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 Ca^{2+} influx, Akt is phosphorylated at T308 in neuroblastoma-glioma cells by Ca^{2+} Calmodulin (CaM) mediated Kinase Kinase (CaMKK) [9]. A CaMKK-dependent activation of Akt was also demonstrate in prostate cancer (PCa) cells upon stimulation of Ca^{2+} 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 Ca^{2+} 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.

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 (OVCAR-3) 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.

RNA interference and transfection

18-24h before transfection, cells were plated in 60-mm plate at the density of 3 X 10^5 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 25^oC 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 10_5 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 10_5 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: *, $P < 0.05$; **$P < 0.01$, *** $P < 0.001$.