A novel activation-induced suicidal degradation mechanism for Akt by selenium

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Abstract. Selenium has been associated with an anti-cancer effect via the modulation of Akt. In order to investigate whether selenium modulates Akt by hitherto unidentified molecular mechanisms, we examined the effect of selenium on the stability and activity of Akt. Selenium induced destabilization of Akt which is coupled to its own enzyme activation. Mutation of T308 and S473 of Akt to alanine as well as the inhibition or depletion of upstream kinases for Akt activation blocked Akt degradation. These features of Akt degradation are reminiscent of the 'activation-induced suicidal degradation' mechanism. PTEN was also required for Akt destabilization as Akt activation alone was unable to elicit Akt degradation in the absence of PTEN. Conversely, PTEN introduction in PTEN-null prostate cancer cells restored the ability to degrade Akt upon selenium treatment. Collectively, selenium seems to achieve ultimate negative regulation of Akt signaling by destabilizing the protein, and this regulation mechanism might provide a paradigm for the anti-cancer activity of selenium.

Introduction

Selenium has been associated with an anti-cancer effect. Epidemiological data show an inverse relationship between the risk of cancer and selenium intake (1). This cancer preventive effect is significant in prostate, lung and colon cancers.

One of the possible mechanisms for the anti-cancer effect of selenium involves modulation of Akt signaling (2). AKT is a serine/threonine kinase and mediates cellular processes such as control of cell growth, proliferation, and survival. AKT is activated via a multi-step process (3). Phosphatidylinositol 3-kinase (PI3K) converts phosphatidylinositol-4,5-biphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3 then recruits phosphoinositide-dependent protein kinase-1 (PDK1) and Akt to the cell membrane. In the plasma membrane, Akt residues, threonine 308 in the catalytic site and serine 473 in the hydrophobic motif, are subsequently phosphorylated by PDK1 and a 'Ser473 kinase' respectively. Phosphorylation of both Thr308 and Ser473 seems to be critical for full activation of Akt.

Many human tumors show hyperactivation of Akt or deregulation of the Akt signaling cascade which are implicated in tumor aggressiveness (4). PTEN (phosphatase and tensin homologue deleted on chromosome 10) inhibits activation of Akt by dephosphorylating PIP3, thus leading to attenuated cell proliferation signaling. The mutation that impairs the PTEN function results in a significant increase in cellular PIP3, and constitutive activation of Akt leading to increased cell proliferation and tumor formation (5).

Considering the roles of Akt in cell survival, proliferation and tumor formation, blocking of the signaling could impede proliferation of cancer cells and induce apoptosis.

We investigated the response of Akt to seleno-L-methionine (SeMet) treatment in lung and prostate cancer cells. We found a novel negative regulation mechanism for Akt following SeMet treatment. Our findings revealed that selenium is able to destabilize Akt via a unique activation-dependent mechanism and that the process requires certain genetic components.

Materials and methods

Cell lines and treatment. Human lung cancer cell line NCI-H460 and two prostate cancer cell lines, PC-3 and DU145 were obtained from the American Type Culture Collection repository (Manassas, VA). Cells were cultured in RPMI-1640 media supplemented with 10% FBS and glutamine. SeMet, cycloheximide and LY294002 were purchased from Sigma (St. Louis, MO) and MG-132 from Calbiochem (San Diego, CA). Lipofectamine Reagent (Invitrogen, Carlsbad, CA) was used for transfection of cells.

RT-PCR analysis. Standard RT-PCR analysis with GAPDH as a control was performed. The following Akt primers

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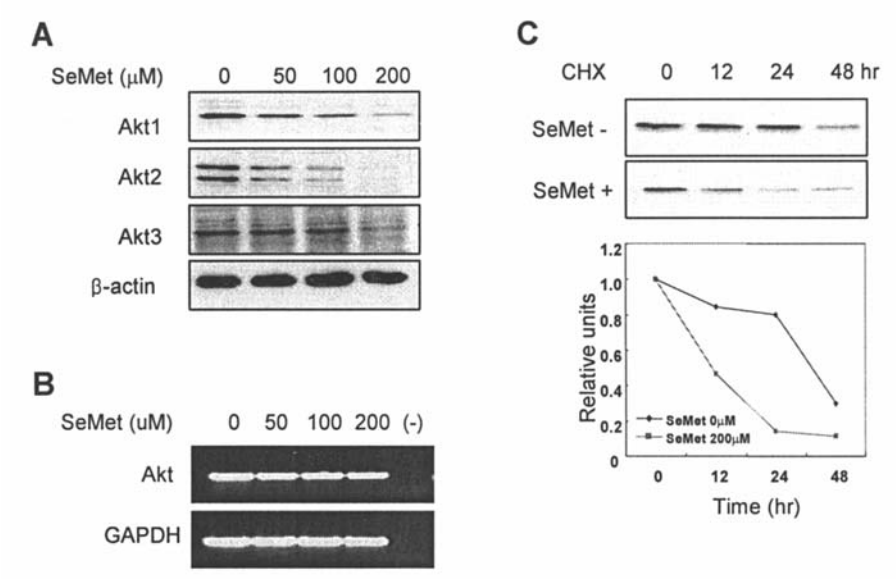

Figure 1. Destabilization of Akt by selenium. (A) Protein levels of Akt1, 2, and 3 were determined by Western blot analysis following a 48-h treatment of NCI-H460 cells with various doses of seleno-L-methionine (SeMet). ß-actin was used as a loading control. (B) The Akt transcript level was determined by RT-PCR analysis. Total RNA prepared from NCI-H460 cells that were treated for 48 h with SeMet was used. GAPDH was used as a control. -, reverse transcription reaction without reverse transcriptase serving as a negative control. (C) Akt stability in NCI-H460 cells upon SeMet treatment was determined by measuring the protein half-life. CHX, cycloheximide treatment. Graphical representation of the result is also shown. The amount of protein at zero time point for cycloheximide treatment was set to 100% (1.0) and others were normalized accordingly. An arbitrary unit was used.

generated the 380-bp RT-PCR product: forward primer, 5'-CCTGGACTACCTGCACTCGGAGAA-3'; and reverse primer, 5'-TTGCTTTCAGGGCTGCTCAAGAAGG-3'. The GAPDH primers are as follows: forward primer, 5'-CAT GGAGAAGGCTGGGGCTCATTT-3'; and reverse primer, 5'-CGCCAGTAGAGGCAGGGATGATGT-3'. Oligonucleotides were purchased from GenoTech (Daejeon, Korea).

DNA constructs. AKT and PTEN cDNA were kindly provided by Dr J.K. Chung at KAIST (Daejeon, Korea). The following primers were used to create DNA constructs. All constructs were inserted into *Eco*RI or *Bam*H1 restriction sites of the pCDNA3 vector; for Flag-tagged Akt construct, Akt Flag 5', 5'-CCGGAATTCATGGACTACAAGGACG ACGATGACAAGGGAAACGACGTAGCC-3'; and Akt 3', 5'-CCGGGATCCGGCTGTGCCACTGGCTGAGTA. To construct the Akt mutation at amino acid 308 (T308A construct), two internal primers (Akt308mt 3' and Akt308mt 5') with mutated sequences were used together with Akt Flag 5' and Akt 3'; Akt308mt 5', 5'-GTGCCACTATGAAG GCATTCTGCGGAACGCC-3' and Akt308mt 3', 5'-GGCGTT CCGCAGAATGCCTTCATAGTGGCAC-3'. The serine to alanine mutation at the Akt amino acid residue 473 (S473A) was constructed using Akt Flag 5' and Akt473mt 3' primer set; Akt473mt 3', 5'-CCGGAATTCTCAGGCTGTGCCAC TGGCTGAGTAGGCGAACTGGGGGAA-3'. The ΔPEST Akt construct was generated by using Akt Flag 5' and Akt 3' ΔPEST primers; Akt 3' ΔPEST, 5'-CCGGAATTCTCAGGC TGTGCCACTGGCTGAGTAGGAGAACTGGGGGAAGT G-3'. The PESTfind analysis program was used to identify putative PEST motif within Akt (embl.bcc.univie.ac.at/ embnet/tools/bio/PESTfind).

Protein analysis and silencing of gene expression by siRNA. Cell extract preparation, Western blot analysis and immunoprecipitation were performed as described previously (6). Antibodies were purchased from the following sources: Akt (catalog no. 9272), P-Akt (Thr308, Ser473), PGSK-3ß, PARP, caspase-3, ILK1, HA and PTEN antibodies were from Cell Signaling (Beverly, MA). Akt1, 2, 3 and ubiquitin antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). ß-actin and Flag antibodies were from Sigma.

The half-life of Akt was measured after treating cells with 100 μg/ml of cycloheximide in the presence or absence of SeMet. To examine the effect of the PI3K inhibitor on Akt stability, LY294002 was added to the cell plates which were treated as above. At specified time points, cells were harvested and processed for Western blot analysis. Densitometer (Bio-Rad Fluor-S™ MultiImager, Hercules, CA) measurement of the Akt protein band was plotted to determine the Akt half-life. The Akt amount at the zero time point for each treatment condition was set to 100% (1.0), and the other points were normalized to the value.

Akt kinase activity was measured by using the Akt kinase assay kit (#9840; Cell Signaling). ILK1-specific small interfering RNA was used to silence ILK (Signalsilence ILK1 siRNA kit, #6200, Cell Signaling). Both non-specific and a non-targeted negative control were used to monitor specificity of ILK siRNA. To determine transfection efficiency, fluorescein-labeled non-targeted siRNA control was monitored using a fluorescence microscope.

Results

Rapid turnover of Akt upon selenium treatment. SeMet treatment of H460 lung cancer cells decreased Akt1, 2 and 3

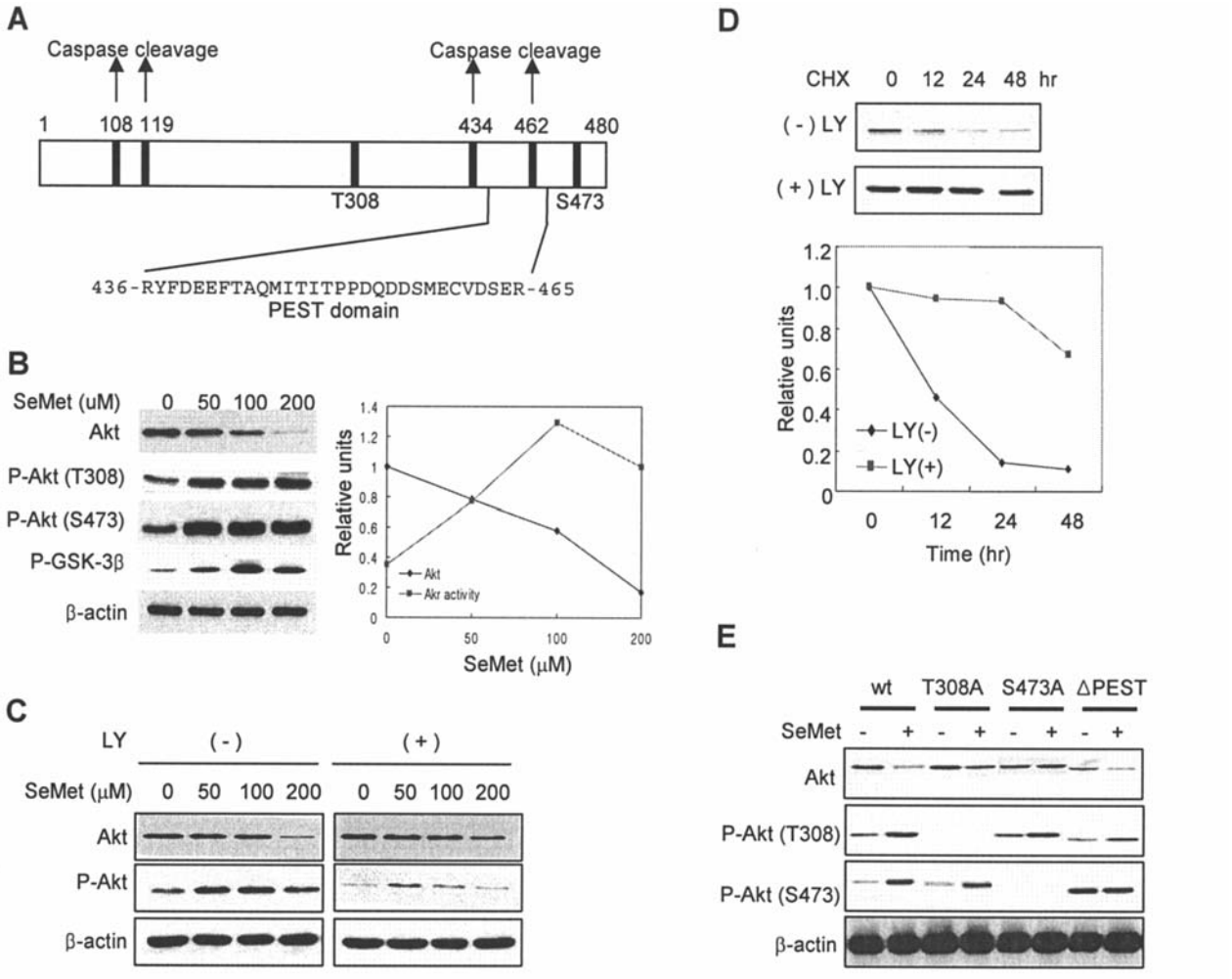

Figure 2. Activation-dependent destabilization of Akt. (A) Sequence motifs that are implicated in Akt stability are shown. (B) Protein levels, phosphorylation and activity of Akt were determined following a 48-h SeMet treatment. Akt and phospho-Akt were detected by Western blot analysis using corresponding antibodies. Akt kinase activity was measured using Akt immunoprecipitate in a kinase reaction with GSK-3ß fusion protein as a substrate. Graphical representation of the inverse relationship between Akt protein level and Akt activity is shown. For Akt protein levels, that of the SeMet-untreated (0 μM) sample was set to 100% (1.0) whereas Akt activity of the 200 μM SeMet-treated sample was set to 100% (P-GSK-3ß). Other samples were normalized accordingly. Units are arbitrary. (C) Effect of PI3K inhibitor LY294002 on Akt stability. NCI-H460 cells were pretreated with LY294002 (30 μM) 2 h before SeMet addition and were further incubated for 48 h before harvest. Western blot analysis was carried out to detect Akt and phospho-Akt (S473). (D) Effect of LY294002 treatment on Akt half-life was measured as described in Materials and methods. Akt protein levels were determined by Western blot analysis. For graphical representation, the amount of Akt protein at the zero time point for each treatment condition was set to 100% (1.0) and others were normalized. An arbitrary unit was used. (E) Protein levels and phosphorylation of Akt mutants were determined by Western blot analysis in the absence or presence of SeMet (200 μM). NCI-H460 cells were transiently transfected with the Akt constructs and treated with SeMet (200 μM) for 48 h. Akt levels were determined by Western blot analysis using antibody to Flag tag as the Akt constructs are Flag-tagged. T308 and S473 phosphorylation was also detected by Western blot analysis using corresponding antibodies. wt, wild-type Akt construct.

protein levels whereas control actin was constant (Fig. 1A). Gene expression was unlikely to be the cause of the protein decrease as RT-PCR analysis of Akt1 revealed similar levels of Akt1 transcript (Fig. 1B). Rather protein stability seemed to be responsible for the decreased Akt levels. The half-life of Akt protein was significantly shortened by SeMet treatment compared to the untreated control (Fig. 1C).

Normally Akt is a very stable protein with an ~36-h half-life (7). SeMet treatment (200 μM) of NCI-H460 cells reduced the half-life of Akt from ~36 h to <12 h (Fig. 1C, graph). Therefore the results suggest that Akt undergoes rapid turnover by SeMet treatment which leads to an attenuated Akt protein level.

Activation-dependent destabilization of Akt by selenium. Akt has several regulatory sequences that are critical for its activity or stability (Fig. 2A). To confirm whether the decreased Akt protein level was correlated with its activity, we examined the Akt level, phosphorylation and enzyme activity (Fig. 2B). Notably, while the Akt protein level decreased, Akt enzyme activity temporarily increased by SeMet treatment to a specific point (100 μM) and then decreased as assessed using GSK-3ß as a substrate. Phosphorylation of Akt threonine 308 (T308) and serine 473 (S473) also increased. The levels of Akt had an inverse relationship with the activity (Fig. 2B, graph). At the highest concentration of SeMet (200 μM), Akt activity began to decline probably due to the decreased Akt protein level even though Akt phosphorylation remained high.

To determine whether Akt activation by SeMet was coupled to its destabilization, we treated cells with LY294002, a potent inhibitor of PI3K. Pretreatment of NCI-H460 cells

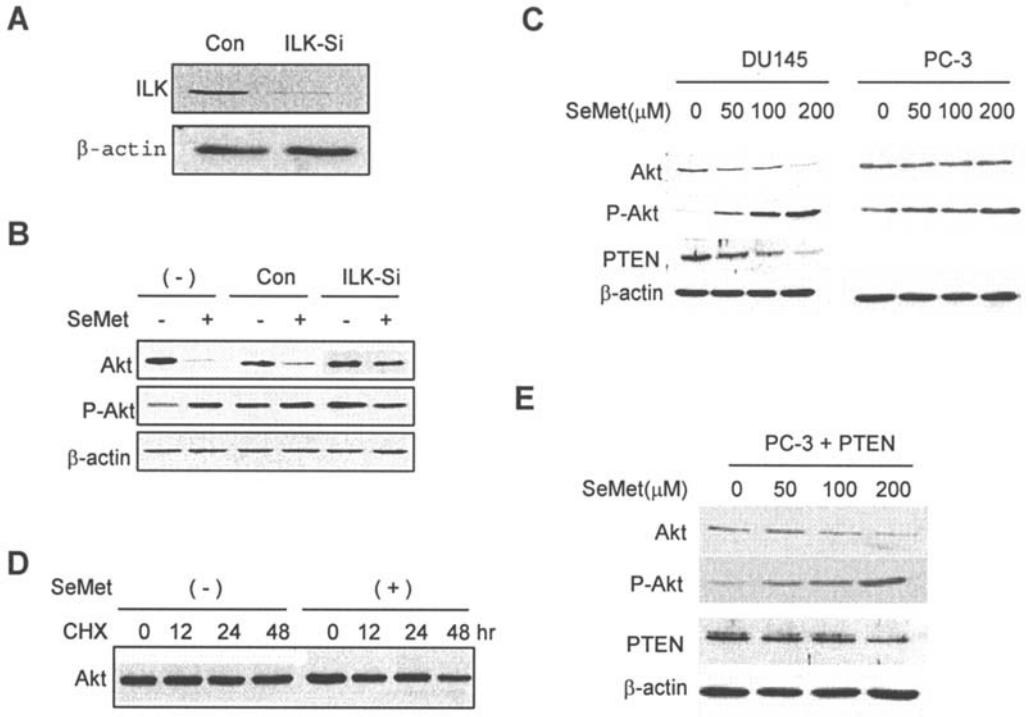

Figure 3. Regulators of Akt destabilization upon selenium treatment. (A) Depletion of ILK1 was achieved by siRNA and confirmed by Western blot analysis. Con, control siRNA-treated sample. (B) Effect of ILK1 depletion on SeMet-induced Akt destabilization was assessed by treating ILK1-depleted NCI-H460 cells with SeMet (200 μM) for 48 h. Control siRNA-treated (Con) and untreated control (-) samples are also shown. Akt protein levels and S473 phosphorylation (P-Akt) were detected by Western blot analysis. (C) Levels of Akt, S473 phosphorylation (P-Akt) and PTEN in DU145 and PC-3 cells were determined by Western blot analysis following a 48-h SeMet treatment. (D) Akt stability in PTEN-negative PC-3 cells was determined by measuring its half-life following SeMet (200 mM) treatment. (E) PTEN expression-dependent Akt destabilization was demonstrated by transfecting PC-3 cells with PTEN followed by SeMet (200 μM) treatment for 48 h. Akt, Akt S473 phosphorylation (P-Akt) and PTEN proteins were detected by Western blot analysis.

with the PI3K inhibitor decreased SeMet-induced phosphorylation of Akt S473 compared to the untreated control (Fig. 2C). More importantly, SeMet-induced decrease of Akt protein was attenuated by the treatment. Furthermore, LY294002 treatment prolonged the half-life of Akt when compared to the control samples (Fig. 2D). Thus the results support the notion that SeMet-induced Akt activation leads to its destabilization.

To determine the Akt sequence requirement for its stability, T308 and S473, and a putative PEST motif were mutated. The T308A and S473A mutants were unable to be phosphorylated at the corresponding residues (Fig. 2E). Compared to wild-type Akt, more T308A and S473A mutant Akt protein remained suggesting involvement of the residues in modulating Akt stability upon SeMet treatment. Particularly, the S473A mutant showed an almost intact protein level suggesting that phosphorylation at S473 might be critical for the destabilization of Akt.

Deletion of the putative PEST motif (ΔPEST) was unable to prevent SeMet-induced Akt destabilization (Fig. 2E, ΔPEST lanes). The ΔPEST Akt level was decreased by SeMet treatment. Therefore, the motif might be dispensable for SeMet-induced Akt destabilization.

ΔPEST induced phosphorylation at T308 upon SeMet treatment whereas S473 phosphorylation was constitutively high with no additional increase following the treatment (Fig. 2E). Although the mechanism for the high constitutive S473 phosphorylation is unclear, the results corroborate the notion that S473 phosphorylation is critical for the modulation of Akt stability upon SeMet treatment.

Regulators of Akt destabilization upon selenium treatment. Considering the attenuation of selenium-induced Akt destabilization by the PI3K inhibitor and by the S473 mutation, an S473 kinase which is PI3K-dependent might be involved in the process. Among the kinases that are able to phosphorylate Akt S473, ILK activity is stimulated *in vitro* by PIP3, and PI3K activates ILK in *vivo* (8). Thus we examined the role of ILK in the destabilization of Akt.

Depletion of ILK1 by siRNA was confirmed (Fig. 3A). ILK1-undepleted cells (- and Con lanes in Fig. 3B) showed a decrease in Akt following SeMet treatment with concomitant increase in S473 phosphorylation, whereas the ILK1-depleted cells (ILK-Si lane) had less S473 phosphorylation with consequent attenuation of destabilization. Compared to the result with the S473A mutation (Fig. 2E), ILK1 depletion resulted in a partial inhibition of Akt destabilization. Incomplete ILK depletion and the presence of redundant S473 kinases might underlie the response.

These data suggest that upon SeMet treatment, ILK1 might be involved at least in part in S473 phosphorylation and consequent Akt destabilization.

Since the lack of PTEN function led to an elevated concentration of PIP3 and consequent constitutive activation of Akt, the status of PTEN might determine Akt stability. We used two prostate-derived tumor cell lines, DU145 (PTEN-

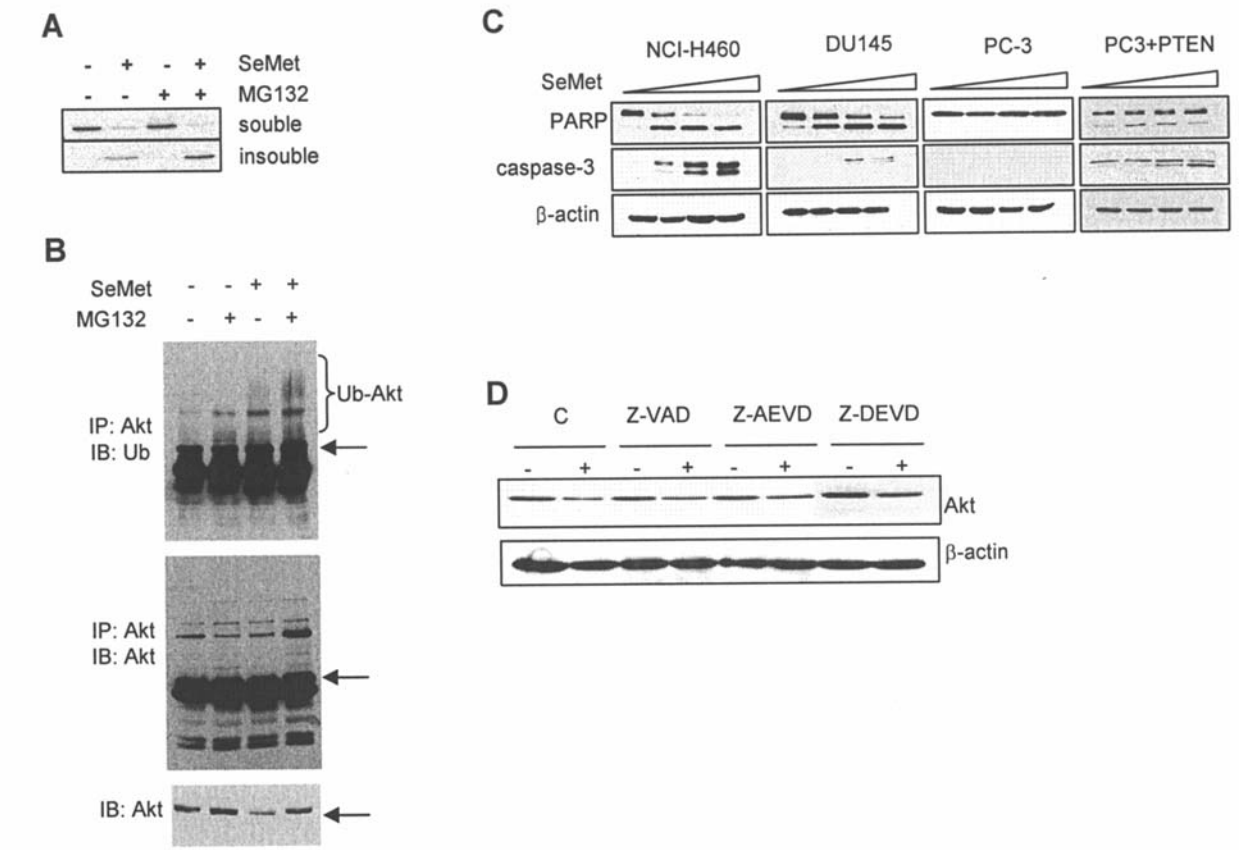

Figure 4. Proteasome- and caspase-dependent pathways for Akt degradation upon selenium treatment. (A) Proteasomal degradation of Akt upon selenium treatment. MG132 (100 μM) was added to NCI-H460 cells 12 h before harvest of either treated or untreated cells with SeMet (200 μM). The levels of Akt in the soluble and insoluble fraction were assessed by Western blot analysis. (B) Increased ubiquitination of Akt upon selenium treatment. NCI-H460 cells were transfected with HA-tagged ubiquitin and treated with proteasome inhibitor MG132 (100 μM) in the presence or absence of SeMet (200 μM). To detect ubiquitinated Akt, Akt was immunoprecipitated and subjected to Western blot analysis using ubiquitin antibody. The amount of immunoprecipitated Akt as well as the total Akt protein in whole cell extract was detected by Western blot analysis (bottom panel). Arrows indicate Akt protein bands. Ubiquitinated Akt (Ub-Akt) is also indicated. (C) SeMet-induced caspase-3 activation was determined by PARP and procaspase-3 cleavage following 0, 50, 100 and 200 μM SeMet treatment for 48 h. NCI-H460, DU145 and PC-3 cells were used for the treatment. Cleaved fragments of the proteins were detected by Western blot analysis using corresponding antibodies. PTEN-dependent caspase-3 activation was demonstrated by treating PTEN-transfected PC-3 cells with SeMet and processed as above. (D) Inhibition of SeMet-induced Akt degradation by caspase inhibitors. NCI-H460 cells were treated with various caspase inhibitors such as Z-VAD (pan-caspase inhibitor, 5 μM), Z-AEVD (caspase-10 inhibitor, 10 μM) and Z-DEVD (caspase-3 inhibitor, 30 μM) and maintained for 48 h with or without SeMet. Akt levels were determined by Western blot analysis.

positive) and PC-3 (PTEN-null), to elucidate the role of PTEN in the Akt response upon SeMet treatment.

SeMet treatment decreased the Akt level of DU145 as well as H460 cells, whereas PTEN-null PC-3 cells exhibited an intact Akt level (Fig. 3C). Akt S473 phosphorylation was significantly increased in DU145 cells upon selenium treatment whereas that of PC-3 cells was constitutively high even without SeMet treatment (Fig. 3C). Moreover, PC-3 cells showed only a slight decrease in Akt stability following SeMet treatment (Fig. 3D). As expected from the PTEN genotype, PTEN-wild-type DU145 cell lines had PTEN expression whereas PTEN-null PC-3 cells did not (Fig. 3C). Notably, the PTEN level was also decreased in the DU145 cell line. This result implies that the SeMet-induced PTEN decrease might be coupled to a significant increase of Akt phosphorylation in DU145 cells.

Comparison of the results from the two prostate cancer cell lines suggests that S473 phosphorylation is necessary but not enough for Akt destabilization following SeMet treatment.

To demonstrate whether PTEN was required for the destabilization of Akt, we introduced PTEN into the PTEN-null PC-3 cell line. Expression of PTEN in PC-3 cells decreased Akt protein with a concurrent significant increase in Akt S473 phosphorylation upon SeMet treatment (Fig. 3E). PTEN expression in PC-3 cells recapitulated SeMet-induced Akt destabilization as in the DU145 cells (Fig. 3E). The results suggest that SeMet-induced destabilization of Akt requires PTEN expression as well as S473 phosphorylation.

Akt degradation by selenium treatment is mediated by proteasome and caspases. We examined whether proteasomal or caspase-dependent degradation was involved in Akt destabilization by SeMet treatment. Treatment with SeMet alone decreased soluble Akt with a moderate increase in insoluble Akt, whereas treatment of H460 cells with both SeMet and a proteasome inhibitor MG132 showed decreased soluble Akt with a concurrent significant increase in insoluble Akt suggesting that Akt could be degraded via the proteasomal pathway (Fig. 4A).

Since the proteasomal pathway of protein degradation requires ubiquitination of the target protein, we examined ubiquitination of Akt following SeMet treatment. Addition of MG132 to SeMet-treated cells increased ubiquitinated Akt supporting the notion that the ubiquitin-dependent proteasomal degradation pathway is involved (Fig. 4B).

Since Akt is cleaved by caspase-3 (9,10), we examined caspase-3 activation in several cell lines following SeMet treatment. PTEN-positive cell lines such as H460 and DU145 induced caspase-3 activation following SeMet treatment as assessed by cleavage of PARP and procaspase-3 while PTEN-null PC-3 cells did not (Fig. 4C). Expression of PTEN in PC-3 cells restored the ability to cleave procaspase-3 and PARP suggesting that PTEN expression is necessary for caspase-3 activation and the subsequent Akt degradation. Moreover, various caspase inhibitors attenuated Akt destabilization (Fig. 4D).

Taken together, Akt seems to be degraded by SeMet treatment via several different pathways which involve at least the proteasome- and caspase-dependent pathways.

Discussion

We found a novel molecular mechanism for Akt destabilization by selenium. The mechanisms required two conditions for Akt degradation: i) transient phosphorylation and activation of Akt following selenium treatment and ii) the presence of a tumor suppressor PTEN.

SeMet treatment caused a transient increase in phosphorylation of T308 and S473 with consequent activation of Akt, whereas the amount of Akt was concomitantly decreased.

The behavior of Akt degradation upon SeMet treatment is reminiscent of the so-called model, 'activation-induced suicidal degradation' of kinases. This model describes that the destabilization of certain protein kinases is coupled to its own enzyme activation, and agonist-induced kinase activation leads to degradation of the proteins. For example, genotoxic stress induces phosphorylation and activation of Chk1 kinase followed by polyubiquitination and proteasome-dependent degradation (11). PKC also shows similar activation-triggered degradation upon its agonist treatment (12).

Activation of Akt by S473 phosphorylation has been reported to occur via a disorder to order the transition of αC helix in the N-terminal lobe with restructuring of the activation segment (13). We speculate that such conformational changes upon Akt activation might render Akt susceptible to various protein degradation machineries in SeMet-treated cells leading to its degradation.

Coupling of kinase activation to its degradation might prevent the activated kinase from accumulating and inappropriately affecting cellular signaling. Thus Akt degradation upon SeMet treatment might serve as a negative feedback control mechanism.

Selenium treatment might affect the AGC family kinases which includes PKA, PKC, SGK, and the p70/p90 S6 kinases as well as Akt (3). The kinases share numerous functional similarities with Akt such as i) conservation of key phosphorylation sites, ii) dependence on phosphorylation for activity and iii) similar structural changes upon hydrophobic motif phosphorylation similar to that of S473 of Akt (13). Therefore, it is worthy of attention whether SeMet affects the AGC kinases with similar molecular mechanisms as that of Akt.

SeMet-induced Akt degradation also required a tumor suppressor PTEN. The PTEN-mediated cellular process for Akt degradation appears to be caspase activation. This is not without precedent as PTEN introduction has been known to sensitize PTEN-null prostate cancer cells to apoptosis which involves caspase-3 and caspase-8 activation (14). Therefore, in addition to the conventional role of the negative regulator in Akt activation as a lipid phosphatase, PTEN seems to achieve an additional way of negative regulation by its involvement in Akt degradation upon certain stress such as SeMet treatment.

SeMet treatment degraded Akt via both caspase- and proteasome-mediated processes (Fig. 4). The two mechanisms have been implicated in Akt degradation by various conditions. For example, Hsp90 inhibitor 17AAG or platelet-derived growth factor (PDGF) treatment increases Akt ubiquitination and subsequent degradation by proteasome (7,15). Cytokine withdrawal or VP-16 treatment resulted in cleavage of Akt by caspase-3 (9,10). To note, SeMet treatment seems to achieve inhibition of Akt activity by employing multiple degradation pathways.

Selenium-induced degradation of signaling protein has been reported in prostate cancer cells. Selenium increases androgen receptor (AR) protein degradation with subsequent disruption of AR signaling (16). Thus, degradation of proteins involved in cell proliferation signaling might serve as a paradigm for selenium-mediated antiproliferation and apoptosis.

Studies indicate that, unlike normal cells, tumor cells might require Akt activation for survival and that tumors with increased Akt activity are sensitive to inhibition of Akt activity (17). Therefore, the targeting and destabilization of Akt by selenium may be an appealing therapeutic intervention in cancer. However, for a potential clinical application, the proper dose of selenium should be determined. SeMet is metabolized in the liver into a variety of metabolites (18), which might induce the observed Akt response. The cell lines that we used in this investigation may have low capacity to generate the selenium intermediates, thus the doses we used for the treatment of cells might be substantially higher than those required for an *in vivo* study. Determination of proper doses of effective selenium compounds should lead to improvements in tumor control.

Taken together, the present results showed that selenium treatment induced transient activation-dependent Akt destabilization serving as a negative feedback mechanism to modulate kinase activity.

Acknowledgements

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