

Altered Protein Kinase C (PKC) Isoforms in Non-Small Cell Lung Cancer Cells: PKC δ Promotes Cellular Survival and Chemotherapeutic Resistance

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Abstract

Drugs that target protein kinase C (PKC) are now being evaluated in patients with non-small cell lung cancer (NSCLC), but the role of PKC in NSCLC cells remains unclear. We report here that NSCLC cell lines show enhanced phosphorylation and altered expression of specific PKC isoforms compared with normal lung epithelial cells. PKC inhibition variably increased apoptosis, with rottlerin, a PKC δ inhibitor, being most effective and potentiating chemotherapy-induced apoptosis, especially with trastuzumab. Consistent with PKC δ being anti-apoptotic in NSCLC cells, transient transfection of a kinase-dead mutant of PKC δ increased apoptosis and potentiated chemotherapy-induced apoptosis. Our studies provide a rationale for targeting PKC isoforms in NSCLC cells, especially PKC δ .

Introduction

The inadequacy of standard chemotherapy in advanced NSCLC² has driven the development of novel, targeted therapeutic approaches such as those directed against the epidermal growth factor receptor. Recently, our laboratory identified two signaling pathways downstream of growth factor receptors such as epidermal growth factor receptor that are constitutively active in the majority of NSCLC cell lines and promote cellular survival and resistance to chemotherapy and radiation. These pathways are the PI3K/Akt pathway and the MEK/ERK pathway (1, 2). Although we described in a subset of NSCLC cell lines an inverse relationship between these pathways that was possibly related to c-Raf phosphorylation (2), regulatory mechanisms between these pathways are likely to be complex and are likely to involve other kinases.

In the present report, we characterize PKC isoform expression and function in NSCLC cells. One motivation was the known role of PKC isoforms in regulation of the PI3K/Akt and MEK/ERK pathways. PKC isoforms such as PKC α , β , δ , and ζ may be activated in concert with Akt because PDK1, which phosphorylates and activates Akt, also phosphorylates and activates PKC α , β , δ , and ζ (3). Once activated, PKC isoforms may regulate the PI3K/Akt pathway positively (4) or negatively (5). A PKC isoform of particular interest with regard to Akt is PKC δ . PKC δ is proapoptotic in most systems (reviewed in Ref. 6). PKC δ is activated by both PDK1 and mammalian target of rapamycin (mTOR) (7), a downstream substrate of Akt. PKC δ and Akt are activated by cellular stress (8) as well as by the products of PI3K, phosphatidylinositol 4,5-bisphosphate, and phosphatidylinositol 3,4,5-trisphosphate (9). Akt can physically associate with the regulatory domain of PKC δ (10), and Akt

may phosphorylate PKC δ under certain conditions (11). PKC δ can also mediate activation of the MEK/ERK pathway caused by phorbol esters or growth factors.

A second motivation for analysis of PKC isoforms in NSCLC cells is that therapeutic approaches targeting PKC are being evaluated in clinical trials. These trials are investigating drugs such as bryostatin or ISIS 3521, that through discovery or design inhibit PKC (12, 13), as well as drugs such as staurosporin or UCN-01 that incidentally inhibit PKC. Thus far, however, little is known about PKC isoforms *in vitro* or in NSCLC tumor specimens to rationally support studies in patients with NSCLC.

In the present studies, we demonstrate that at least six PKC isoforms are expressed in a panel of NSCLC cell lines that we previously characterized for Akt and ERK activation. Compared with normal lung epithelial cells, NSCLC cells exhibit selective expression of certain PKC isoforms in an active state and exhibit selective sensitivity to PKC inhibitors. A PKC δ inhibitor, rottlerin, was most effective at increasing apoptosis and dramatically sensitized NSCLC cells to the effects of trastuzumab, as well as other chemotherapeutic agents. These effects were recapitulated when NSCLC cells were transfected with a PKC δ kinase-dead mutant but not with wild-type PKC δ . Our biochemical and genetic data suggest that PKC δ promotes cellular survival and chemotherapeutic resistance in NSCLC cells, in contrast to its proapoptotic role in many systems.

Materials and Methods

Materials

NSCLC cells were obtained and grown as described previously (1). NHBE cells and materials were purchased from BioWhittaker Inc. (Walkersville, MD) and grown according to the manufacturer's instructions. BEAS-2B cells were a generous gift from Dr. C. Harris (Bethesda, MD) and grown as described previously (14). LY294002 was obtained from Alexis Biochemicals (San Diego, CA). Protease inhibitor mixture, cisplatin, and α -tubulin antibodies were from Sigma Chemical Co. (St. Louis, MO). Protein assay kits were purchased from Bio Rad (Hercules, CA). Protran pure nitrocellulose membranes were from Schleicher & Schuell (Keene, NH). Rottlerin, calphostin C, chelerythrine chloride, and paclitaxel were purchased from Calbiochem (La Jolla, CA). Trastuzumab was from Genentech (San Francisco, CA). Gemcitabine was obtained from Eli Lilly (Indianapolis, IN). Docetaxel was from Aventis Pharmaceuticals Products Inc. (Collegeville, PA). All native PKC isoform antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). All phospho-specific antibodies, horseradish peroxidase-conjugated secondary antibodies, and the PARP antibodies were purchased from Cell Signaling (Beverly, MA). P-PKC β_{II} (S660) and P-PKC α/β_{II} (T638/T641) antibodies are directed against autophosphorylation sites that are necessary for PKC activity. P-PKC ζ/λ (T410/403), P-PKC δ (T505), and P-PDK-1 (S241) antibodies are directed against residues in the activation loop that are necessary for PKC ζ , PKC δ , and PDK-1

Received 7/25/02; accepted 1/7/03.

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² The abbreviations used are: NSCLC, non-small cell lung cancer; PKC, protein kinase C; PI3K, phosphatidylinositol 3'-kinase; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/ERK kinase; PDK, phosphoinositide-dependent kinase; GFP, green fluorescent protein; FBS, fetal bovine serum; DAG, diacylglycerol; PARP, poly (ADP-ribose) polymerase; CF, catalytic fragment.

activity, respectively. PKC ζ (T410) and P-PKC δ (T505) are sites for PDK-1 phosphorylation. Antibodies against GFP were purchased from Zymed Laboratories, Inc. (South San Francisco, CA). Construction of wild-type PKC δ -GFP and K376R (kinase-dead) PKC δ -GFP was described previously.

Methods

Pharmacological Treatments. To determine the effects of LY294002 on PKC phosphorylation, cells were plated at 9×10^5 to 1.5×10^6 cells/25-cm² flask to achieve ~70% confluence. After attachment, medium was changed to DMEM with 0.1% FBS overnight. LY294002 (25 μ M) was added 2 h before harvesting. To study the effect of PKC inhibitors on apoptosis, cells were plated at 1×10^5 or 1.5×10^5 in 24- or 12-well plates, respectively, and allowed to attach. Medium was changed to DMEM with 0.1% FBS with or without rottlerin (10 μ M), Gö6976 (100 nM), calphostin C (1 μ M), or chelerythrine chloride (10 μ M). Cells were incubated for 48 h and harvested for measurement of apoptosis. To assess the effects of rottlerin on chemotherapy-induced apoptosis, rottlerin (10 μ M) was added simultaneously to the indicated agents in DMEM with 0.1% FBS for 48 h.

Immunoblotting. To examine PKC isoform expression in NSCLC cells and NHBE and BEAS2B cells, confluent 75-cm² flasks were utilized. Preparation of cell extracts, protein assays, and immunoblotting were performed as described previously (1). Equivalent loading was confirmed by staining membranes with fast green as described previously. All PKC isoform and phospho-specific PKC antibodies were used at 1:1,000 dilutions. Cleaved PARP antibody was used at 1:1,500, and GFP monoclonal antibody was used at 1:1,000. Secondary antibodies were used at 1:2,000 to 1:10,000 dilutions. All immunoblotting experiments were repeated at least three times.

Flow Cytometry. Floating cells were collected, and adherent cells were harvested by trypsinization and centrifuged for 5 min. Cells were fixed in ice-cold 70% methanol added dropwise and then incubated at -20°C for 30 min. Cells were centrifuged and incubated with propidium iodide (25 μ g/ml) supplemented with RNase A (30 mg/ml) for 30 min at room temperature. Quantification of sub-2N DNA was determined by flow cytometry analysis using a Becton-Dickinson FACSort and by manual gating using CellQuest software. Gating was performed on blinded triplicate samples.

Transient Transfections. Cells were plated at 3×10^5 cells/well in 6-well dishes (approximately 70% confluence) and transfected in triplicate with plasmids encoding wtPKC δ or K376R PKC δ using the Superfect Transfection Reagent protocol (Qiagen, Valencia, CA). Cells were allowed to recover from transfection for 24 h, and then medium was changed to DMEM with 0.1% FBS or 10% FBS \pm chemotherapeutic agents as indicated for 48 h. Whole cell lysates from ungated samples were harvested for immunoblotting to assess GFP levels and PARP cleavage. Samples were also fixed, stained with propidium iodide, and prepared for flow cytometry. Apoptosis was quantified by gating on GFP-positive cells and performing cell cycle analysis. Experiments were repeated at least three times.

Statistical Analysis. Statistical comparison of mean values was performed using Student's *t* test. All *P*s are two tailed.

Results

PKC family members are grouped based on structural similarities that determine dependence on activation by Ca²⁺ and DAG (classical isoforms), DAG alone (novel isoforms), or neither (atypical isoforms; reviewed in Ref. 15). To determine the extent of PKC isoform expression in NSCLC cells, we performed immunoblotting with isoform-specific antibodies. Multiple PKC isoforms within each PKC family were expressed in each of five NSCLC cell lines previously analyzed for Akt and ERK activity, including α and β in the classical family, δ and ϵ in the novel family, and μ and ζ in the atypical family (Fig. 1A). PKC λ and η were not examined in these studies. PKC γ was barely detectable in all cell lines. H1155 cells, which have the highest levels of Akt activity, had the lowest expression of PKC α , β , and ϵ . PKC δ levels were lowest in the H157 cells, and the *M_r* 40,000 catalytic fragment (CF) of PKC δ was only detected in A549 and H1355 cells, which have the highest levels of ERK activity.

Because multiple PKC isoforms were expressed in each NSCLC cell line and because PKC activation can contribute to cellular transformation, we compared PKC isoform expression and phosphorylation in primary human lung epithelial cells (NHBE), NHBE cells immortalized with SV40 large T antigen (BEAS-2B; Ref. 14), and a NSCLC cell line (H1355). Compared with NHBE, H1355 cells had increased expression of PKC α , β , ϵ , and μ (Fig. 1B, left panels). PKC δ expression was decreased in H1355 cells, but expression of the PKC δ CF was increased. Immortalized BEAS-2B cells expressed levels of PKC α , β , ϵ , and μ comparable with those of H1355 cells but had levels of PKC δ comparable with NHBE. Levels of PKC ζ did not differ among these three cell types. Although these three cell types are not isogenic, these studies suggest that transformation of lung epithelial cells may be accompanied by changes in PKC isoform expression. Because the levels of PKC α , β , ϵ , and μ were increased in H1355 cells compared with NHBE, but were similar among the five NSCLC cell lines, increased PKC isoform expression may be a general feature of NSCLC cells.

To assess whether altered protein expression of PKC isoforms in H1355 cells was associated with increased phosphorylation of PKC isoforms, we performed immunoblotting with phospho-specific antibodies that recognize PKC isoforms in their active states (Fig. 1B, right panels). Phosphorylation of PKC α , β , δ , and ζ was greatest in H1355, intermediate in BEAS-2B, and low or absent in NHBE. Increased PKC α and β phosphorylation mirrored increased protein expression, but increased PKC δ and ζ phosphorylation occurred independently of increased protein expression. Notably, increased PKC δ phosphorylation was observed despite a decrease in protein level. The fact that the highest levels of PKC isoform phosphorylation were observed with H1355 cells prompted us to evaluate PKC phosphorylation in the other NSCLC cell lines.

To determine whether PKC isoforms were maintained in an active state with serum starvation and to determine whether PKC phosphorylation was PI3K dependent (because PKC α , β , δ , and ζ are all substrates of the PI3K-dependent kinase, PDK-1), NSCLC cells were serum starved in the absence or presence of a PI3K inhibitor, and phosphorylation of PKC α , β , δ , and ζ ; PDK-1; and Akt was measured. Fig. 1C shows that in NSCLC cell lines with high levels of Akt activity, Akt phosphorylation was maintained with serum deprivation but was inhibited by a PI3K inhibitor, LY294002. PDK-1 phosphorylation was also maintained with serum deprivation, but it was not affected by LY294002. Likewise, phosphorylation of PKC α , β , ζ , and δ at the PDK-1 sites was maintained under conditions of serum deprivation, but no inhibition was observed with LY294002. In fact, phosphorylation of PKC α , β , and ζ was slightly increased in the H157 cells by LY294002 treatment. Together, these data showed that Akt, PDK-1, and multiple PKC isoforms were maintained in an active state when NSCLC cells were serum deprived but that only Akt phosphorylation was PI3K dependent.

Maintenance of four PKC isoforms in active states under conditions of serum starvation suggested that these PKC isoforms might contribute to NSCLC cell survival. To test this hypothesis, we treated NSCLC cell lines with four widely used PKC inhibitors (16): (a) rottlerin, a selective PKC δ inhibitor; (b) calphostin C, which inhibits binding of DAG to both classical and novel isoforms; (c) chelerythrine, which also inhibits classical and novel isoforms; and (d) Gö6976, which selectively inhibits classical isoforms. As shown in Fig. 2, each NSCLC cell line responded to at least one PKC inhibitor with at least a 6-fold induction of apoptosis. Apoptosis was confirmed by assessing morphological changes in the NSCLC cells and by assessing the binding of annexin V to apoptotic cells via flow cytometry (data not shown). The NSCLC cell lines differed slightly in sensitivity to the PKC inhibitors, but rottlerin, in particular, increased apoptosis in all

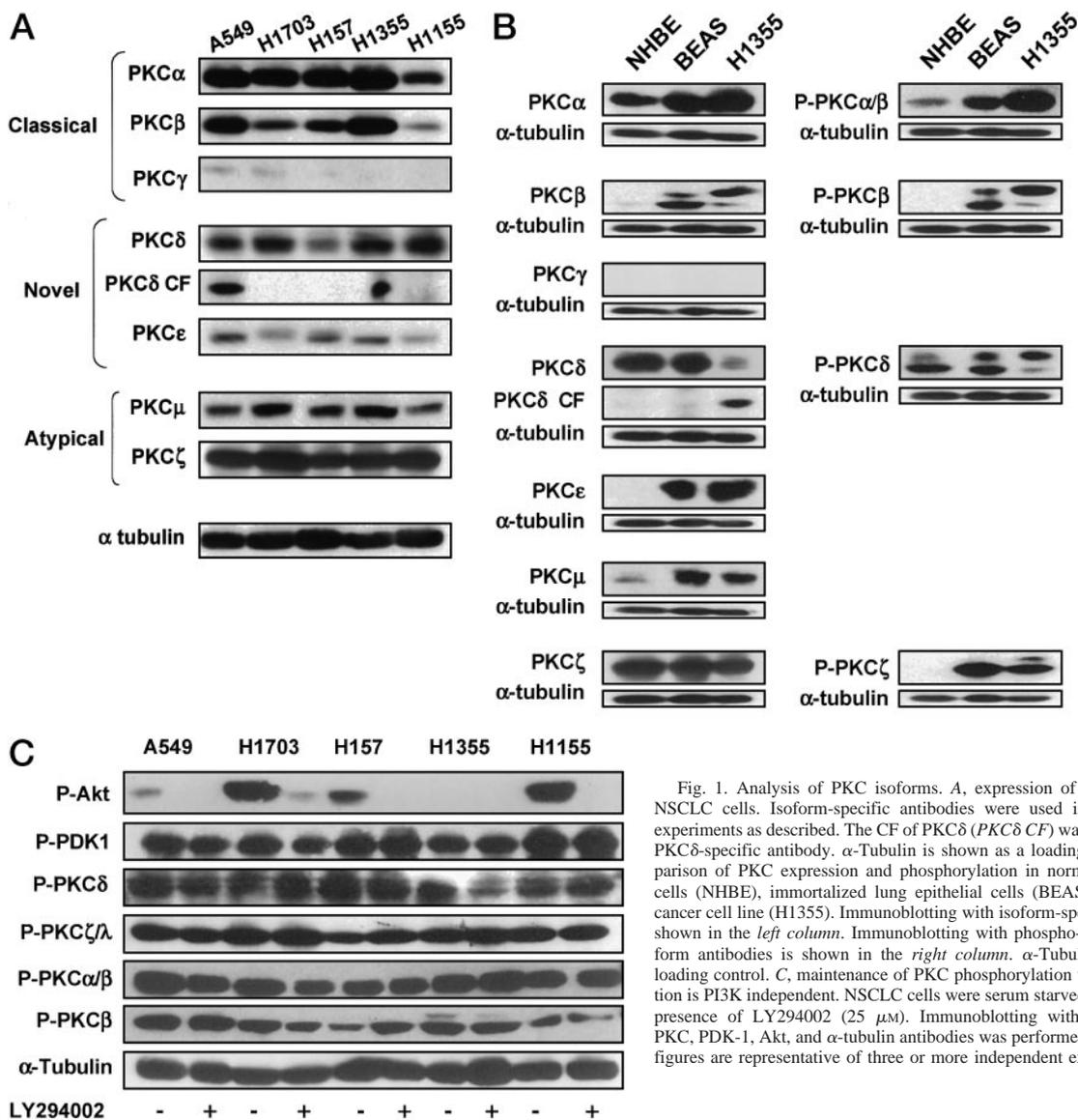


Fig. 1. Analysis of PKC isoforms. *A*, expression of PKC isoforms in NSCLC cells. Isoform-specific antibodies were used in immunoblotting experiments as described. The CF of PKC δ (*PKC δ CF*) was detected with the PKC δ -specific antibody. α -Tubulin is shown as a loading control. *B*, comparison of PKC expression and phosphorylation in normal lung epithelial cells (NHBE), immortalized lung epithelial cells (BEAS-2B), and a lung cancer cell line (H1355). Immunoblotting with isoform-specific antibodies is shown in the *left column*. Immunoblotting with phospho-specific PKC isoform antibodies is shown in the *right column*. α -Tubulin is shown as a loading control. *C*, maintenance of PKC phosphorylation with serum starvation is PI3K independent. NSCLC cells were serum starved in the absence or presence of LY294002 (25 μ M). Immunoblotting with phospho-specific PKC, PDK-1, Akt, and α -tubulin antibodies was performed as described. All figures are representative of three or more independent experiments.

five NSCLC cell lines and induced the most apoptosis in four of five NSCLC cell lines. Chelerythrine also increased apoptosis in all five NSCLC cell lines, and calphostin C increased apoptosis in all but the H157 cells. Gö6976 was ineffective, even at higher doses (data not shown). The induction of apoptosis by chelerythrine and calphostin C, but not by Gö6976, suggested that a novel (but not classical) PKC isoform might be responsible for promoting NSCLC cell survival. Moreover, the induction of NSCLC cell apoptosis by rottlerin suggested that PKC δ might be the novel isoform promoting NSCLC cell survival. Genetic evidence supporting this conclusion will be presented below.

Because of the robust apoptotic response induced by rottlerin and the fact that other modes of PKC inhibition are being combined with standard chemotherapy in ongoing clinical trials, we combined rottlerin with chemotherapeutic agents used in the treatment of NSCLC and measured apoptosis. In H1703 cells, combining rottlerin with gemcitabine, cisplatin, docetaxel, or trastuzumab resulted in greater-than-additive induction of apoptosis (Fig. 3A). Similar results were observed when gemcitabine, docetaxel, or trastuzumab was combined with rottlerin in the H1155 cells (Fig. 3B). In both cell lines, combining rottlerin with paclitaxel was ineffective, but combining rottlerin with a different taxane, docetaxel, resulted in greater-than-

additive increases in apoptosis. Rottlerin alone was quite effective in H1355 (Fig. 3C) and H157 (Fig. 3D) cells, but combining chemotherapeutic agents with rottlerin in these cells did not increase apoptosis, with the exception of trastuzumab, where greater than additive effects were seen. In H1355 cells, combining cisplatin and rottlerin or docetaxel and rottlerin was antagonistic to apoptosis caused by rottlerin alone. This may be due to cell cycle conflict because both cisplatin and docetaxel alone induced a G₂-M-phase arrest, but in combination with rottlerin, H1355 cells underwent a G₀-G₁ arrest (data not shown).

To confirm the proapoptotic effects of rottlerin and the putative role of PKC δ , we transiently transfected three NSCLC cell lines with plasmids encoding GFP-tagged wild-type PKC δ or a GFP-tagged PKC δ kinase-dead mutant (K376R). After recovery from transfection, cells were serum starved in the absence or presence of chemotherapy, and apoptosis was assessed in the GFP-positive cells using flow cytometry. (In these experiments, basal levels of apoptosis were higher than those in Fig. 3 because of the nonspecific toxic effects of the transfection reagent.) In H1355 cells transfected with wild-type PKC δ , trastuzumab did not increase apoptosis, as assessed by either flow cytometry or PARP cleavage (Fig. 4A). In contrast, transfecting H1355 cells with K376R decreased PKC δ phosphorylation, doubled

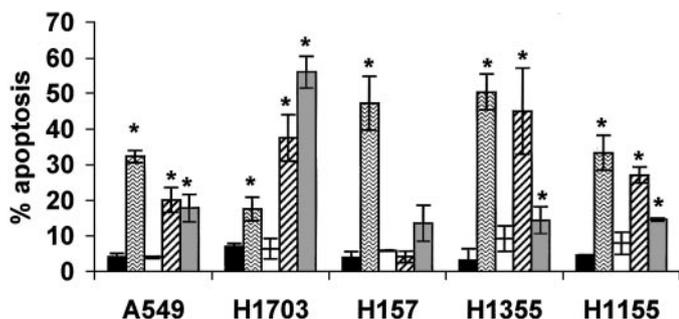


Fig. 2. Effect of PKC inhibitors on NSCLC apoptosis. NSCLC cells were plated and serum starved in the absence (black bars) or presence of PKC inhibitors [rottlerin (10 μ M; horizontal hatched bars), Gö6976 (100 nM; white bars), calphostin C (1 μ M; diagonally hatched bars), or chelerythrine (10 μ M; gray bars)] for 48 h. Cells were harvested, and apoptosis was quantified via flow cytometry as described. Bars are the means \pm SE from three independently performed experiments. P s \leq 0.05 are shown with *.

serum starvation-induced apoptosis, and increased trastuzumab-induced apoptosis. To address whether the apoptosis resulting from transfection of K376R was an artifact due to cellular fragility caused by serum starvation and exposure to the transfection reagent, we repeated transfection of H1355 cells with wild-type PKC δ or K376R and kept the serum concentration at 10% throughout the experiment. We chose to test cisplatin with wild-type PKC δ or K376R in these experiments because rottlerin did not potentiate trastuzumab-induced apoptosis in 10% FBS (see below). Consistent with the data obtained under conditions of 0.1% FBS, transfecting K376R decreased PKC δ phosphorylation and increased apoptosis with and without chemotherapy (Fig. 4B).

Similar results were observed when we transfected H1155 cells or H157 cells, even when we chose chemotherapeutic agents that were not effective in combinations with rottlerin. In H1155 cells (Fig. 4C), cisplatin slightly increased apoptosis in cells transfected with wild-type PKC δ , but transfection of K376R decreased PKC δ phosphorylation and increased apoptosis caused by serum starvation or serum starvation and cisplatin. In H157 cells (Fig. 4D), paclitaxel did not significantly increase apoptosis in cells transfected with wild-type PKC δ , but in cells transfected with K376R, decreased PKC δ phosphorylation and increased basal and paclitaxel-induced apoptosis were observed. Consistent with these data, we also observed a pro-apoptotic function of K376R when we attempted to generate NSCLC cells stably transfected with wild-type PKC δ or K376R, and we found that we were unable to expand any clones expressing K376R (data not shown). Together, these results show that pharmacological or genetic inhibition of PKC δ increases NSCLC apoptosis caused by serum starvation and administration of chemotherapy, and suggest that PKC δ promotes NSCLC cell survival.

Discussion

Our study is the first to evaluate PKC isoform expression in cell lines derived from NSCLC, the most lethal human cancer. Despite differences in status of *p53*, *K-ras*, *Rb*, tumor histology, Akt activity, and ERK activity (1, 2), a panel of five NSCLC cell lines expressed similar levels of at least six isoforms of PKC (α , β , δ , ϵ , μ , and ζ). PKC γ was not expressed in any of these NSCLC cell lines, which is consistent with a previously noted lack of PKC γ expression in lung tissue (17). PKC λ and η were not examined. Multiple PKC isoforms have also been described in other tumor cell systems derived from small cell lung cancer (18), breast (19), colon (20, 21), and brain (22) tumors. Differences in PKC isoform expression and phosphorylation were observed when we compared normal human lung epithelial cells (NHBE), immortalized but nontumorigenic human lung epithelial

cells (BEAS-2B), and a NSCLC cell line (H1355). When compared with NHBE, H1355 cells expressed higher levels of PKC α , β , ϵ , and μ and the CF of PKC δ . BEAS-2B also expressed increased levels of PKC α , β , ϵ , and μ , suggesting that immortalization with SV40 large T antigen is associated with increased expression of these isoforms and that increased PKC expression might be associated with lung tumorigenesis. Of note, Webb *et al.* (17) have observed expression patterns of PKC isoform expression in normal lung tissues that are closer to what we observed in H1355 cells rather than NHBE. Unfortunately, the tissues used by Webb *et al.* (17) were either denuded of epithelium (trachea) or a mixture of multiple cell types (lung), so direct comparison is not possible. Similar data correlating increased PKC isoform expression with increased tumor grade or degree of transformation have been observed by Mandil *et al.* (22), who showed

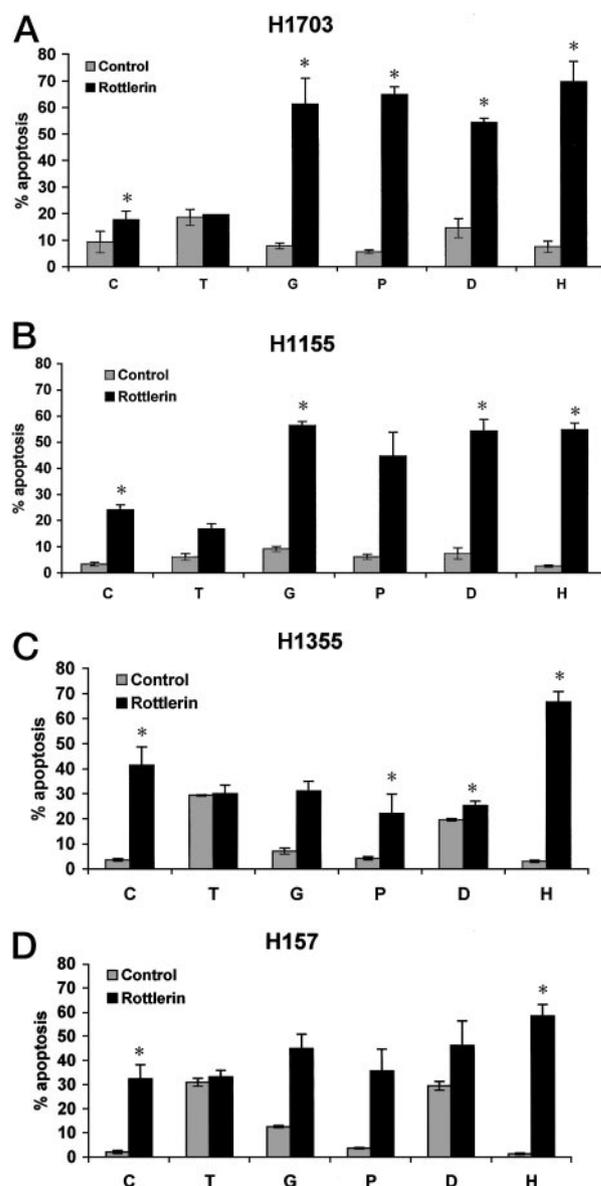


Fig. 3. Effect of rottlerin on chemotherapy-induced apoptosis. H1703 (A), H1155 (B), H1355 (C), or H157 (D) cells were plated at 1×10^5 cells/well and placed in 0.1% FBS. Rottlerin (10 μ M) and/or chemotherapeutic agents were added simultaneously, and cells were incubated for 48 h. Apoptosis was quantified as described. Bars: C, control (no chemotherapy); T, paclitaxel (1 μ M); G, gemcitabine (100 μ M); P, cisplatin (20 μ M); D, docetaxel (1.16 μ M), and H, trastuzumab (10.5 μ M). Bars are the means \pm SD of a representative experiment done in triplicate from five independently performed experiments for each cell line. $P \leq$ 0.05 comparing chemotherapy + rottlerin with rottlerin alone are shown with *.

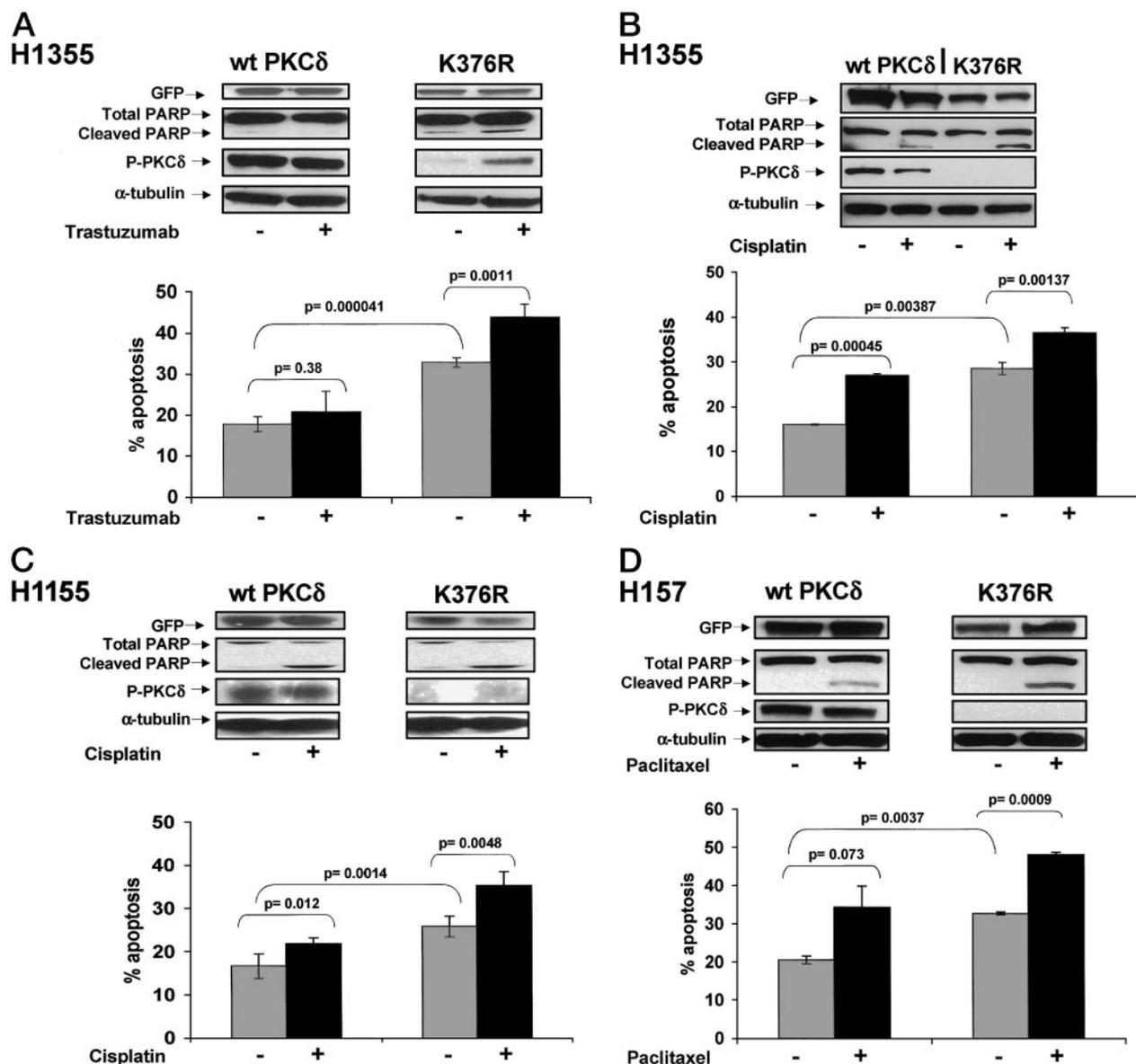


Fig. 4. Dominant negative PKC δ increases NSCLC apoptosis. NSCLC cells (H1355, A and B; H1155, C; H157, D) were plated at 2×10^5 cells/well and transiently transfected with plasmids encoding wild-type PKC δ (wt PKC δ ; gray bars) or dominant negative PKC δ (K376R; black bars) as described. Cells were allowed to recover from transfection for 24 h and then exposed to serum starvation \pm chemotherapy for 48 h (except B, where 10% FBS was substituted for 0.1% FBS). Parallel samples from each transfection were harvested for immunoblotting to evaluate GFP, PARP, phospho-PKC δ , and α -tubulin expression. Samples were also fixed, stained with propidium iodide, and prepared for flow cytometry. Apoptosis was quantified by gating on GFP-positive cells and performing cell cycle analysis. *P*s for comparing apoptosis under various experimental conditions are shown. A representative experiment from three independent experiments performed with each cell line is shown.

increased levels of PKC α and ϵ expression in high-grade brain tumors (glioblastomas) compared with low-grade tumors (astrocytomas).

In addition to increased protein expression, we observed progressively increased phosphorylation of PKC α , β , δ , and ζ in NHBE, BEAS-2B, and H1355 cells, respectively. The phosphorylation sites we assessed on PKC δ and ζ are sites for PDK-1 phosphorylation, and the sites we assessed on PKC α and β become phosphorylated only after phosphorylation by PDK-1. Surprisingly, under conditions where we hypothesized phosphorylation would be decreased (namely, serum starvation \pm the addition of a PI3K inhibitor), maintenance of phosphorylation was observed in all cell lines. LY294002, which has previously been shown to decrease phosphorylation of PDK-1-dependent PKC isoforms in many studies, did not decrease phosphorylation in our studies. Although one report has shown that PKC α and β can be phosphorylated by PDK-1 in a PI3K-independent manner in mouse or monkey cells (23), this is the first report in any tumor cell

system to show that LY294002 decreases Akt phosphorylation without affecting PDK-1 or PDK-1-dependent PKC isoform phosphorylation. Our studies suggest that PDK-1 and PKC function may be PI3K independent in these NSCLC cells, which is a subject of current investigation. Moreover, they raise the possibility that increased phosphorylation of PKC isoforms may accompany increased PKC expression in the process of lung tumorigenesis.

Our observation that PKC inhibitors increased NSCLC cell apoptosis suggests that PKC isoforms contribute to NSCLC survival and support the concept of targeting PKC in NSCLC. Currently, the only isoform-specific approach against PKC in lung cancer clinical trials is ISIS 3521, which is an antisense approach against PKC α . ISIS 3521 has not been reported to decrease PKC α levels in patients' tissues, which raises the possibility that its mechanism of action may be unexpected. In our studies, the classical PKC inhibitor, Gö6976, which has been demonstrated to inhibit PKC α in other studies, was

ineffective compared with calphostin C, chelerythrine, or rottlerin. Rottlerin increased apoptosis at least 4-fold in all NSCLC cell lines and was the most effective PKC inhibitor tested in four of five NSCLC cell lines. Rottlerin potentiated chemotherapy-induced apoptosis in a cell line- and drug-specific manner, although antagonism was observed in H1355 cells when rottlerin was combined with cisplatin or docetaxel. The most consistent potentiation occurred with trastuzumab, where quantitatively similar, greater-than-additive effects were observed in all NSCLC cell lines tested. Because H1703 cells have much higher levels of erbB2 protein expression compared with the other NSCLC cell lines (data not shown), potentiation of trastuzumab-induced apoptosis by rottlerin does not appear to correlate with erbB2 expression, and the mechanism responsible for this potentiation is unclear.

Interestingly, the potentiation of chemotherapy-induced apoptosis by rottlerin was observed when experiments were performed in 0.1% FBS, but not 10% FBS. These differences may have been due to serum-derived, growth factor-induced changes in cell cycle distribution under the different culture conditions. In 0.1% FBS, approximately 5% of cells were in S phase at 48 h, as assessed by flow cytometry, which closely agrees with the observed growth fraction of solid tumor cells *in vivo* (24). In 10% FBS, approximately 20% of the NSCLC cells from each cell line were in S phase. Because a 20% growth fraction is rarely observed in NSCLC tumors, we believe data derived from NSCLC cells grown in 0.1% FBS may better reflect conditions *in vivo*. In spite of the potentially decreased relevance of performing experiments with NSCLC cells in 10% FBS, however, we assessed potentiation of chemotherapy-induced apoptosis by rottlerin in the H1355 or H1155 cells in 10% FBS (data not shown). Although potentiation of chemotherapy-induced apoptosis was not observed, rottlerin was equally toxic in 10% FBS, and cisplatin was more toxic in 10% FBS.

The fact that rottlerin effectively killed NSCLC cells independent of serum concentration suggests that rottlerin is an interesting agent. Evaluation of rottlerin in the National Cancer Institute 60 cell line screen showed that rottlerin inhibited the growth of 57 of 60 cancer cell lines at a concentration of 10 μM .³ Whether inhibition of PKC δ is responsible for this widespread cytotoxicity is unknown, however, because rottlerin can also promote apoptosis in a PKC δ -independent manner through potentiation of tumor necrosis factor α -induced apoptosis (25). Cytotoxicity across multiple cancer cell lines raises the possibility that rottlerin may have utility in many types of tumor cell systems and that combining rottlerin with trastuzumab in cancer cells where trastuzumab has been shown to be effective (such as breast cancer) might yield interesting results.

Because rottlerin is similar to other kinase inhibitors in that it has nonspecific effects and its activity may depend upon the cellular system in which it is evaluated (26), we confirmed the putative role for PKC δ in promoting NSCLC survival by transfecting NSCLC cells with wild-type or a kinase-dead mutant of PKC δ . Compared with wild-type PKC δ , K376R PKC δ decreased PKC δ phosphorylation and increased apoptosis in three NSCLC cell lines. K376R increased apoptosis irrespective of serum concentration. K376R PKC δ also potentiated chemotherapy-induced apoptosis, but the level of potentiation was less than that observed with rottlerin combined with chemotherapy, possibly because of effects of rottlerin unrelated to PKC δ or a previously noted artifact of transient transfection experiments due to gating on GFP-positive cells, which underestimates apoptosis (1).

In many systems, PKC δ promotes rather than inhibits apoptosis

(reviewed in Ref. 6). However, it is clear that the role of PKC δ is tissue and system dependent. For example, in glial cells, overexpression of PKC δ inhibits apoptosis induced by Sindbis virus (27), although it enhances apoptosis in the same cells in response to etoposide (28). Likewise, PKC δ is involved in the antiapoptotic action of basic fibroblast growth factor in PC12 cells (29). Further evidence that supports PKC δ as a suppressor of apoptosis includes the dependence of insulin-like growth factor I receptor-mediated transformation on active PKC δ (30), the activation of PKC δ in response to platelet-derived growth factor-mediated transformation (31), and the induction of anchorage-independent growth and increased metastatic potential of breast cancer cells that overexpress PKC δ (32, 33).

Specificity of PKC δ function may be related to different mechanisms of activation of PKC δ in different systems or differential availability of downstream substrates that mediate PKC δ activity in different cell types. Other factors that might play a role in determining the apoptotic potential of PKC δ are the generation of ceramide and the translocation of PKC δ to various intracellular organelles (34). Ceramide, a sphingolipid second messenger, is synthesized in response to cellular stresses such as chemotherapy. Generation of ceramide may stimulate PKC δ activation or may occur as a consequence of PKC δ activation. Translocation of PKC δ to the plasma membrane, mitochondria, or nucleus after exposure to cytotoxic stresses has been reported and interference with translocation may inhibit the induction of apoptosis by PKC δ (35). Translocation of PKC δ to different cellular compartments might contribute to the different cellular effects of PKC δ because PKC δ may interact with and/or phosphorylate different proteins in these specific compartments. The contribution of ceramide generation and/or cellular translocation to the response of NSCLC cells to inhibition of PKC δ , with or without chemotherapy, is unclear.

The results of our studies on the role of PKC δ in NSCLC cells are consistent with a recent evaluation of PKC δ in lung tissue performed by Lounsbury *et al.* (36). These investigators showed that PKC δ expression and phosphorylation of a downstream substrate were increased in murine lung tissues exposed to asbestos, a lung carcinogen. Increased levels of PKC δ expression and downstream substrate phosphorylation were associated with increased asbestos-stimulated growth of murine lung epithelial cells. This study raises the possibility that increases in PKC δ expression or activity may be involved in asbestos-related lung tumorigenesis.

In conclusion, our studies support the ongoing efforts targeting PKC isoforms in NSCLC therapy but suggest that current approaches may be limited in two ways. First, if increases in PKC isoform expression and/or phosphorylation can be detected in NSCLC tumor specimens compared with precursor lesions, then targeting PKC isoforms may have utility in NSCLC prevention as well as treatment. Secondly, current clinical approaches targeting PKC isoforms are not specifically directed at PKC δ . Our biochemical and genetic data suggest that the role of PKC δ in NSCLC should be reconsidered.

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