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Activation of ERK during DNA damage-induced apoptosis involves protein kinase Cδ ☆

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Abstract

We have previously shown that protein kinase C (PKC) acts upstream of caspases to regulate cisplatin-induced apoptosis. Since extracellular signal-regulated kinases (ERKs) have also been implicated in DNA damage-induced apoptosis, we have examined if ERK signaling pathway acts downstream of PKC in the regulation of cisplatin-induced apoptosis. PKC activator PDBu induced ERK1/2 phosphorylation which was inhibited by general PKC inhibitor bisindolylmaleimide and Gö 6983 as well as the MEK inhibitor U0126 but not by the PKCδ inhibitor rottlerin. Cisplatin caused a concentration-dependent activation of ERK1/2 in HeLa cells. The level of ERK2 was decreased in HeLa cells that acquired resistance to cisplatin (HeLa/CP). The MEK inhibitor U0126 inhibited cisplatin-induced ERK activation and attenuated cisplatin-induced cell death. Inhibition of PKCδ by rottlerin or depletion of PKCδ by siRNA inhibited cisplatin-induced ERK activation. These results suggest that cisplatin-induced DNA damage results in activation of ERK1/2 via PKCδ.

Keywords: PKCδ; ERK; Cisplatin; Apoptosis; DNA damage; Drug-resistance; Rottlerin; U0126; HeLa cells; siRNA

cis-Diamminedichloroplatinum(II) (cisplatin or CP) is one of the most important anticancer drugs used for the treatment of solid tumors, including ovarian, testicular, and cervical cancer [1]. Acquisition of resistance by tumor cells to chemotherapeutic drugs, however, poses a serious problem in anticancer therapy [1]. The anticancer activity of cisplatin is believed to be due to its ability to damage DNA [2]. Cisplatin-induced DNA damage results in activation of caspases, followed by the cleavage of critical cellular proteins, including PARP, DNA-dependent protein kinase and lamin B, resulting in apoptotic cell death [3,4]. The efficacy of the chemotherapeutic drugs depends not only on their ability to detect damage but also on the cell's ability to detect and respond to DNA damage [5]. Cisplatin-induced genotoxic stress activates multiple signal transduction pathways, which can contribute to cisplatin-induced apoptosis or to cellular protective responses.

The protein kinase C (PKC) signal transduction pathway plays an important role in influencing cisplatin-induced apoptosis [4]. PKC is a family of lipid-dependent serine/threonine kinases that have been subdivided into 3 groups: conventional (α, βI, βII, and γ), novel (δ, ε, η, and θ), and atypical (ζ, ι/λ) [6–9]. PKCδ is a substrate for caspase-3 and proteolytic activation of PKCδ has

☆ *Abbreviations:* BIM, bisindolylmaleimide II; bryo, bryostatin 1; CP, *cis*-diamminedichloroplatinum(II); CF, catalytic fragment; ERK, extracellular signal-regulated kinase; JNK, c-jun N-terminal kinases; MAPK, mitogen-activated protein kinase; MEK, MAP kinase kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PDBu, phorbol 12,13-dibutyrate; PKC, protein kinase C; aPKC, atypical PKC; cPKC, conventional PKC; nPKC, novel PKC; Rot, rottlerin; SAPK, stress-activated protein kinase; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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been directly associated with DNA damage-induced apoptosis [10]. PKC can interact with other signaling pathways to elicit its biological responses.

Mitogen-activated protein kinases (MAPK) are a family of structurally related serine/threonine kinases that coordinate various extracellular signals to regulate cell proliferation, differentiation, and cell survival [11–13]. They are also activated in response to various stresses [12]. The MAPK subfamilies, including the extracellular signal-regulated kinases (ERK), the c-jun N-terminal kinases (JNK)/stress-activated protein kinases (SAPK), and the p38 MAP kinases have been implicated in DNA damage-induced apoptosis [14–23]. There are controversies whether activation or inhibition of ERKs is important for cisplatin-induced cell death [14,15,17,18,20,24].

There have been several reports that suggest that PKC acts upstream of the ERK signaling pathway [25–30]. ERK is activated via a linear cascade consisting of Ras, Raf, MEK, and ERK, and the classical Raf/MEK/ERK pathway transduces signals from the plasma membrane to the cell nucleus [11]. Several PKC isozymes, including PKCδ, have been shown to activate ERK via activation of Raf [25,28,29]. However, how DNA damage induces ERK activation is incompletely understood. Since PKCδ plays an important role in DNA damage-induced apoptosis, we have examined if ERK acts downstream of PKCδ to influence cisplatin sensitivity. Our results show that activation of ERK was associated with cisplatin-induced apoptosis. Furthermore, cisplatin-induced DNA damage results in the activation of ERK1/2 via PKCδ.

Materials and methods

Materials. PDBu were purchased from LC Service Corporation (Woburn, MA). Rottlerin, Gö 6983, Gö 6976, U0126, and SB203580 were purchased from EMD Biosciences (La Jolla, CA). Cisplatin and MTT were from Sigma (St. Louis, MO). Polyclonal antibodies to tubulin and PKCδ, and siRNAs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody to ERK2 was from Transduction Laboratories (Lexington, Kentucky). Monoclonal antibody to phospho-p42/44 MAPK (Thr202/Tyr204) was purchased from Cell Signaling Technology (Beverly, MA). Monoclonal antibody to PARP was from Pharmingen (San Diego, CA). Horseradish peroxidase conjugated donkey anti-mouse and anti-rabbit antibodies were obtained from JacksonImmunoResearch Lab. (West Grove, PA). Poly(vinylidene difluoride) membrane was from Millipore (Bedford, MA) and enhanced chemiluminescence detection kit was from Amersham (Arlington Heights, IL).

Cell culture. Human cervical carcinoma HeLa cells and its cisplatin-resistant variants (HeLa/CP) were maintained in Dulbecco's modified minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mM glutamine, and kept in a humidified incubator at 37 °C with 95% air and 5% CO_2.

Assessment of cell viability by MTT assay. The exponentially growing cells were plated in microtiter plates and incubated at 37 °C in 5% CO_2. The following day, cells were pretreated with vehicle DMSO or with kinase inhibitors and then with different concentrations of cisplatin. The number of viable cells was determined using the dye MTT as previously described [31].

Immunoblot analysis. Equal amounts of total cell extracts were electrophoresed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred electrophoretically to poly(vinylidene difluoride) membrane. Immunoblot analyses were performed as described before [3]. Intensities of immunoreactive proteins were quantified using a laser densitometer (Molecular Dynamics) and Image Quant software.

Knock-down of PKCδ. Control siRNA or siRNA targeted against PKCδ was introduced into HeLa cells using Lipofectamine 2000 (Invitrogen) and manufacturer's protocol.

Results

Effects of PKC modulators on ERK1/2 activation

Activation of ERK requires dual phosphorylation of Thr and Tyr residues by MEK1 and MEK2. To determine if ERK activation is mediated by PKC, we examined the effect of PDBu on the activation of ERK1/2 by monitoring phosphorylation of ERK1/2 using an antibody that specifically recognizes Thr202/Tyr204 phosphorylation sites. Cells were serum-starved for 4 h to decrease the basal phosphorylation status of ERK1/2. Fig. 1 shows that PDBu caused an increase in ERK1/2 phosphorylation without any change in the total ERK2 level. We were unable to detect any ERK1 using the ERK2 antibody that recognizes both ERK1 and ERK2. The MEK inhibitor U0126 prevented PDBu-induced ERK1/2 activation, suggesting that PDBu acts via MEK to activate ERK. The general PKC inhibitors bisindolylmaleimide and Gö 6983 that inhibit both conventional and novel PKCs also inhibited PDBu-induced ERK activation but the PKCδ inhibitor

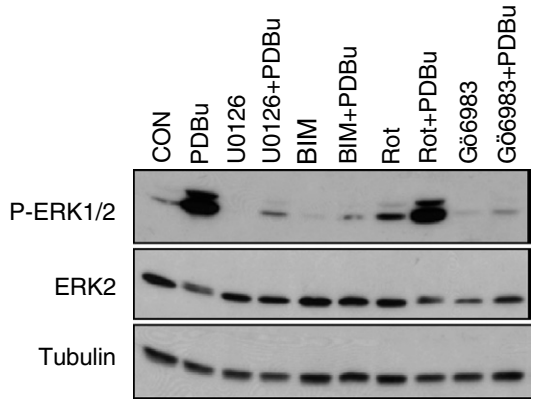

Fig. 1. Effects of PKC inhibitors on PDBu-induced ERK activation. Cells were serum-starved for 4 h and treated with 10 μM U0126, 3 μM BIM, 10 μM rottlerin, and 1 μM Gö 6983 for 1 h. Cells were then treated with 1 μM PDBu for 15 min and Western blot analysis was performed with indicated antibodies. Results are representative of at least two experiments.