus, loss of mitochondrial integrity and overproduction of ROS may account for activation of apoptosis; also, excess of ROS and activation of the TSC2/mTOR/S6 kinase/eEF-2 kinase pathway (Fig. 3B) both may contribute to induction of autophagy, which appears to favor cell survival. Of note, when autophagy was blunted by inhibition of eEF-2 kinase, degradation of the damaged mitochondria was
retarded (Fig. 5G), and this might further increase the accumulation of ROS. Indeed, MK-2206 activated a mitochondrial autophagy, as an upregulation of the autophagy marker, LC3 II, was only detected in the mitochondrial fractions of the treated cells (Fig. 5H). On the contrary, inhibiting eEF-2 kinase causes a decline in cellular ATP content due to the upregulation of protein synthesis (16, 17), and depletion of the cellular ATP can also impair mitochondrial function and facilitate ROS generation, which, consequently, can elicit more apoptotic cell death. This may explain the augmentation of MK-2206 cytotoxicity by inhibiting eEF-2 kinase, which was observed in this study. It is likely that ROS serves as a mediator in these cellular processes.
processes, as NAC, a ROS scavenger, could block the autophagy (Fig. 5E) and apoptosis (Fig. 5F) activated by MK-2206, although not completely, probably due to the dual effects of ROS on autophagy and apoptosis. On the basis of the results of this study, we believe that eEF-2 kinase could act as an essential regulator controlling the switch between autophagy and apoptosis. On the basis of the results of this study, we believe that eEF-2 kinase could act as an essential regulator controlling the switch between autophagy and apoptosis.

Because of the central role of Akt in oncogenic signaling and in tumor progression, Akt is considered one of the major targets for development of anticancer drugs. In fact, several inhibitors of Akt are being tested in clinic as promising cancer therapeutic agents (24–26); nevertheless, these compounds seem to have limited activity as a single agent. Strategies for reinforcing the activity of the Akt inhibitors would make this type of drugs more valuable in treatment of cancer. Our findings that suppression of the eEF-2 kinase–mediated autophagy can promote apoptotic cell death and augment the cytotoxicity of MK-2206, even under hypoxic condition, may provide a rationale for designing new and more effective therapeutic intervention for treatment of malignant tumors such as gliomas, that is, combining an Akt inhibitor with an eEF-2 kinase inhibitor to shuttle cells into more reliable and permanent pathways of cell death. Evaluation of this strategy in preclinical...
glioma and other tumor models is underway. Considering that MK-2206 is now under clinical development as a new anticancer agent, and that we have already identified an inhibitor of eEF-2 kinase (14), the results reported here may have translational potentials and a direct impact on cancer therapy.

In summary, the results reported here show that suppressing autophagy and augmenting apoptosis via inhibition of eEF-2 kinase can modulate sensitivity of tumor cells to Akt inhibition, and suggest that eEF-2 kinase may be utilized as an effective target for reinforcing the antitumor efficacy of Akt inhibitors such as MK-2206.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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