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AKT phosphorylation sites of Ser473 and Thr308 regulate AKT degradation

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ABSTRACT

Protein kinase B (AKT) is a serine-threonine kinase that mediates diverse cellular processes in a variety of human diseases. Phosphorylation is always the best studied posttranslational modification of AKT and a connection between phosphorylation and ubiquitination has been explored recently. Ubiquitination of AKT is an important step for its phosphorylation and activation, while whether phosphorylated AKT regulated its ubiquitination status is still unknow. In the present study, we mimic dephosphorylation of AKT by using mutagenesis techniques at both Thr308 and Ser473 into Alanine (AKT-2A). After losing phosphorylation activity, AKT enhances its degradation and prevents itself release from the plasma membrane after insulin stimulation. Fourthermore, AKT-2A is found to be degraded through ubiquitin-proteasome pathway which declared that un-phosphorylation of AKT at both Ser473 and Thr308 sites increases its ubiquitination level. In conclusion, AKT phosphorylated at Ser473 and Thr308 sites have a significant effect on its ubiquitination status.

Abbreviations: AKT: Protein kinase B; Ser: serine; Thr: threonine; IF: immunofluorescence; Epo: Epoxomicin; Baf: Bafilomycin; PBS: phosphate buffer solution

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Protein kinase B (AKT) is the major component of the insulin signaling pathway that plays several crucial roles in diverse aspects in the body, such as regulation of metabolism, energy homeostasis, and cell proliferation and death, especially in liver, muscle, kidney, and brain tissues [1–3]. When the signaling pathway is triggered, AKT is phosphorylated at Thr308 by PDK1. In this process, AKT bind to phosphatidylinositol-3,4,5-triphosphate (PIP3) that results in a conformational change and exposes the kinase domain to its upstream kinase PDK1 in the cell membrane [4,5]. For maximal activation, phosphorylated at another site Ser473 by mTORC-2 is essential to further increase its activity [6,7]. Thus comparing of the basal level, phosphorylated at both Thr308 and Ser473 sites extremely augments AKT activity in response to growth factor stimulation, and usually these two phosphorylation sites are considered as the markers of AKT activity. After AKT is phosphorylated at Thr308 and Ser473, then executes diverse biological actions by phosphorylating a range of downstream intracellular proteins, such as the FoxO family members, GSK-3β, and mTOR, among others [8,9].

Ubiquitination is a prominent posttranslational modification that covalently contacts ubiquitin protein to lysine residues of the target protein [10,11]. Two different ubiquitination systems for AKT have been reported: Lys48-linked ubiquitination, which targets phosphorylated AKT for degradation whereras ubiquitination of Lys63 promotes the activation and phosphorylation of AKT [12,13]. Yang et al [14] reported that TRAF6 was a direct E3 ligase and essential for ubiquitination of AKT. In response to growth factor stimulation, TRAF6 ubiquitinate AKT in the PH domain, results in membrane recruitment and phosphorylation of AKT. Thus, AKT ubiquitination is an important step for its phosphorylation and activation. Compared to ubiquitination regulating phosphorylation is widely disscussed, whether acyivity of AKT could regulate its degradation remains uncertain. Thus, after investigating the correlation between phosphorylation and ubiquitination of AKT, we show that AKT dephosphorylation could accelerate its degradation and degraded through the ubiquitin-proteasome pathway.

Materials and methods

Cell culture

HEK 293T and Hela cells were obtained from Chinese Type Culture Collection (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) at 37℃ humidified incubator with 5% CO2.

Antibodies and reagents

Used antibodies included anti-HA was purchased from Abcam (Cambridge, MA, USA), anti-GAPDH

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and anti-ubiquitin were obtained from SantaCruz (CA, USA).

Insulin, Epoxomicin (Epo) and Bafilomycin (Baf) used in this study were purchased from Sigma (St. Louis, MO, USA). Epo and Baf were scale-diluted with dimethylsulfoxide (DMSO) solvent, and then were stored at −80℃. Cycloheximide (CHX) used was abtained from Selleck (Houston, Texas, USA), and was dilluted with DMSO solvent in a storage concentration.

Plasmids construction and DNA mutagenesis

AKT tagged with hemagglutinin (HA) was cloned into the pCI-Neo vector via the XhoI and SalI sites. Mutation of AKT at Thr308 or Ser473 to Alanine were achieved by using the QuickChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) with the following forward and reversed primers: 5′-ggtgccaccatgaagGccttttgc ggcacacctg-3′ and 5′-caggtgtgccgcaaaaggCcttcatg gtggcacc-3′ for Thr308 to Ala (AKT-T308A), and 5′-ccacttcccccagttcGcctactcggccagcggc-3′ and 5′-gccgctggccgagt aggCgaactgggggaagtgg-3′ for Ser473 to Ala (AKT-S473A). The mutation at Thr308 and Ser473 to Ala (AKT-2A) were produced by using PCR from vector pCI-Neo-HA-AKT-T308A with the same forward primers used for Ser473 to Ala. In the same way, mu tation of AKT at Thr308 or Ser473 to Asp aragine used the following forward and reversed primers: 5′-ggtgccaccatgaagTccttttgcggcacacctg-3′ and 5′-caggtgtgccgcaaaa ggActtcatggtggcacc-3′ for Thr308 to Asp (AKT-T308D), and 5′-ccacttcccccagttcAcct actcggccagcggc-3′ and 5′-gccgctggccgagtaggGgaactgggggaa gtgg-3′ for Ser473 to Asp (AKT-S473D). The mutation at Thr308 and Ser473 to Asp (AKT-2D) were produced by using PCR from vector pCI-Neo-HA-AKT-T308D with the same forward primers used for Ser473 to Asp. All the mutations were confirmed by DNA sequencing.

Plasmids transfection

Transfection of cells were performed using LipofectamineTM3000 (Invitrogen, Carlsbad CA, USA) in 24-well plates. After cells were maintained for 24 h, plasmids at a final concentration of 0.5 μg were transfected into cells mixed with 2 μl Lipo3000 regeant and 2 μl Lipo p3000 regeant for incubation another 48 h.

Western blot analysis

Cells were lysed with SDS sample buffer directly and then quantified by BCA Protein Assay Kit (Merck, Darmstadt, Germany). For analysis, cell lysates were separated with SDS-PAGE and transferred onto PVDF membrane (Millipore, Schwalbach, Germany). Then the membrane was blocked with 5% skim milk for at least 30 min and subsequently incubated with the antibodies specific to HA (1:1000), GAPDH (1:500) and ubiquitin (1:500) at 4℃ overnight. Next day, after washed 3 times with 1× TBS-T buffer, the membrane was incubated with HRP-conjugated goat anti-mouse/rabbit secondary antibody for 2 h at the room temperature. Finally, each sample was detected using an ECL system (ThermoFisher Scientific, MA, USA).

Cells immunofluorescent (IF) assay

Cells were fixed with 4% paraformaldehyde for 30 min, followed by incubation with a primary antibody specific to HA (1:500) at 4℃ overnight in TBS which containing 5% goat serum and 0.1% TritonX-100. After washing with TBS, the sections were incubated with Alexa 488-conjugated goat anti-mouse IgG (1:1000) plus TO-PRO-3 in TBS at room temperature for 1 h. The immunostaining was analyzed by using a laser scanning confocal microscope (TCS-SP2, Leica, Germany).

Co-immunoprecipitation (co-ip) assay

In the present study, Co-IP technique was performed to detect the occurrence of spontaneous ubiquitination in WT- and mutant Akts. At 48 h after transfection with plasmids of HA-tagged Akts, cells were treated with Epo for 12 hours to induce ubiquitination. The protein A/G beads (ThermoFisher Scientific, MA, USA) were incubated with antibodies against HA, at 4℃for 6 h, respectively. After washed with TBS three times, the beads which combined with antibodies were incubated with the cell lysates prepared from ultrasonic fragmentation at 4℃overnight. Next, the beads were also washed with TBS, centrifuged at 1,3000 rpm for 5 min. After discarding the supernatant, the equivoluminal SDS buffer was added into the beads. Finally, the beads were boiled for 5 min, and the relevant antibodies were used to detect the target proteins by Western blot analysis with anti-Ub antibody. 20 μL cell lysates used for IP was analyzed as controls by Western blot with anti-HA antibody.

Statistical analysis

Data were presented as means± SD from experiments repeated at least three times independently. Statistical analysis was performed by SPSS 19.0 software. Differences between individual groups were analyzed by student's t test. P values< 0.05 were considered to have statistically significance. Graphs were created with GraphPad Prism 5.0 software.

Results

The mutation of AKT ser473 and thr308 into alanine (AKT-2A) decreases AKT protein level

After transfected AKT constructs with various mutants where either or both of Ser473 and Thr 308 were mutated to alanine to prevent AKT from activation in HEK293T cells, we notably found that the AKT protein level dramatically decreased in cells transfected with the double mutants AKT-2A, but not when transfected with only single mutant AKT-T308A or AKT-S473A, when compared to AKT wide type (Figure 1(a)). Then we asked whether AKT-2A with double mutants affects its transcriptional level. The regular RT-PCR or quantitative-PCR showed there was no any difference in the mRNA level between four constructs, suggesting AKT-2A did not affect its transcription (Figure 1(b,c)).

The mutation of AKT ser473 and thr308 into alanine (AKT-2A) enhances AKT degradation

Next, we treated cells with the translational inhibitor cycloheximide (CHX) 24 hours after transfection of constructs, and then examined the AKT protein levels at different time points. We observed that AKT-2A degraded much faster than the others, indicating the double mutants in AKT-2A enhanced the protein degradation (Figure 2(a,b)). We also mutated AKT ser473 and/or Thr308 into Asparagine as mimics of phosphorylated serine or threonine and found that the asparagine mutation did not affect the protein level (Figure 2(c)). Thus, we concluded that unphosphorylation of AKT at both serine and threonine sites enhances protein degradation.

Mutation of AKT ser473 and thr308 into alanine prevents AKT release from the plasma membrane after insulin stimulation

When cells are stimulated by growth factor insulin, AKT is recruited from the cytosol to the membrane by interaction with phosphoinositide docking sites, so that it can be fully activated. Once active, AKT translocates from the plasma membrane to the cytosol and nucleus, where many of its substrates reside. Next we investigated whether the mutants of AKT affect the classic AKT activation process after the stimulation of insulin. Interestingly, WT-AKT, AKT-T308A, and AKT-S473A located in the nucleus abundantly. By contrast, however, AKT-2A was exclusively located in the cytosol, suggesting that, at the basal level, most of WT-AKT, AKT-T308A, and AKT-S473A had been activated and translocated in the nucleus, but as for AKT-2A, in which both T308 and S473 sites could not be phosphorylated, AKT-2A stayed in the cytosol as an inactive form (Figure 3(a–c)).

After 15 min of insulin stimulation, immunofluroscence staining showed most of WT-AKT, AKT-T308A, and AKT-S473A were still in the nucleus, and only a few were observed in the plasma membrane, indicating after insulin stimulation, AKT recruitment to plasma membrane and release was a very quick process. In 15min, most of AKT had been activated and released to the cytosol and the nucleus. By contrast, most of AKT-2A was observed in plasma membrane (Figure 3(b–d)). In addition, nuclear extraction and western blotting assays demonstrated the AKT localization which confirmed the above immunofluorescent results (Figure 3(e,f)). Therefore, mutation of AKT Ser473 and Thr308 into Alanine did not affect the AKT recruitment to the plasma membrane, but double mutation prevented its release from the membrane to the cytosol and the nucleus.

AKT-2A is degraded through ubiquitin-proteasome pathway

Most intracellular proteins are degraded by the ubiquitin-proteasome pathway, and extracellular proteins and some cell surface proteins are taken up by endocytosis and degraded within lysosomes. However, recently accumulated evidence shows a large number of intracellular proteins can also be degraded through the autophagy- lysosome pathway. In addition, the finding that AKT-2A remained in the plasma membrane after insulin stimulation led us to

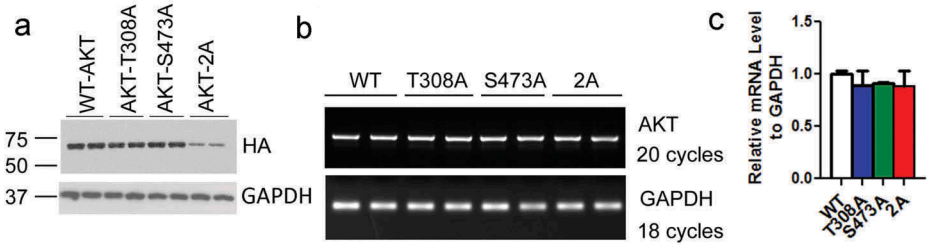

Figure 1. The mutation of AKT Ser473 and Thr308 into Alanine (AKT-2A) decreases AKT protein expression. (a) WT-AKT, AKT-T308A, AKT-S473A, AKT-2A plasmids tagged HA were transfected into HEK293T cells, and protein expressions of AKT were detected by Western blots. WT-AKT, AKT-T308A, AKT-S473A, AKT-2A plasmids were transfected into HEK293T cells, expressions of mRNA were detected by regular RT-PCR (b) or quantitative-PCR (c).

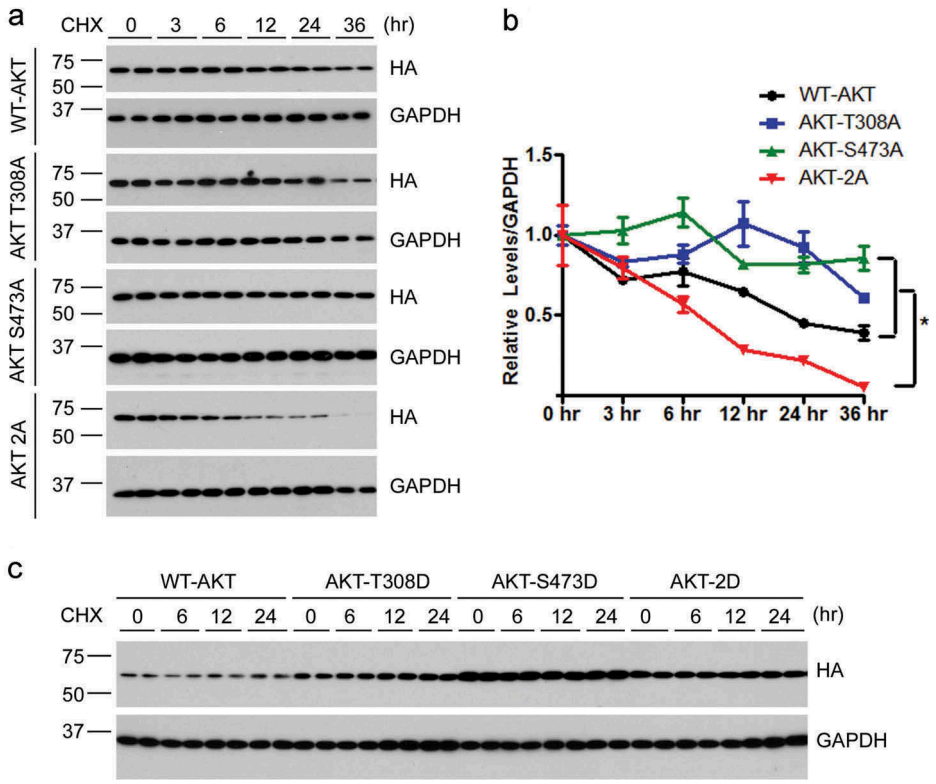

Figure 2. The mutation of AKT Ser473 and Thr308 into Alanine (AKT-2A) enhances AKT degradation. (a and b) Treated cells with the translational inhibitor cycloheximide (CHX) 24 hours after transfection of WT-AKT, AKT-T308A, AKT-S473A, AKT-2A constructs, and then examined the AKT protein levels at different time points (n = 3, *P< 0.05). (c) Mutated AKT ser473 and/or Thr308 into Asparagine to mimics of phosphorylated serine or threonine, and detected protein levels of WT-AKT, AKT-308D, AKT-473D, AKT-2D after transfection of constructs.

investigate whether AKT-2A is degraded through proteasome or lysosome pathways. We transfected the AKT-2A construct into HEK293T cells followed by treatment, after 24 hours, with 12.5 nM proteasome inhibitor Epoxomicin (Epo) or 100 nM lysosome inhibitor Bafilomycin (Baf) respectively, together with a translational inhibitor CHX to minimalize the side effects from the translation for 12 hours. Epo increased ubiquitin level and Baf increased the ratio of LC3II/LC3I, compared to the control, confirming that the Epo inhibited the proteasome pathway, and that Baf inhibited the lysosome pathway (Figure 4(a) and 4(b)). Protein level of AKT-2A was increased with the proteasome inhibitor Epo, but not lysosome inhibitor Baf (Figure 4(c,d)). Thus, we concluded that AKT-2A was degraded through the ubiquitin-proteasome pathway. Modification of a target protein by ubiquitin serves as a recognition signal that allows proteasome to degrade. Next, we examined whether the alanine mutation of AKT at both Ser473 and Thr308 sites, thereby keeping it unphosphorylated, would affect its ubiquitination modification. For this part, we detected the occurrence of ubiquitination in WT- and mutant Akts. We transfected with plasmids of HA-tagged Akts, immunoprecipitated with anti-HA antibody and performed Western blot with anti-Ub antibody. At 24 hours after transfection, cells were treated with Epo for 12 hours to induce ubiquitination. The results showed that the un-phosphorylated of AKT at both Ser473 and Thr308 sites would increased its ubiquitination modification (Figure 4(e)).

Discussion

AKT activation mediates a wide array of cellular processes such as survival, cell proliferation, protein translation and metabolism [15,16]. As we know, the precise activation and timely degradation of AKT play important roles in maintenance diverse biological responses [17,18]. In some cases, degradation of proteins has been shown to be induced by phosphorylation and dephosphorylation, and the ubiquitin- proteasome pathway has been invloved [19]. Here, we disscussed the inactivation of AKT is a well-characterized example of coupling between phosphorylation and ubiquitination. We showed that ubiquitination of AKT is regulated by its dephosphorylation at both Thr308 and Ser473. This ubiquitination pathway for AKT degradation is illustrated in Figure 4.

Phosphorylated AKT translocates from the plasma membrane to intracellular compartments, including the cytoplasm and nucleus where it phosphorylates

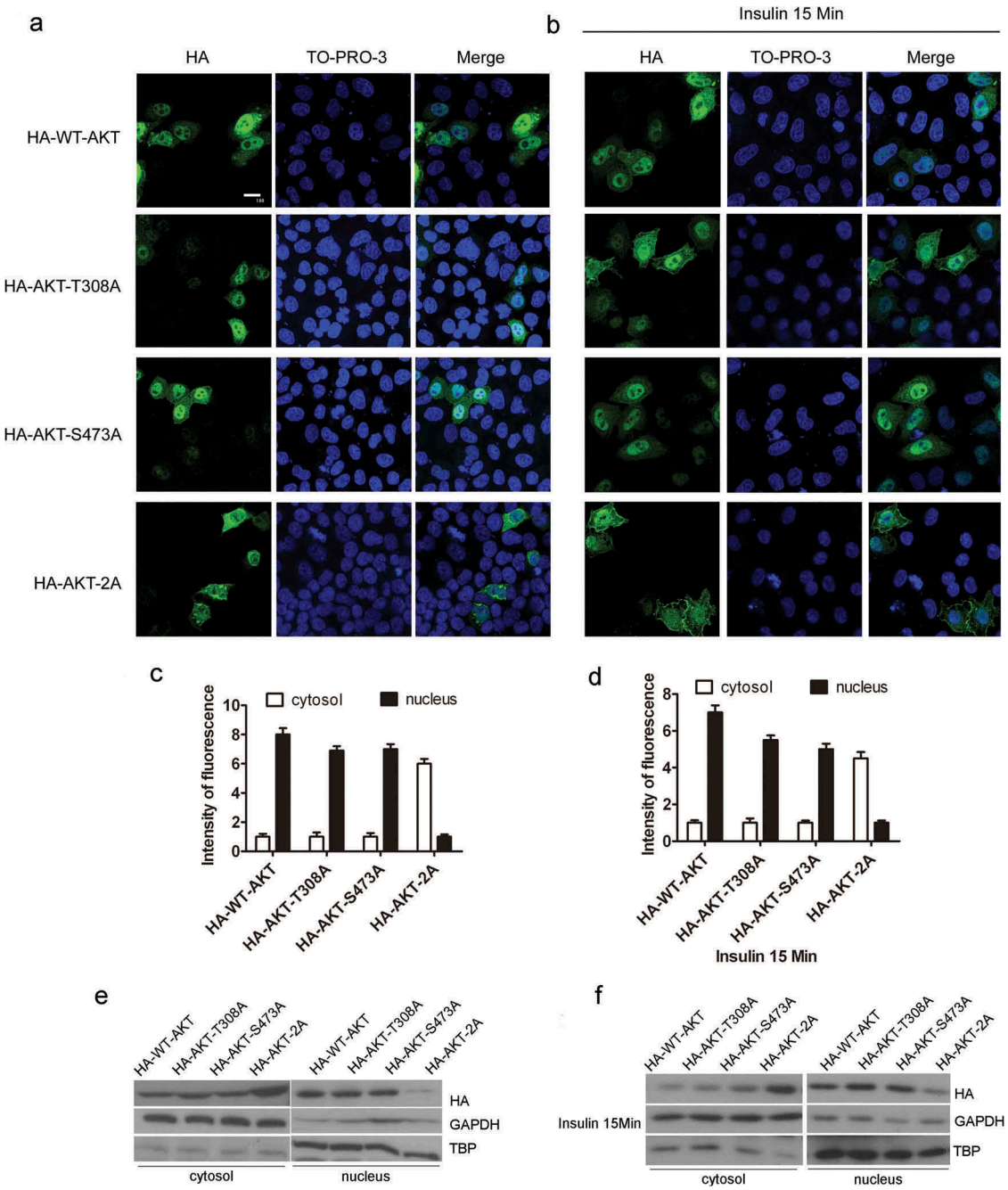

Figure 3. Mutation of AKT Ser473 and Thr308 into Alanine prevents AKT release from the plasma membrane after insulin stimulation. (a–c) After transfected with constructs, the location of WT-AKT, AKT-T308A, AKT-S473A and AKT-2A was detected by immunofluorescence technology in Hela cells. AKT was stained green, and nuclei stained with TO-PRO-3 were blue (Bar = 70 μm). (b–d) After 15 min of insulin stimulation, immunofluroscence staining showed most of WT-AKT, AKT-T308A, and AKT-S473A were also detected by immunofluorescence technology. AKT was stained green, and nuclei stained with TO-PRO-3 were blue (Bar = 70 μm). (e) After transfected with constructs, nuclear extraction and western blotting assays determined the distribution of WT-AKT, AKT-T308A, AKT-S473A and AKT-2A. GAPDH and TATA box binding protein (TBP) were used as loading control. (f) After 15 min of insulin stimulation, the distribution of WT-AKT, AKT-T308A, AKT-S473A and AKT-2A was also detected by nuclear extraction and western blotting assays. GAPDH and TATA box binding protein (TBP) were used as loading control.

downstream substrates [2,20]. However, the mechanisms underlying transportation of the active form of AKT to its action sites remain unclear. In the previous studies, ubiquitination regulated AKT protein stability while nondegradable ubiquitination could positively affect AKT plasmamembrane affinity and subsequently activated AKT for translocation to the nucleus [21]. Here, our findings reveal a new step of AKT ubiquitination that is regulated by phosphorylation as well as AKT nuclear trafficking.

Crosstalk between phosphorylation and ubiquitination occurs at several levels. Phosphorylation can promote or inhibit ubiquitination, which in turn leads to proteasomal degradation or regulate intracellular

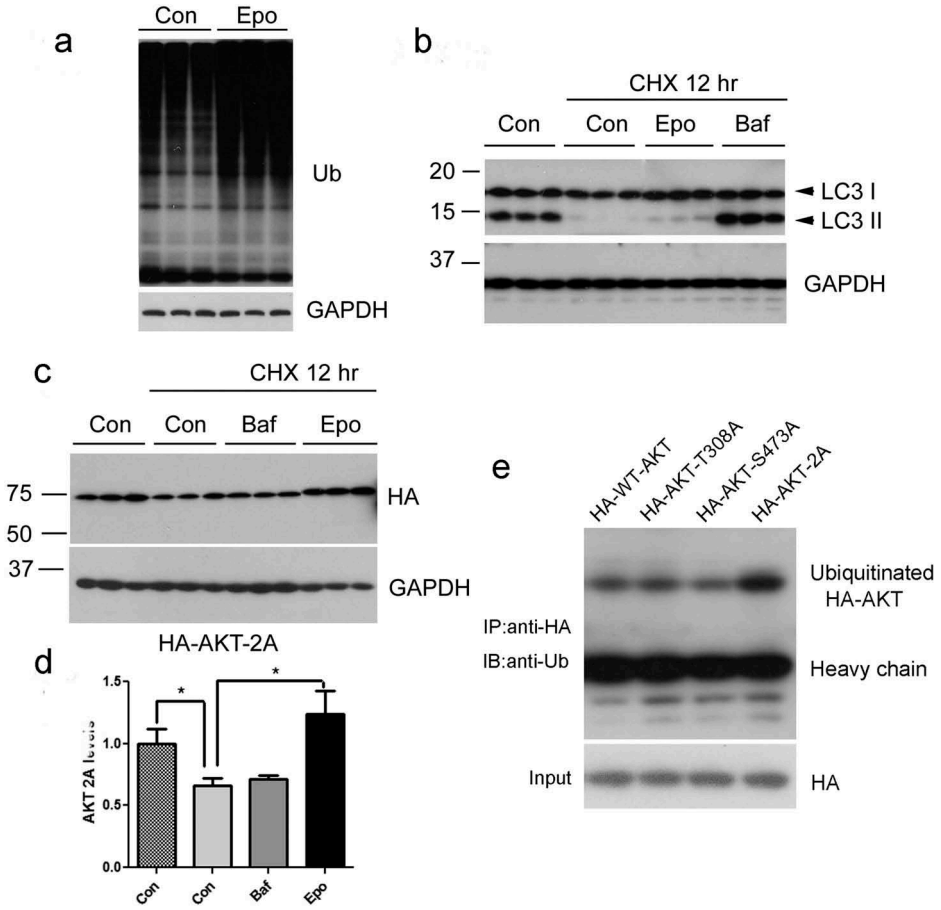

Figure 4. AKT-2A is degraded through Ubiquitin-proteasome pathway. (a and b) The AKT-2A construct was transfected into HEK293T cells followed by treatment, after 24 hours, with 12.5 nM proteasome inhibitor Epo or 100 nM lysosome inhibitor Baf respectively, together with the translational inhibitor CHX for 12 hours. Ubiquitin level and the ratio of LC3II/LC3I were detected by Western blots. (c and d)The AKT-2A construct was transfected into HEK293T cells followed by treatment, after 24 hours, with 12.5 nM Epo or 100 nM Baf respectively, protein level of AKT-2A was detected by Western blots (n = 3, $*P< 0.05$). (e) Ubiquitination status in WT- and mutant Akts. Transfection, immunoprecipitation and Western blot were performed as described in Materials and methods. At 48 h after transfection, cells were treated with Epo for 12 hours to induce ubiquitination. 20 μL cell lysates used for IP was analyzed as controls by Western blot with anti-HA antibody.

trafficking of membrane proteins [22,23]. In our study, ubiquitination of AKT is promoted by dephosphorylation at Thr308 and Ser473 which stands for a new style of crosstalk between phosphorylation and ubiquitination. Su, C.H. et al [21] suggested that Thr308 and Ser473 phosphorylations prompt Akt to enter the CHIP mediated ubiquitin-proteasome pathway. Mutation at either Thr308 or Ser473 dampened its ability to bind to the U-box E3 ligase CHIP. In the present study, we mimic dephosphorylation of AKT by using mutagenesis techniques at both Thr308 and Ser473 into Alanine (AKT-2A), and AKT enhances its degradation after losing phosphorylation activity. However, single mutant AKT-T308A or AKT-S473A had no significant change which was different with Su's research. Risso, G. et al. [24] have described detailedly the relationship between phosphorylation and ubiquitination of AKT. They uneviled two types of ubiquitination: degradative and non-degradative which mean that phosphorylation of AKT possibly is bidirectional to its ubiquitination. In addition, Akt degradation was reported to be prevented by the translation-coupled phosphorylation event at Thr450 mediated by mTORC2. Lack of this phosphorylation rendered Akt unstable [25]. As a result, interrelation between AKT phosphorylation and degradation is intricate which needs to be further investigated.

Deregulation of AKT signalling is associated with a variety of human diseases, revealing AKT-dependent pathways as an attractive target for therapeutic intervention [26,27]. We put forward for the first time that AKT dephosphorylation sites of Ser473 and Thr308 promote AKT degradation which provide a new approach to inactive AKT.

Conclusion

In summary, our research demonstrates that AKT dephosphorylation at both Ser473 and Thr308 sites could accelerate its degradation and degraded

through the ubiquitin-proteasome pathway. This new style of crosstalk between phosphorylation and ubiquitination of AKT provide a new way to cut down its expression and activity.

Author contribution

Xiaoxia Jin conceived and designed the experiments. Yingze Wei, Jianyun Zhou and Xiaoxia Jin performed the experiments. Yingze Wei analyzed data. Xiaoxia Jin wrote the paper.

Disclosure statement

No potential conflict of interest was reported by the authors.

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