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Research Article

PKCη is a negative regulator of AKT inhibiting the IGF-I induced proliferation

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ABSTRACT

The PI3K-AKT pathway is frequently activated in human cancers, including breast cancer, and its activation appears to be critical for tumor maintenance. Some malignant cells are dependent on activated AKT for their survival; tumors exhibiting elevated AKT activity show sensitivity to its inhibition, providing an Achilles heel for their treatment. Here we show that the PKCη isoform is a negative regulator of the AKT signaling pathway. The IGF-I induced phosphorylation on Ser473 of AKT was inhibited by the PKCη-induced expression in MCF-7 breast adenocarcinoma cancer cells. This was further confirmed in shRNA PKCη-knocked-down MCF-7 cells, demonstrating elevated phosphorylation on AKT Ser473. While PKCη exhibited negative regulation on AKT phosphorylation it did not alter the IGF-I induced ERK phosphorylation. However, it enhanced ERK phosphorylation when stimulated by PDGF. Moreover, its effects on IGF-I/AKT and PDGF/ERK pathways were in correlation with cell proliferation. We further show that both PKCη and IGF-I confer protection against UV-induced apoptosis and cell death having additive effects. Although the protective effect of IGF-I involved activation of AKT, it was not affected by PKCη expression, suggesting that PKCη acts through a different route to increase cell survival. Hence, our studies show that PKCη provides negative control on AKT pathway leading to reduced cell proliferation, and further suggest that its presence/absence in breast cancer cells will affect cell death, which could be of therapeutic value.

Introduction

Tumor formation is currently viewed as a multistage process, in which several mutations in growth-enhancing oncogenes or growth-inhibiting tumor suppressor genes are acquired, leading to deregulation of specific signaling pathways [1]. Many tumors, unlike normal cells, appear to be highly dependent on the constitutive activation of specific genes, which led to the notion that cancer cells are addicted to certain signaling pathways, thus providing an Achilles heel for the treatment of cancer [2]. The elucidation of the molecular mechanisms that result in these aberrant changes in tumor cells, in particular the signaling processes of cell proliferation and cell survival, will enable us to better predict the most appropriate targets for preferential tumor killing and cancer therapy.

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Abbreviations: PKB, protein kinase B; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; IGF, Insulin-like growth factor; PDGF, platelet derived growth factor; PDK-1, Phosphoinositide-dependent kinase-1; PI3K, phosphatidylinositol 3-kinase; shRNA, short hairpin RNA.

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The PI3K-AKT pathway is frequently activated in human cancers, and AKT activation appears to be critical for tumor maintenance. Moreover, many studies indicate that malignant cells may depend on activated AKT for survival, and that tumor cells exhibiting elevated AKT activity are sensitive to the inhibition of the AKT pathway [3]. Notably, increased AKT kinase activity has been reported in ~40% of breast and ovarian cancers [4].

Stimulation of many cells with a variety of extracellular agonists initiate signaling pathways that culminate in the recruitment and activation of AKT [5]. Full activation of AKT is phosphatidylinositol-3-kinase (PI3K) dependent and requires both recruitment to the plasma membrane and phosphorylation on two key regulatory sites, Thr308 by PDK1 (an effector kinase downstream to PI3K) and on serine473 by autophosphorylation or by PDK2, recently implicated as mTOR/rictor [6]. Several key pro-apoptotic proteins are targets for AKT phosphorylation including BAD [7], caspase 9 [8] and FKHR [9]. In addition, AKT alters cell cycle control by phosphorylating and inactivating $p21^{WAF1}$ or regulating the transcription of cyclin D1 and $p27^{KIP1}$ phosphorylation and stability [10,11]. Different mechanisms were described that contribute to AKT hyperactivation in human cancer; inactivation of PTEN (a tumor suppressor phosphatase that dephosphorylates phosphatidylinositol (3,4,5)-triphosphate (PIP3)), exhibiting deletions and mutations in many types of cancer leading to AKT activation [12]. *PIK3CA* and *Ras* mutations were shown to lead to AKT activation and occur frequently in human cancers [13], and PHLPP and PML also regulate the AKT pathway in tumorigenesis [14,15]. Thus, it appears that AKT activation plays a pivotal role in the genesis of cancer.

Many oncoproteins and tumor suppressors intersect with the AKT pathway, deregulating cellular functions by interfering with signal transduction and metabolic control [4]. The involvement of PKC enzymes in the regulation of the PI3K-AKT/PKB pathway was recently suggested. Protein kinase C (PKC) represents a family of Serine/Threonine kinases implicated in a variety of cellular responses including proliferation, differentiation, gene expression, membrane transport, secretion and transformation. The early observations that PKC isoenzymes are activated by the tumor-promoting phorbol esters suggested a key role for PKC in tumor promotion and progression, thus being considered as targets for cancer therapy [16]. The PKC isoforms are classified into classical PKCs (α, βI, βII and γ), that require Ca+2 and DAG for activation, novel PKCs (δ, ε, η and θ), that are Ca+2 independent but respond to DAG, and atypical PKCs (ζ and ι/λ), that are insensitive to both Ca+2 and DAG [17]. Although these enzymes share similar structural domains, they differ with respect to their tissue distribution and sub-cellular localization. Each of the PKC isoforms appears to execute specific functions since several PKCs are usually expressed within the same cell, although it is likely that some functional redundancy also exists [18]. Moreover, the functions of PKC isoforms in proliferation or apoptosis may be opposing; of the ten family members of PKC, PKCα and PKCε were implicated in cell proliferation, while PKCδ and PKCη were associated with differentiation and control of apoptosis [16]. Although, in breast cancer cells and in glioblastomas, PKCη was also shown to regulate proliferation [19,20].

A cross-talk between the PI3K and PKC pathways was recently suggested as one of the mechanisms regulating cellular proliferation and apoptosis. PDK1, downstream of PI3K, phosphorylates and activates both AKT and PKC [17]. Several PKC isoforms showed both positive and negative effects on AKT phosphorylation and activation [20–26]. Here we show that the PKCη isoform is a negative regulator of the AKT pathway in MCF-7 breast adenocarcinoma cancer cells. The IGF-I or insulin stimulated phosphorylation of AKT was inhibited by the induced expression of PKCη in these cells. The reduced phosphorylation on AKT, observed in response to IGF-I stimulation in cells expressing PKCη, was in correlation with inhibition of cell proliferation. We further show that both PKCη and IGF-I confer protection against UV-induced apoptosis, having an additive effect. Although the protective effect of IGF-I against UV-induced cell death involved activation of AKT, it was not affected by PKCη expression, suggesting that PKCη acts through a different route to increase cell survival.

Materials and methods

Cell culture and reagents

MCF-7 cells inducibly expressing PKCη (MCF21.5) or MCF-7 cells inducibly expressing PKCδ (MCFD44) were previously described [19]. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM 1-glutamine and 10% Fetal Bovine Serum (FBS) in a 5% CO_2 humidified atmosphere at 37 °C. The expression of PKCη or PKCδ was induced by removal of tetracycline (2 μg/ml) from their growth medium. For growth factors stimulation, sub-confluent cells were transferred to serum-free medium for overnight followed by their stimulation with insulin like growth factor (IGF-I) in 0.1% serum, insulin in serum-free medium supplemented with 0.2% BSA or platelet-derived growth factor BB (PDGF-BB) in 0.1% serum. For the irradiation studies, the medium was removed and the cells were exposed to UVC (254 nm), 2 J/m^2 per second for 6 s (12 J/m^2). IGF-I and PDGF-BB were purchased from Cytolab (Israel). Insulin, Okadaic Acid (OA) and tetracycline were purchased from Sigma-Aldrich (USA). PD98059 (PD) and Bisindolylmaleimide I (BIM) were purchased from Alexis (USA) and LY294002 (LY) from Cell Signaling Technology (USA).

Knock-down of PKCη with short hairpin RNA

Cells were transfected with two pre-designed PKCη short hairpin RNA (shRNA) vectors or scrambled vector (SureSilencing, SA Biosciences USA), according to the manufacturer's instructions. To isolate neomycin-resistant colonies, 1 mg/ml Geneticin selection was initially applied and later reduced to 400 μg/ml. Silencing of PKCη expression was confirmed by reverse transcription-PCR analysis and immunoblot. Transient PKCη knocked-down MCF-7 cells were generated using the pSuper vector (from R. Agami) as previously described [27]. MCF-7 cells were transfected with the plasmid containing the silencing insert (shPKCη) or with a control plasmid (pSuper) using the jetPEI™ reagent (Poly transfection, France) according to the manufacturer's instructions.

Cell lysis and immunoblot analysis

Cell lysates were prepared using RIPA lysis buffer containing 10 mM Tris pH 8.0, 100 mM NaCl, 5 mM EGTA, 0.1% SDS, 1% NP-40, 45 mM β−mercaptoethanol, 50 mM NaF. Protease inhibitors (1 mM PMSF, 10 μg/ml aprotinine and 10 μg/ml leupeptin) and phosphatase inhibitors (100 μM sodium orthovanadate, 50 mM