**Genotoxic stress causes activation of Akt via non-canonical Ca2/calmodulin-dependent protein kinase kinase 2 (CaMKK2) in ovarian cancer cells**

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**Abstract**

**Introduction**

**Role of PI3K/Akt in oncogenic signaling**

The PI3K/ Akt pathway is a major signaling network for control of growth and survival of normal and neoplastic cells and is oncogenic for multiple cancer types, including Ovarian Cancer (OVCa) [1]. Therefore, there have been clinical trials to suggest targeting this pathway at levels up and downstream of Akt, as well as at the level of Akt itself [4,5]. PI3K synthesizes phosphatidylinositol 3,4,5-trisphosphate, which recruits Akt and phosphoinositide-dependent kinase 1 (PDK1) to the plasma membrane via their pleckstrin homology (PH) domains, resulting in PDK1 phosphorylation of Akt at its activation loop site Thr-308. Once phosphorylated at Thr-308, Akt phosphorylates SIN1 of the mechanistic target of rapamycin (mTOR) complex 2 (mTORC2), which activates mTORC2, resulting in phosphorylation of Akt at Ser-473 [6]. Phosphorylation of Akt at both Thr-308 and Ser-473 is required for maximal activation. As previously mentioned, the activation of Akt causes promotion of protein translation, cell growth and cell survival. The translation of protein is mediated by the phosphorylation of PRAS40 (proline-rich Akt substrate 40) which leads to release of mTORC1 from a state of inhibition allowing for its phosphorylation of p70 ribosomal protein S6 kinase (S6) and eukaryotic initiation factor 4E-binding protein (4E-BP1) [7]

**Relevance of Ca2+ Calmodulin mediated kinase kinase 2 (CaMKK2) activity in Akt signaling**

Initially, PDK1 was concluded to be the sole upstream kinase for Akt (T308) in the findings where PDK1-/- embryonic stem cell (ES) did not show growth factor-responsive Akt phosphorylation at T308 [8]. However, this conclusion did not consider the possibilities that there may be other kinase(s) that are not expressed at the ES cell stage, and are not activated by growth factors but make use of alternate stimuli, or are overexpressed in cancer. These may also be responsible for phosphorylation of Akt activity in cells. In 1998, it was reported that in response to Ca2+ influx, Akt is phosphorylated at T308 in neuroblastoma-glioma cells by Ca2+ Calmodulin (CaM) mediated Kinase Kinase (CaMKK) [9]. A CaMKK-dependent activation of Akt was also demonstrate in prostate cancer (PCa) cells upon stimulation of Ca2+ influx [1]. Therefore, there is significance in the role of CaMKK with respect to its property of stimulating Akt activation. CaMKK exists as two paralogues, 1 and 2 , with closely related structures and similar enzymatic properties [11-13]. CaMKK1 and CaMKK2 activate both CaMKI and CaMKIV by phosphorylating their activation loop sites (Thr-177 and Thr-200, respectively). CaMKK2 is also an upstream-activating kinase for 5-AMP-activated kinase (AMPK) [14-15]. We observed that CaMKK2 is highly expressed in high-grade serous ovarian cancer such as Ovarian cancer cells (OVCa). Knockdown or pharmacological inhibition of CaMKK2 decreased Akt phosphorylation at Thr-308 and Ser-473 thus, controlling cell growth, proliferation, protein synthesis and stability. In agreement with this effect, Akt activation phosphorylation was also significantly inhibited by intracellular Ca2+ chelation (BAPTA-AM) and CaM inhibition (W-7) [1]

**The Role of Akt activation in response to Genotoxic Stress**

Resistance to chemotherapy is a major cause of treatment failure. There have been accumulating evidence that has indicated that the acquisition of resistance to chemotherapeutic drugs involves the activation of the PI3K/Akt pathway[16]. There is mounting evidence that Akt-induced resistance to chemotherapy and radiotherapy is mediated by involvement in DNA repair. This may occur by co-localization to H2AX nuclear foci after DNA damage and recruitment to sites of double strand breaks through association with DNA-PKcs. Therefore, therapy designed to specifically target Akt and other components of the pathway are now being developed for clinical use as single agents and in combination with chemotherapy to overcome therapeutic resistance [17].

Many chemotherapeutic drugs mainly act by causing genotoxic stress in the cell. There are evidence of genotoxic treatments such as Carboplatin, Doxorubicin and Gemcitabine that activate Akt but the mechanism by which Akt activation occurs on response to DNA damage and its downstream consequences is unclear. Etoposide also acts by the mechanism of DNA damage. It functions by binding to topoisomerase II, an enzyme that can relax DNA supercoiling, insert or remove knots, and catenate or decatenate DNA. It catalyzes an ATP dependent cycle in which both strands of one DNA helix are broken, followed by passage through this break of a second helix and religation of the breaks in the first. By inhibiting the religation step, etoposide and other anticancer agents, such as doxorubicin, create double-strand breaks. They can also cause trapping of complexes in which tyrosine residues of the topoisomerase II [18]. Treatment with

etoposide can cause an induction of PI3K/Akt activity in gastric cancer cells and NIH 3T3 fibroblast cell lines. This causes a decrease in the sensitivity to chemotherapy via prevention of apoptosis. However, a mechanistic connection between PI3k/Akt activation and the actions of etoposide remains to be established [16, 20, 21]

Thus, from the above information we understand that there is a co-relation between activation of Akt and development of resistance to chemotherapy in cancer cells. Also, CaMKK2 phosphorylates and activates Akt. CaMKK2 activation of Akt is seen in many downstream targets known to mediate Akt’s regulation of cell growth, proliferation, apoptosis, protein synthesis, and protein stability. Akt phosphorylation was significantly inhibited by intracellular Ca2+ chelation or CaM inhibition.

The research in this thesis verifies these information and tries to establish a relation between Akt activation and the functions of etoposide. It focuses on following specific aims:

1. Determining if Akt is activated on treatment with DNA damaging chemotherapeutic drug-Etoposide.
2. Determine if CaMKK2 is responsible for activation of Akt on treatment with DNA damaging chemotherapeutic drug-Etoposide.
3. Determine if the response to DNA damaging agents are dependent on intracellular Ca2+
4. Determine if the effects of DNA damaging agents are CaM dependent.

**Materials and Methods**

***Chemicals***

Etoposide ( # E1383) and Carboplatin were purchased from Sigma. W-7 and BAPTA-AM (# 196419) were purchased from Calbiochem. Antibodies to p-Akt T308 (# 13038), p-Akt S473 (# 4060) and Akt (# 4691) were purchased from Cell Signaling. GAPDH (# 0411) antibody was purchased from Santa Cruz. Hanks Balanced Salt Solution (HBSS) (# 21-022) was obtained from Corning Life Sciences.

***Cell Culture***

NIH: OVCAR-3 The human epithelial ovarian cancer cell line OVCAR-3 (#HTB-161) cells were purchased from American Type Culture Collection (ATCC) and authenticated by ATCC by short tandem repeat DNA analysis. OVCAR-3 cells were cultured in RPMI media 1640 supplemented with 20% FBS (Gemini, # 900-108), 10 mM HEPES (Corning, # 25-060), 1 mM sodium pyruvate (Corning, # 25-000-CI), 1 X penicillin/streptomycin, 2mM L-glutamine, 2.4 mg/ml D-glucose (Sigma-Aldrich, # G8270), and 1% (Sigma-Aldrich, # I9278),. Cells were maintained in a humidified atmosphere of 5% CO2 at 37 °C. RPMI-1640 medium as powder (# 31800-022) was purchased from Gibco. Cells were fed with fresh +serum media twice per week and passaged on a weekly basis.

***RNA interference and transfection***

18-24h before transfection, cells were plated in 60-mm plate at the density of 3 X 105 in antibiotic and serum free medium. Cells were transfected using following siRNAs: NS control, CaMKK2 (#2) Silencer Select, 5`-GCAUCGAGUACUUACACUAtt-3`with Lipofectamine 2000 (Invitrogen) as the transfection agent. The NS control siRNA was used at the same concentrations as its targeting siRNA. After 6h, medium was replaced with anti-biotic free, serum-containing medium and cells were allowed to grow for 18hrs.

***Western blotting***

Cells were harvested in Harvest buffer and protease inhibitor. Samples were sonicated, centrifuged and heated for 5minutes. SDS-PAGE gels were loaded with equal volumes from each samples, GAPDH was used as loading control and all signals were normalized to GAPDH before analysis was performed. Typically electrophoresis was performed in 10% polyacrylamide gels made freshly. The same volume from each of the samples were loaded onto the gels and electrophoresis was performed at 200 V for 45 min to 1 h. Separated proteins were transferred to PVDF membranes by electrophoresis at 100 V for 1.5 h. The membranes were blocked by incubation with blocking buffer, consisting of 5% milk at 25oC for 1h. Overnight incubation at 4°C in PBS with 0.1% triton X-100 and 3% non-fat milk was carried out for primary antibodies.Western blotting was performed using primary antibodies against phosphorylated- Akt at T308 (1:500, Cell signaling) ,S473 (1:1000, Cell signaling), PRAS40 (1:1000, Cell signaling), CaMKK2 (1:500, Cell signaling). After extensive washing with PBS and 0.1% tween X-100, Secondary antibodies (1:1000, Cell signaling) were used for signal detection with the ECL system (Super Signal® West Pico, Pierce, Rockford, IL). The level of protein loading was determined by antibodies against total Akt (T-Akt, 1:1000, Cell Signaling), total PRAS40 (PRAS40, 1:1000, Cell Signaling) and GAPDH (1:10,000). The intensity of protein level obtained from within the linear range of exposures were quantified after local background subtraction using Image Lab and shown in figures with representative blots.

***Treatment of cells with Etoposide***

Etoposide is poorly soluble in water but soluble in organic solvents such as ethanol, methanol and DMSO to different extents. A 50 mM solution was prepared in dimethyl sulfoxide as a stock solution. OVCa cells were plated at a density of 3 X 105 in a 60-mm plate. The cells were allowed to attach and reach 50-60% confluency and thus plated for 18-24h.This was followed by treatment with 100uM Etoposide or DMSO (vehicle) for 18h in media containing 10% (one-half) the concentration of FBS as their normal growth media.

***Treatment of cells with BAPTA-AM and W-7***

OVCAR-3 cells were plated at a density of 3 X 105 in 60-mm plates. Cells were washed with Hanks’ balanced Salt Solution (HBSS) (Corning). The following treatments were conducted. For BAPTA-AM, cells were incubated in RPMI 1640 medium (Invitrogen) for 2 h and then treated with 15uM BAPTA or vehicle (0.3% DMSO) in HBSS for the times indicated.

***Presentation of Data and statistical Analysis***

Significances of differences were determined using GraphPad Prism 6, using Student t-test, one-way ANOVA with Sidak’s multiple comparison test as appropriate. Numbers of replicates (n) refer to the biological replicates, that is, the number pf independent cell cultures analyzed. Significance of differences are indicated as follows: \*, P <0.05; \*\*P < 0.01, \*\*\* P < 0.001.

**RESULTS**

1. **Etoposide activates Akt in ovarian cancer cells**

AKT is a critical effector of serine/threonine kinase in the receptor tyrosine kinase/phosphatase and controls a multitude of cellular functions. Activation of AKT not only supports tumor growth and progression but also contributes to tumor-cell evasion of the cytotoxic effects of cancer therapy through many avenues including the promotion of anti-apoptosis, proliferation, and migration and regulation of the cell cycle. Accumulating evidence has implicated AKT as a direct participant in the DNA damage response and repair induced by commonly used genotoxic agents [19]. We have previously mentioned that etoposide causes activation of Akt in gastric cancer cells Therefore, here we verified if Etoposide causes activation of Akt following its induction in OVCAR-3 cells.

In OVCAR-3 cells, Akt activation following addition of 30uM and 50uM Etoposide was either insignificant or modest. Infusion of high-dose etoposide in humans results in peak plasma concentrations ranging from 50 to 200uM/L [19]. Akt activation was observed following treatment of OVCAR-3 cells at 9h, 18h and 24h with 100uM Etoposide (drug) or 100uM DMSO (control). This was associated with the phosphorylation of Akt at Thr308, by -------- fold for 9h, 1.6 fold for 18h (p<0.01) and 2 fold for 24h (mean +\_ SEM, n=5).

We then explored the effects of Etoposide on the activities of downstream Akt target regulating protein translation, apoptosis and cell cycle progression. Akt regulates protein translation by direct phosphorylation of PRAS40 at Thr-246. This releases the inhibition of raptor, resulting in mTORC1 phosphorylation of S6K and 4E-BP1 [7]. Etoposide significantly increased the phosphorylation of PRAS40 (Thr246) by almost 1.8 fold.

1. **Etoposide activated Akt in a CaMKK2 dependent manner.**

CaMKK2 knockdown in OVCAR-3 cells decreased phosphorylation of Akt at its primary activation site, Thr-308 by 65% (siRNA #1) and 78% (siRNA #2) [1]. Thus, CaMKK2 played a significant role in maintaining Akt phosphorylation in OVCa cells. The mechanism by which Etoposide causes Akt activation was still unknown. Since, CaMKK2 regulates Akt activation in OVCa cells, we wanted to verify if CaMKK2 played any active role in causing activation of Akt due to the genotoxic stress caused by addition of Etoposide.

To determine the role of CaMKK2 in regulation of Akt activity by Etoposide of OVCa cells, CaMKK2 knockdown in OVCAR-3 cells was conducted using siRNAs that target distinct region of the CaMKK2 mRNA (siRNA #2). Western blot results show that we obtained effective CaMKK2 knockdown that blocked the Etoposide’s effect of activation of Akt relative to non-specific (NS #2) control. These results show that CaMKK2 plays a role in causing activation of Akt caused due to Etoposide. In order to further strengthen our finding, we again verified the downstream target of Akt PRAS40 . The knockdown prevented the activation of PRAS40 in response to Etoposide.

Thus, in OVCAR-3 cells , the regulation of Akt by etoposide is CaMKK2 dependent.

1. **Etoposide activates and regulates Akt in a Ca2+/ CaM dependent manner.**

Ca2+ is a critical regulator of a wide range of physiological processes including cell proliferation and survival [23]. In order to progress through the cell cycle, mammalian cells require adequate levels of intracellular Ca2+. The control of these physiological processes by intracellular Ca2+ is often mediated by Calmodulin (CaM). It was reported that CaM binds directly to Akt1 at its PHD [24-26]. A current model for the role of Ca2+/CaM in Akt activation includes growth factor (GF) induced rise in Ca2+i, formation of the Ca2+/CaM complex, displacement of the PHD from the activation loop of Akt, its translocation to the inner leaflet of the plasma membrane (PM) and phosphorylation of the residues T308 and S473 by PDK1 and mTORC2, respectively. At the membrane, competition with PIP3 causes the release of CaM to the cytosol. It was previously reported that WT Akt is activated by CaMKK2 in a Ca2+/CaM-dependent manner in vitro . There are data to indicate that Ca2\_-CaM–CaMKK2 complex promotes Akt activation by direct phosphorylation at Thr-308 [1]



The intracellular Ca2+ chelator BAPTA-AM and the CaM antagonist W-7 decreased p-Akt Thr-308, respectively [1]. Therefore, we next investigated if the phenomenon of Akt activation by Etoposide via CaMKK2 is Ca2+/ CaM dependent. Cells were pre-treated with BAPTA-AM / DMSO for 30 mins followed by treatment with 100uMEtoposide + 10uMBAPTA-AM for 18hrs. We observed that the effect of Etoposide is reduced following chelation of intracellular Ca2+ ions. Effect was also observed for the downstream target PRAS40 of Akt and similar results were obtained which showed that the phenomena by which etoposide activates Akt via CaMKK2 is Ca2+ dependent. In order to determine if the phenomena is CaM dependent, we used CaM inhibitor W-7. Cells were pre-treated with W7/ ddH2O followed by treatment with 100uM etoposide + 15uM W7 for 18 hrs. It was observed that the Akt activation by etoposide was reduced following inhibition of Calmodulin. This shows that the effect is CaM dependent.

Thus, Etoposide activates Akt in a Ca2+/ CaM dependent manner via the CaMKK2 pathway.