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Cyclic GMP/Protein Kinase G Type-Iα (PKG-Iα) Signaling Pathway Promotes CREB Phosphorylation and Maintains Higher c-IAP1, Livin, Survivin, and Mcl-1 Expression and the Inhibition of PKG-Iα Kinase Activity Synergizes With Cisplatin in Non-Small Cell Lung Cancer Cells

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ABSTRACT

Previously, our laboratory showed that nitric oxide (NO)/cyclic GMP (cGMP)/protein kinase G type-Iα (PKG-Iα) signaling pathway plays an important role in preventing spontaneous apoptosis and promoting cell proliferation in both normal cells (bone marrow stromal cells and vascular smooth muscle cells) and certain cancer cells (ovarian cancer cells). In the present study, we investigated the novel role of the cGMP/PKG-Iα pathway in preventing spontaneous apoptosis, promoting colony formation and regulating phosphorylation of cAMP response element binding (CREB) protein and protein expression of inhibitor of apoptosis proteins (IAPs) and anti-apoptotic Bcl-2-related proteins in NCI-H460 and A549 non-small cell lung cancer (NSCLC) cells. 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ), which blocks endogenous NO-induced activation of cGMP/PKG-Iα, induced apoptosis and decreased colony formation. ODQ also decreased CREB ser133 phosphorylation and protein expression of c-IAP1, livin, and survivin. DT-2 (inhibitor of PKG-Iα kinase activity) increased apoptosis by twofold and decreased CREB ser133 phosphorylation and c-IAP1, livin, and survivin expression. Gene knockdown of PKG-Iα expression using small-interfering RNA increased apoptosis and decreased CREB ser133 phosphorylation, and c-IAP1, livin, survivin, and Mcl-1 expression. Inhibition of PKG-Iα kinase activity with DT-2 dramatically enhanced pro-apoptotic effects of the chemotherapeutic agent cisplatin. Combined treatment of DT-2 and cisplatin increased apoptosis compared with cisplatin or DT-2 alone, showing a synergistic effect. The data suggest that the PKG-Iα kinase activity is necessary for maintaining higher levels of CREB phosphorylation at ser133 and protein expression of c-IAP1, livin, survivin, and Mcl-1, preventing spontaneous apoptosis and promoting colony formation in NSCLC cells, which may limit the effectiveness of chemotherapeutic agents like cisplatin. J. Cell. Biochem. 113: 3587–3598, 2012.

KEY WORDS: PROTEIN KINASE G; INHIBITOR OF APOPTOSIS PROTEINS; CAMP RESPONSIVE ELEMENT BINDING PROTEIN; NON-SMALL CELL LUNG CANCER; CYCLIC GMP; APOPTOSIS; CISPLATIN

Lung cancer is the leading cause of cancer-related death worldwide, with 85% diagnosed as non-small cell lung cancer (NSCLC). Despite the rapid developments in new drugs and chemotherapy throughout the years, only about 5% of patients survive for 5 years. Therefore, novel therapeutic approaches are urgently needed.

Our early studies of the nitric oxide (NO)/cyclic GMP (cGMP)/protein kinase G (PKG) pathway have identified PKG as a key mediator of vasodilation and anti-hypertensive effects induced by NO as well as atrial natriuretic peptide [Fiscus et al., 1983, 1985; Fiscus and Murad, 1988; Fung et al., 2005]. Recent studies from our laboratory have shown that basal or moderately elevated PKG

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type-Iα (PKG-Iα) activity plays a cytoprotective role in preventing spontaneous apoptosis and promoting cell survival in many types of mammalian cells, including neural cells [Barger et al., 1995; Fiscus et al., 2001, 2002; Fiscus, 2002; Chew et al., 2003; Johlfs and Fiscus, 2010], human ovarian cancer cells [Fraser et al., 2006; Leung et al., 2008, 2010], primary murine vascular smooth muscle cells [Wong and Fiscus, 2010], and murine bone marrow stromal cells [Wong and Fiscus, 2011].

Recent studies from our laboratory have identified the phosphorylation of certain intracellular proteins phosphorylated by PKG-Iα, including BAD [Johlfs and Fiscus, 2010], VASP [Leung et al., 2010; Wong and Fiscus, 2010, 2011], c-Src (unpublished data), and cAMP response element binding (CREB) (unpublished data), which act as downstream targets for PKG-Iα in promoting cell survival and proliferation. CREB was first shown to be phosphorylated by PKG in vitro by Colbran et al. [1992], which showed that PKG effectively phosphorylates CREB at ser133, although at a slower rate compared with PKA. Interestingly, NO was shown to regulate the c-fos promoter involving soluble guanylyl cyclase (sGC) and PKG [Idriss et al., 1999] in a CREB-dependent manner [Gudi et al., 2000]. The same lab has also shown that transfection of PKG in baby hamster kidney cells activated the c-fos promoter [Gudi et al., 1996], which required nuclear translocation of PKG and phosphorylation of CREB at ser133 by PKG [Gudi et al., 1997, 2000; Chen et al., 2003].

Inhibitor of apoptosis proteins (IAPs) have been shown to regulate apoptosis and tumorigenesis [Deveraux and Reed, 1999]. Eight human IAPs have been identified [Salvesen and Duckett, 2002] and are shown to suppress induction of apoptosis [LaCasse et al., 1998]. c-IAP1 and c-IAP2 possess a caspase recruitment domain [Hofmann et al., 1997], and c-IAP1, c-IAP2 and XIAP are shown to directly inhibit activated caspase-3 and caspase-7 [Roy et al., 1997; Eckelman et al., 2006]. Elevated expression of IAP proteins is shown in almost all human cancers and has been implicated as therapeutic targets [Vucic and Fairbrother, 2007]. Particularly, XIAP is shown to play a predominant role in the inactivation of apoptosome in NSCLC NCI-H460 cells [Yang et al., 2003]. Survivin-specific siRNA is shown to increase apoptosis and inhibits cell proliferation in A549 cells through activation of caspase-9 [Chen et al., 2012]. Although how CREB regulates apoptosis through IAPs is largely unknown, it was shown that CREB phosphorylation is a key event in the induction of certain IAPs, c-IAP2, and livin, via multiple protein kinases, PKA, ERK1/2, and p38 MAPK, in colon cancer cells [Nishihara et al., 2003, 2004].

Bcl-2 family proteins play a central role in regulation of apoptosis by regulating the downstream activation of caspases [Cory and Adams, 2002]. The Bcl-2 family proteins consist of anti-apoptotic members (Mcl-1, Bcl-2, and Bcl-xL) and pro-apoptotic members (Bax, Bad, and Bak) [Adams and Cory, 1998]. Mcl-1 (myeloid cell leukemia-1) is localized prominently in mitochondria [Yang et al., 1995] and is shown to inhibit mitochondrial calcium signaling [Minagawa et al., 2005]. Antisense oligonucleotide studies have shown that Mcl-1 is a specific anti-apoptotic factor in myeloma cells and inhibition of Mcl-1 induces apoptosis in human myeloma cells [Derenne et al., 2002]. In colorectal cancer cells, Bcl-xL and Mcl-1 contribute to the resistance to apoptosis [Schulze-Bergkamen et al., 2008]. In NSCLC, Mcl-1, Bcl-2, and Bcl-xL are highly expressed in tissues taken from patients [Borner et al., 1999]. Interestingly, NO has been shown to stabilize Bcl-2 by promoting *S*-nitrosylation of Bcl-2, inhibiting its ubiquitination and subsequent proteasomal degradation, as well as contributing to cisplatin resistance in NSCLC NCI-H460 cells [Azad et al., 2010]. However, a role for the activation of sGC by NO and downstream stimulation of the cGMP/PKG-Iα signaling pathway in NSCLC cells was not determined in their study.

Our recent studies have shown that cisplatin regulates the endogenous expression of nitric oxide synthases (NOSs) in human ovarian cancer cells, upregulating iNOS expression but dramatically downregulating the expression of eNOS and nNOS, which is involved in determining cisplatin resistance in ovarian cancer cells [Leung et al., 2008]. The chemoresistance/cytoprotective effects of endogenous eNOS was later found to involve hyperactivation of PKG-Iα in the ovarian cancer cells [Leung et al., 2010].

The mechanisms by which cGMP and PKG-Iα regulate apoptosis in lung cancer cells are still largely unknown. Therefore, in the present study, we investigated the novel role of the cGMP/PKG-Iα signaling pathway in preventing spontaneous apoptosis, promoting colony formation and CREB ser133 phosphorylation, and regulating the expression of five IAPs (c-IAP1, c-IAP2, XIAP, livin, and survivin) and the anti-apoptotic members (Mcl-1, Bcl-2, and Bcl-xL) of Bcl-2 family proteins in NSCLC cells.

MATERIALS AND METHODS

CELL CULTURES AND REAGENTS

NCI-H460 and A549 lung carcinoma cell lines were purchased from American Type Culture Collection (ATCC). Cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, streptomycin (50 μg/mL), and penicillin (50 units/mL) (all from Lonza). The cells were cultured at 37°C in an atmosphere of 5% CO_2/95% air.

CLONOGENIC CELL SURVIVAL ASSAY

To assess the colony formation ability in NCI-H460 and A549 cells, clonogenic assay was performed. Five hundred cells per well were plated in six-well plates and treated with ODQ and DT-2 (Calbiochem) for 24 h. After the incubation period, the cells were washed with 1× phosphate buffer saline (Lonza) twice and once with fresh media. Fresh media was added and cells were incubated for 7–10 days to form colonies (or until control wells attain confluency), followed by staining with crystal violet (5 mg/mL crystal violet in 95% ethanol).

RADIOIMMUNOASSAY FOR MEASURING INTRACELLULAR LEVELS OF cGMP

To determine the levels of cGMP in cells treated with the sGC inhibitor ODQ, a radioimmunoassay was performed using cyclic GMP RIA kit (Biomedical Technologies). Cells plated in 60-mm tissue culture plates were treated with ODQ (0, 10, 30, and 50 μM) for 20 min and lysed with ice cold 0.1 N HCl. cGMP levels in the lysates were measured using a gamma counter following manufacturer's protocol. Results are represented as cGMP in pmole/million cells.

ASSESSMENT OF APOPTOSIS BY CELL DEATH DETECTION ELISA^{PLUS}

The Cell Death Detection ELISA^{PLUS} assay (Roche Applied Science), based on quantitative sandwich enzyme immunoassay principle with monoclonal antibodies directed against DNA and histones, were used to quantify apoptotic levels in NSCLC cells. Cells at 1×10^4 cells/well were seeded in 96-well plates and treated with ODQ and DT-2 for 24 h. For co-treatment experiments, cells were pretreated with 8-Br-cGMP (Calbiochem) for 3 h followed by addition of cisplatin (Sigma) for 24 h. Procedures followed the manufacturer's protocol, with the following exception. A longer centrifugation time (i.e., 30 min, instead of the recommended 10 min), after cell permeation for releasing apoptotic fragments of DNA, was used in order to obtain a cleaner separation of apoptotic DNA fragments from genomic DNA in cell nuclei. This modification dramatically lowered background interference caused by contaminating genomic DNA and thus allowed better quantification of apoptotic responses.

CASPASE-3/7 ACTIVITY BY THE CASPASE-3/7 CHEMILUMINESCENCE ASSAY

Caspase-3/7 activity was determined using Caspase-3/7 Glo activity kit (Promega). Two thousands cells per well were plated in 384-well plates. The cells were exposed to the sGC inhibitor ODQ (0, 30, and 50 μM) and PKG-Iα inhibitor DT-2 (0, 10, and 30 μM) for 24 h. The cells treated with specific PKG-Iα siRNA were also assessed for caspase-3/7 activity using caspase-3/7 Glo assay following the manufacturer's protocol.

PHOSPHO-CREB (ser133) CELL-BASED FLUOROMETRIC ELISA

To measure the effects of the PKG inhibitor DT-2 on CREB phosphorylation over time, human phospho-CREB (ser133) cell-based ELISA kit was used (R&D System). 1×10^4 cells/well in 96-well plates were exposed to the PKG-Iα inhibitor DT-2 (30 μM) for different time intervals up to 24 h. At the end of the incubation period, phospho- and total-CREB levels were assayed following the manufacturer's protocol. Briefly, the cells were fixed and labeled with phospho- and total-CREB primary antibodies. Enzyme-conjugated secondary antibodies were added, followed by using two spectrally distinct fluorogenic substrates for detection on a plate reader. Normalized relative fluorescence units (RFUs) were determined by dividing the phospho-CREB fluorescence at 600 nm in each well by the total-CREB fluorescence at 450 nm in each well.

PROTEIN EXTRACTION AND WESTERN BLOTTING USING INFRARED IMAGING

For protein extraction, the cells were lysed using 85°C hot 1× sodium dodecyl sulfate (SDS) lysis buffer (50 mmol/L Tris–HCl, pH 6.8, 2% SDS, 10 mmol/L dithiolthreitol and 10% glycerol). The supernatant fractions were collected by centrifugation (15,000×g; 10 min). The total amount of protein in the lysates was calculated from the fluorescence-based protein quantitation kit EZQ (Molecular Probes). Proteins were separated on 4–12% polyacrylamide NuPage gels (Invitrogen) and then transferred to nitrocellulose membrane (Amersham Biosciences) through a wet transfer at 35 V overnight at 4°C. Membranes were blocked (room temperature, 1 h) with blocking buffer (Rockland Immunochemicals) containing 0.05% Tween (Invitrogen) followed by overnight incubation with the primary antibody (PKG-Iα/β [1:1,000], p-[ser239]-VASP [1:500], total VASP [1:1,000], cleaved caspase-3 [1:1,000], Mcl-1 [1:1,000], Bcl-2 [1:1,000], Bcl-xL [1:1,000], p-[ser133]-CREB [1:1,000], total CREB [1:1,000] [all from Cell Signaling Technology], β-actin [1:1,000] [Santa Cruz Biotechnology]), c-IAP1, c-IAP2, XIAP, livin, and survivin antibodies (Chemicon), and subsequently with secondary antibodies labeled with infrared dyes (LI-COR Biosciences) (1:25,000 in blocking buffer; room temperature for 1 h). The membranes were scanned on the Odyssey infrared imaging system (LI-COR Biosciences).

IN VITRO PHOSPHORYLATION REACTION OF RECOMBINANT PKG-Iα AND RECOMBINANT CREB

Interactions between recombinant human PKG-Iα (Upstate) and recombinant rat CREB (Sigma) were determined using in vitro experiments. The recombinant PKG-Iα (50 ng) was incubated with recombinant CREB (25 ng) in a kinase reaction buffer containing Tris buffer (50 mmol/L), $MgCl_2$ (0.1 mol/L), bovine serum albumin (10 mg/mL), dithiolthreitol (10 mmol/L), cGMP (10 μmol/L), and ATP (1 mmol/L) at 30°C for 10 min. As the control, recombinant PKG-Iα was boiled in 95°C for 5 min and incubated with CREB in the same kinase reaction buffer. The samples were analyzed by Western blot analysis.

siRNA-MEDIATED PKG-IA KNOCKDOWN

For siRNA-mediated silencing of gene expression, cells were transfected with 100 nmol/L small interfering RNA Stealth^{TM} RNAi (PKG-Iα siRNA #1, 5′-GAGGAAGACUUUGCCAAGAUU-CUCA-3′ and PKG-Iα siRNA #2, 5′-CGACCUCCGACAGGCAUUCCG-GAAG-3′) for specifically targeting the expression of PKG-Iα (Invitrogen). Transfection was conducted using RNAiMAX (Invitrogen). Non-silencing siRNA (Invitrogen) was used as the negative control. At 72 h after transfection, the culture medium was changed and fresh medium was supplied. The cells were used in experiments 16 h later and levels of apoptosis, caspase-3/7 activity and clonogenic survival were assessed. PKG-Iα knockdown was confirmed by Western blot analysis.

STATISTICAL ANALYSIS

Results were expressed as the mean ± SEM of at least six different samples. Statistical analysis was performed by one-way ANOVA using GraphPad (PRISM software). Differences between experimental groups were determined by the Dunnett's test.

RESULTS

BASAL cGMP LEVELS AND PKG-Iα ACTIVITY PROTECT NCI-H460 AND A549 CELLS AGAINST SPONTANEOUS APOPTOSIS AND STIMULATE COLONY FORMATION

Inhibition of endogenous NO-induced activation of sGC (a cGMP-synthesizing enzyme) using ODQ significantly decreased intracellular cGMP levels in both NCI-H460 and A549 cells (Fig. 1A), which corresponded to significant concentration-dependent increases in apoptosis (Fig. 1B). These data suggest that cGMP and its downstream protein kinase, PKG, may contribute to a cytoprotective

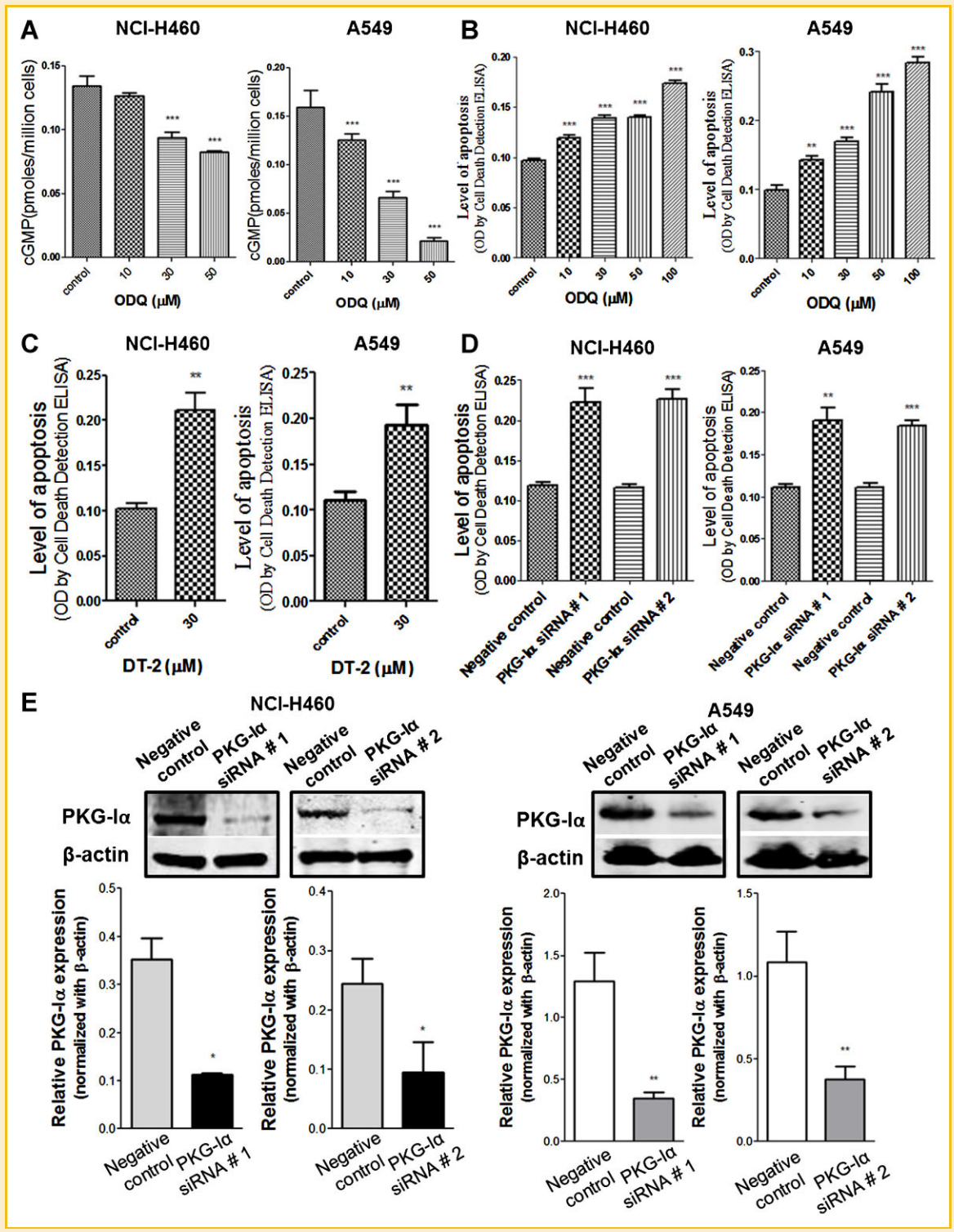

Fig. 1. A: cGMP levels were significantly decreased by ODQ in a concentration-dependent manner in both NCI-H460 and A549 cells. The data represent the mean ± SEM of six observations per treatment group. B: Results from Cell Death Detection ELISA Plus showing effects of ODQ at 10, 30, 50, and 100 μM, showing concentration-dependent induction of apoptosis in both NCI-H460 and A549 cells. C: Inhibition of basal PKG-Iα kinase activity using DT-2 (30 μM) increased apoptosis by twofold in both NCI-H460 and A549 cells. D: Two different PKG-Iα siRNAs both significantly increased apoptosis by approximately twofold in NCI-H460 and A549 cells. E: siRNA mediated gene knockdown of PKG-Iα isoform, using PKG-Iα siRNA #1, decreased PKG-Iα expression levels by about 70%, and PKG-Iα siRNA #2 decreased PKG-Iα expression levels by about 60%, in both NCI-H460 and A549 cells, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

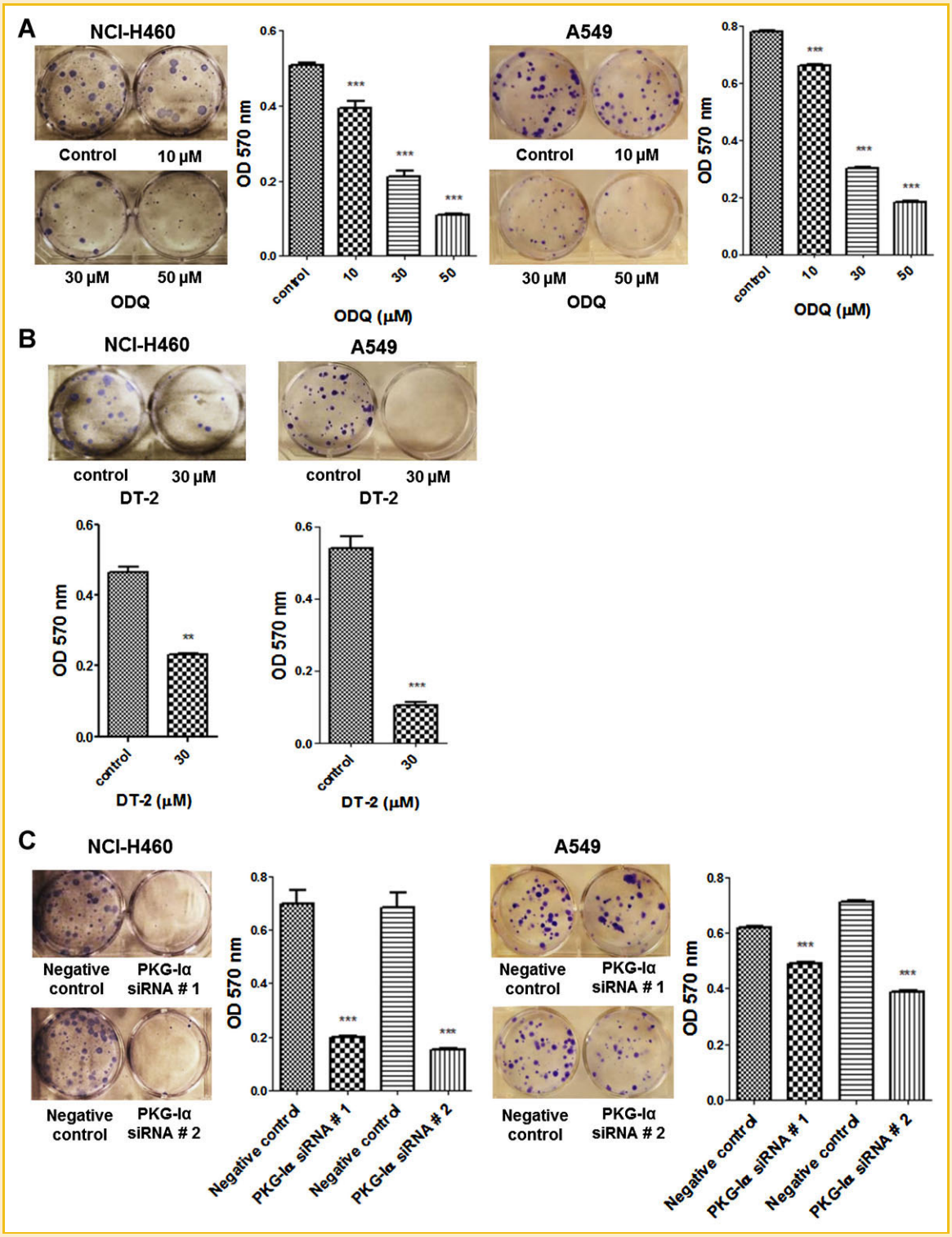

Fig. 2. A: Colony formation assay showing decreased colony formation by inhibition of endogenous NO-induced activation of sGC with ODQ in NCI-H460 and A549 cells. B: Inhibition of basal PKG-Iα kinase activity using DT-2 (30 μM) reduced colony formation by 50% and 80%, in NCI-H460 and A549 cells, respectively. C: Colony formation was significantly decreased in the PKG-Iα siRNA knockdown cells. $^{**}P < 0.01$ and $^{***}P < 0.001$. The photos are representative of six independent experiments and graphs show mean ± SEM from six independent experiments.

effect, preventing spontaneous onset of apoptosis in NSCLC cells. Both NCI-H460 and A549 cells were found to express the PKG-Iα isoform of PKG (see Fig. 1E). To further investigate the anti-apoptotic role of PKG-Iα, a specific PKG-Iα inhibitor, DT-2, was used. Unlike other PKG inhibitors, DT-2 inhibits both stimulated and basal PKG kinase activity [Dostmann et al., 2002]. Figure 1C shows that DT-2 significantly increased apoptosis by twofold ($^{***}P < 0.001$) in both cell lines. Two sets of PKG-Iα siRNA both caused a substantial increase in apoptosis (Fig. 1D). PKG-Iα siRNA #1 lowered PKG-Iα protein expression levels in both cells lines by 70%, and PKG-Iα siRNA #2 lowered PKG-Iα by 60%, as shown by Western blot analysis (Fig. 1E). In addition, ODQ (Fig. 2A), DT-2 (Fig. 2B), and PKG-Iα siRNAs (Fig. 2C) all significantly ($^{***}P < 0.001$) inhibited colony formation in both NCI-H460 and A549 cells.

RECOMBINANT PKG-Iα PHOSPHORYLATES RECOMBINANT CREB AT ser133 IN VITRO

To test whether PKG-Iα directly phosphorylates CREB in vitro, we used recombinant human PKG-Iα and recombinant rat CREB, which were incubated in a kinase reaction buffer containing the necessary ATP and Mg^{2+}. Phosphorylation of CREB at ser133 was determined by Western blot analysis. Figure 3A shows the results of a representative in vitro kinase experiment. Phosphorylation of CREB at ser133 occurred only when both CREB and PKG-Iα were present

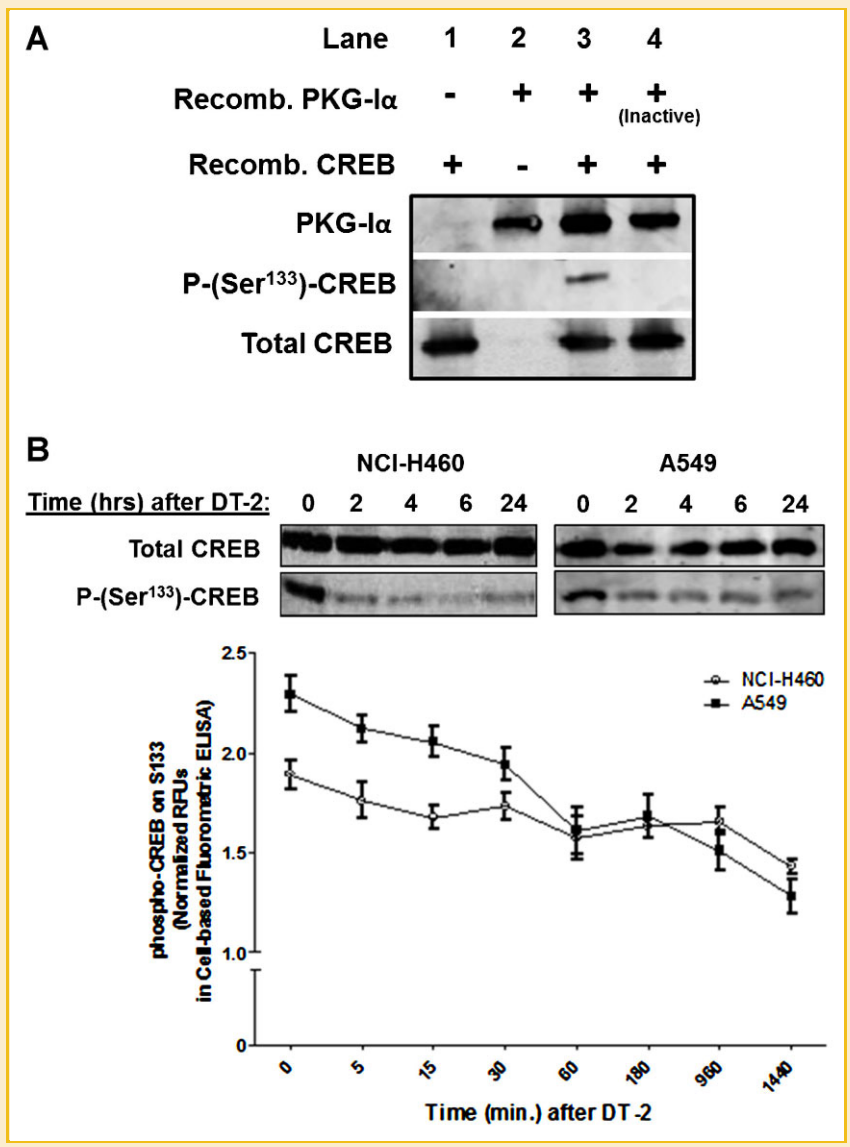

Fig. 3. A: Western blot analysis showing that recombinant human PKG-Iα phosphorylated recombinant rat CREB at ser133 in vitro. Lane 1, recombinant CREB alone; lane 2, recombinant PKG-Iα alone; lane 3, recombinant PKG-Iα phosphorylates CREB at ser133; lane 4, denatured PKG-Iα (boiled in 95°C for 5 min) was used as a negative control. B: Western blot analysis showing PKG-Iα inhibitor DT-2 lowered the phosphorylation levels of CREB at ser133 as early as 2 h in both NCI-H460 and A549 cells (upper panels). Lower panel, time-dependent decrease in phospho-CREB levels at ser133 in both cell lines, expressed as normalized RFUs measured by CREB cell-based fluorometric ELISA.

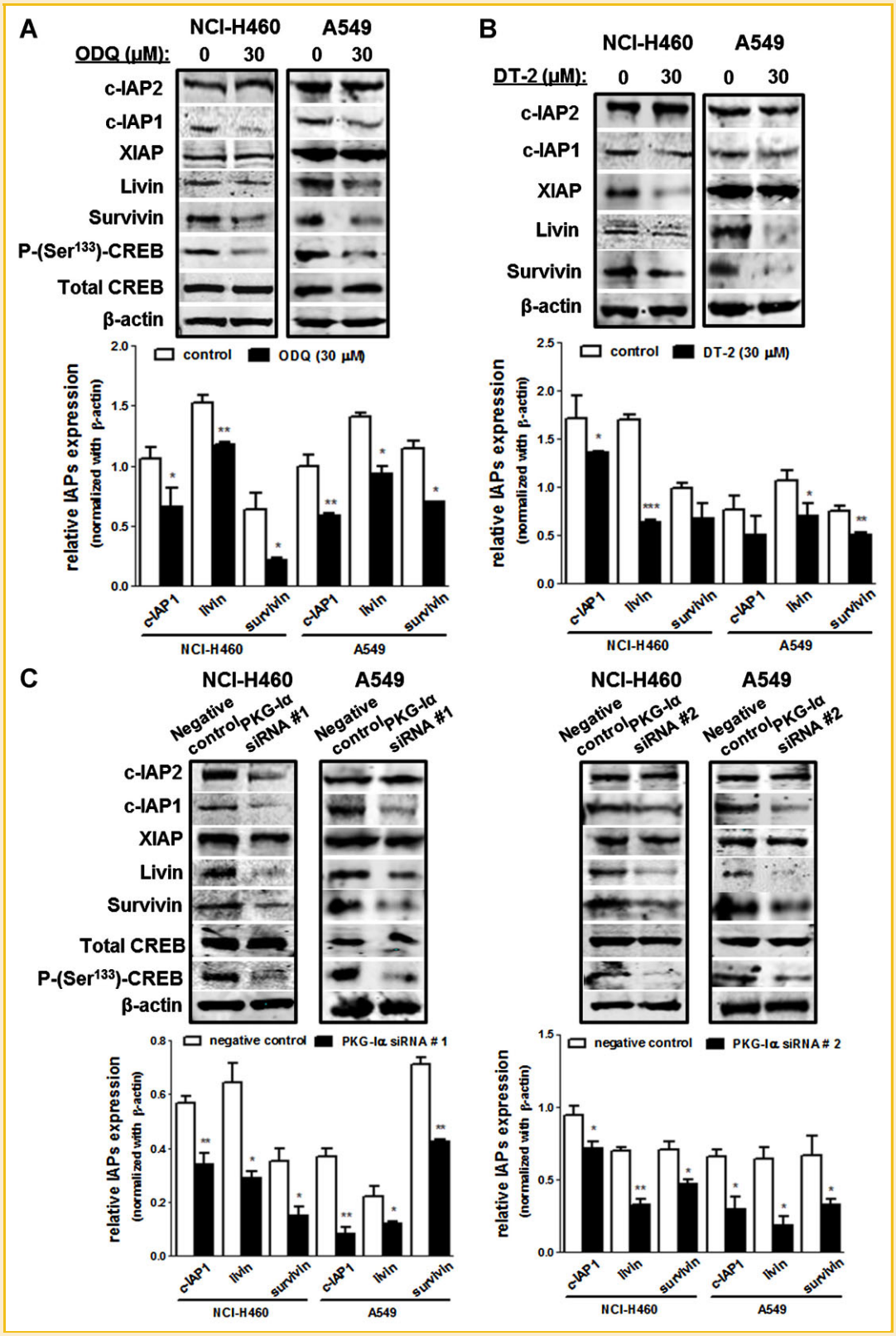

Fig. 4. A: Western blot analysis showing decreased expression levels of various IAPs, including c-IAP1, livin, and survivin, as well as decreased CREB phosphorylation at ser133, by treatment of ODQ (30 μM) for 24 h in both NCI-H460 and A549 cell lines. B: DT-2 (30 μM) also decreased the expression levels of c-IAP1, livin, and survivin in both cell lines. C: Western blot analysis showing decrease in CREB phosphorylation at ser133 and the expression levels of c-IAP1, livin, and survivin by PKG-Iα siRNA gene knockdown, PKG-Iα siRNA #1 and PKG-Iα siRNA #2. The Western blots are representative of four independent experiments and graphs show mean ± SEM from four independent experiments.

(lane 3). When denatured PKG-Iα was used, no phosphorylation of CREB at ser133 was observed (lane 4).

INHIBITION OF THE cGMP/PKG-Iα ACTIVITY DECREASES CREB ser133 PHOSPHORYLATION AND THE EXPRESSIONS OF CERTAIN IAPs

We determined if the phosphorylation of CREB at ser133 is a downstream target of PKG-Iα in NCI-H460 and A549 cells. Inhibition of PKG-Iα kinase activity using DT-2 decreased CREB ser133 phosphorylation as early as 2 h after DT-2 treatment, as well as in a time-dependent manner over 24 h (Fig. 3B), as shown by Western blot analysis (upper panels) and CREB cell-based ELISA (lower panel). Furthermore, we confirmed that cGMP/PKG-Iα activity decreases CREB ser133 phosphorylation in H460 and A549 cells by using the pharmacological inhibitor ODQ and knockdown with PKG-Iα siRNA. Also, we determined the expressions of the five IAPs, c-IAP1, c-IAP2, XIAP, livin, and survivin, are also regulated by PKG-Iα activity. ODQ at 30 μM caused a decrease in CREB ser133 phosphorylation in both cell lines, as well as decreased expressions of c-IAP1, livin, and survivin, by Western blot analysis (Fig. 4A). Figure 4B shows decreased expressions of c-IAP1, livin, and survivin induced by DT-2. The result was further confirmed by PKG-Iα siRNA gene knockdown. Both PKG-Iα siRNA #1 and PKG-Iα siRNA #2 decreased CREB ser133 phosphorylation and decreased expressions of c-IAP1, livin, and survivin (Fig. 4C).

INHIBITION OF THE cGMP/PKG-Iα ACTIVITY INCREASES CASPASE-3/7 ACTIVITY AND DECREASES Mcl-1 EXPRESSION

Because some IAPs (c-IAP1, c-IAP2, and XIAP) have been shown to directly inhibit activated caspase-3/7, we determined whether inhibition of cGMP/PKG-Iα activity affects caspase-3/7 activity and the expression levels of the anti-apoptotic Bcl-2 family proteins (Bcl-2, Bcl-xL, and Mcl-1). ODQ at 30 μM slightly decreased expression of the anti-apoptotic protein Mcl-1, but expressions of Bcl-xL and Bcl-2 remained unchanged (Fig. 5A). Figure 5B shows that PKG-Iα inhibition by DT-2 also slightly decreased Mcl-1 expression. Interestingly, one of the PKG-Iα siRNAs, PKG-Iα siRNA #1, substantially decreased Mcl-1, but also Bcl-2 expressions in both cell lines (Fig. 5C). ODQ at 30 and 50 μM (Fig. 6A), DT-2 (Fig. 6B) and PKG-Iα siRNAs (Fig. 6C), all significantly increased cleaved caspase-3 expression in Western blot analysis (upper panels), as well as caspase-3/7 activity (lower panels). The results further confirm the anti-apoptotic role of cGMP/PKG-Iα signaling pathway in NSCLC cells.

INHIBITION OF PKG-Iα KINASE ACTIVITY WITH DT-2 SYNERGIZES AND ENHANCES THE PRO-APOPTOTIC EFFECT OF CISPLATIN IN A549 CELLS

Cisplatin is widely used as a chemotherapeutic agent for solid tumors to induce apoptosis in many types of cancer, including NSCLC. To investigate the role of PKG-Iα in regulation of cisplatin-

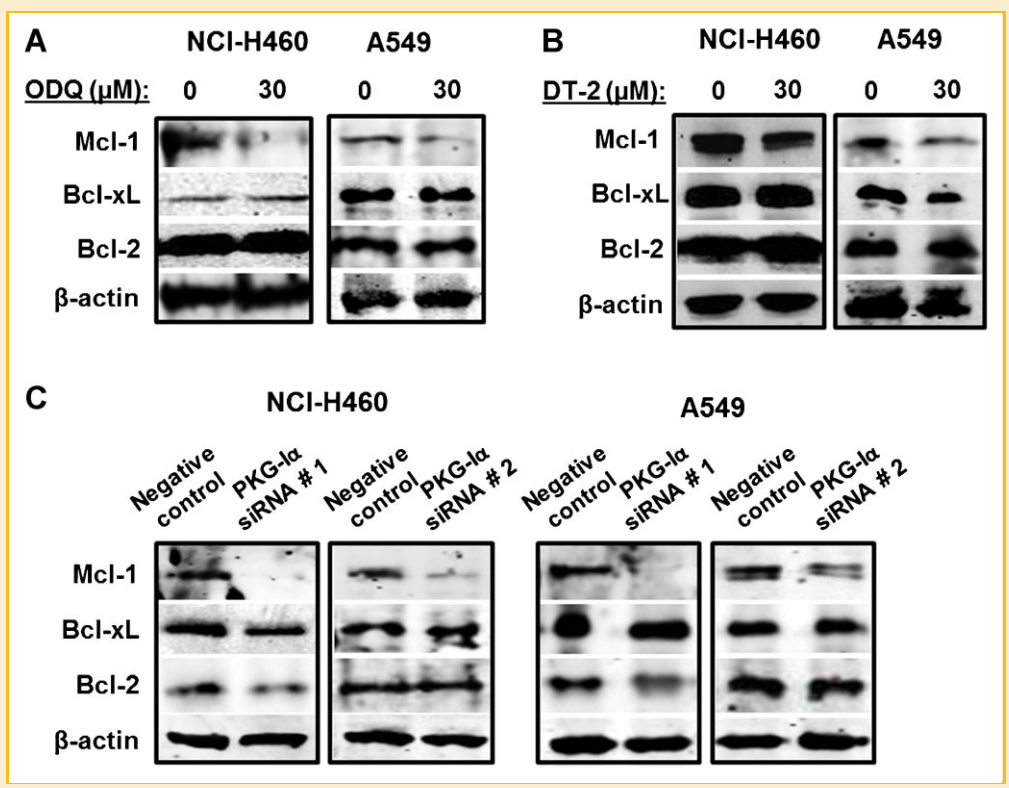

Fig. 5. Western blot analysis showing decreased expression of the anti-apoptotic protein Mcl-1 by ODQ (A), DT-2 (B), and PKG-Iα siRNA knockdown, PKG-Iα siRNA #1 and PKG-Iα siRNA #2 (C), in both NCI-H460 and A549 cells. The Western blots are representative of at least three different experiments.

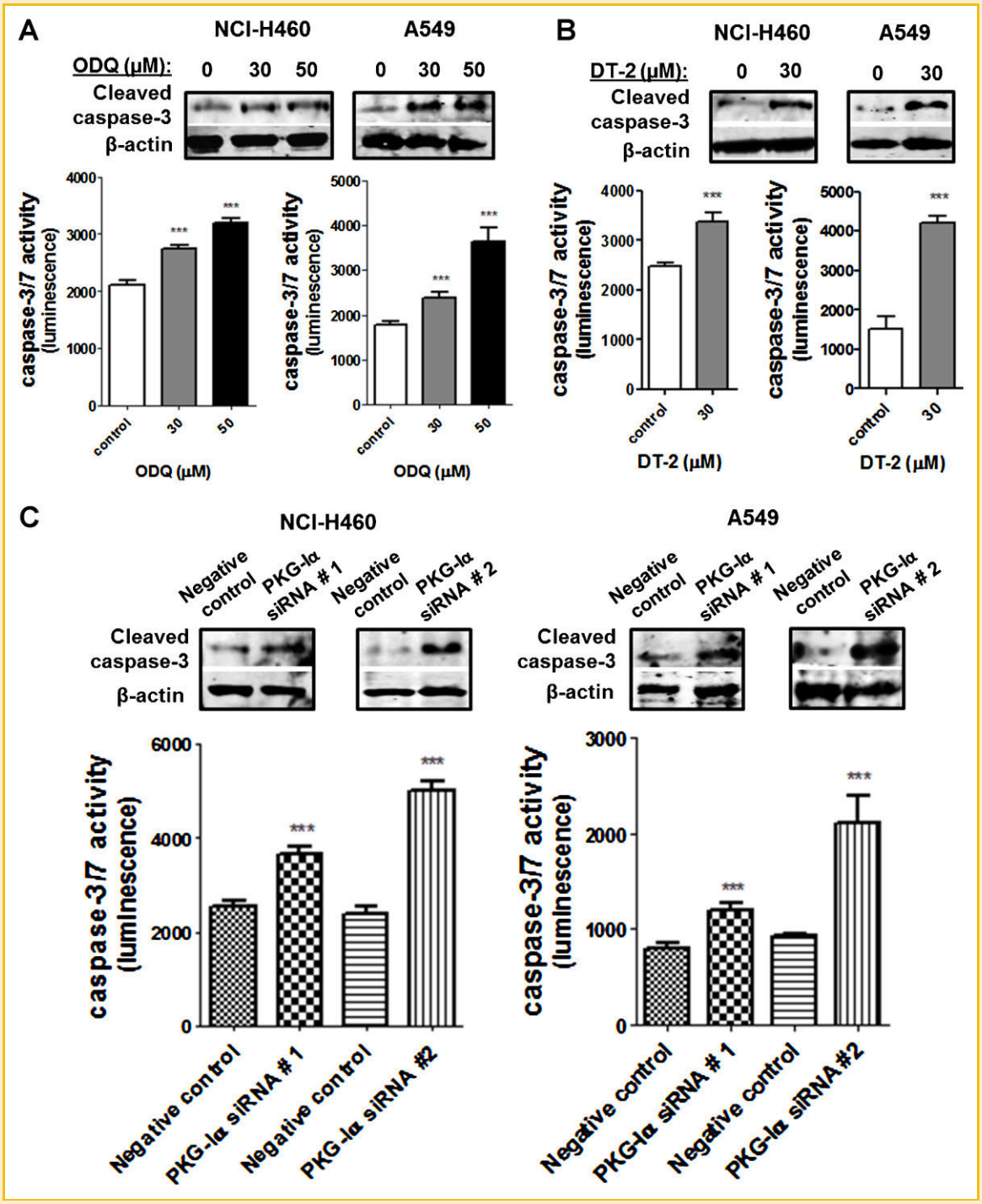

Fig. 6. Western blot analysis (upper panels) showing significant increases in cleaved caspase-3 expressions as well as caspase-3/7 activities (lower panels) by treatments of ODQ at 30 and 50 μM (A), DT-2 at 30 μM (B), and PKG-Iα siRNA knockdown, PKG-Iα siRNA #1 and PKG-Iα siRNA #2 (C), in both NCI-H460 and A549 cells. The Western blots are representative of at least three different experiments. $^{***}P < 0.001$.

induced apoptosis, we first determined a range of concentrations of DT-2 and cisplatin to induce apoptosis on A549 cells. DT-2, starting at 10 μM, significantly ($^{**}P < 0.01$) induced apoptosis (Fig. 7A), while cisplatin significantly ($^{**}P < 0.01$) induced apoptosis starting at 1 μM (Fig. 7B). A simultaneous treatment of DT-2 (5 or 10 μM) and cisplatin (1 or 2 μM) showed synergism in induction of apoptosis in the cisplatin-resistant A549 cells. Figure 7C shows combined treatment of DT-2 (5 or 10 μM) and cisplatin (1 μM) significantly ($^{***}P < 0.001$) increased apoptosis by 1.5- and 2-folds, respectively, compared with cisplatin (1 μM) alone. Similarly, DT-2

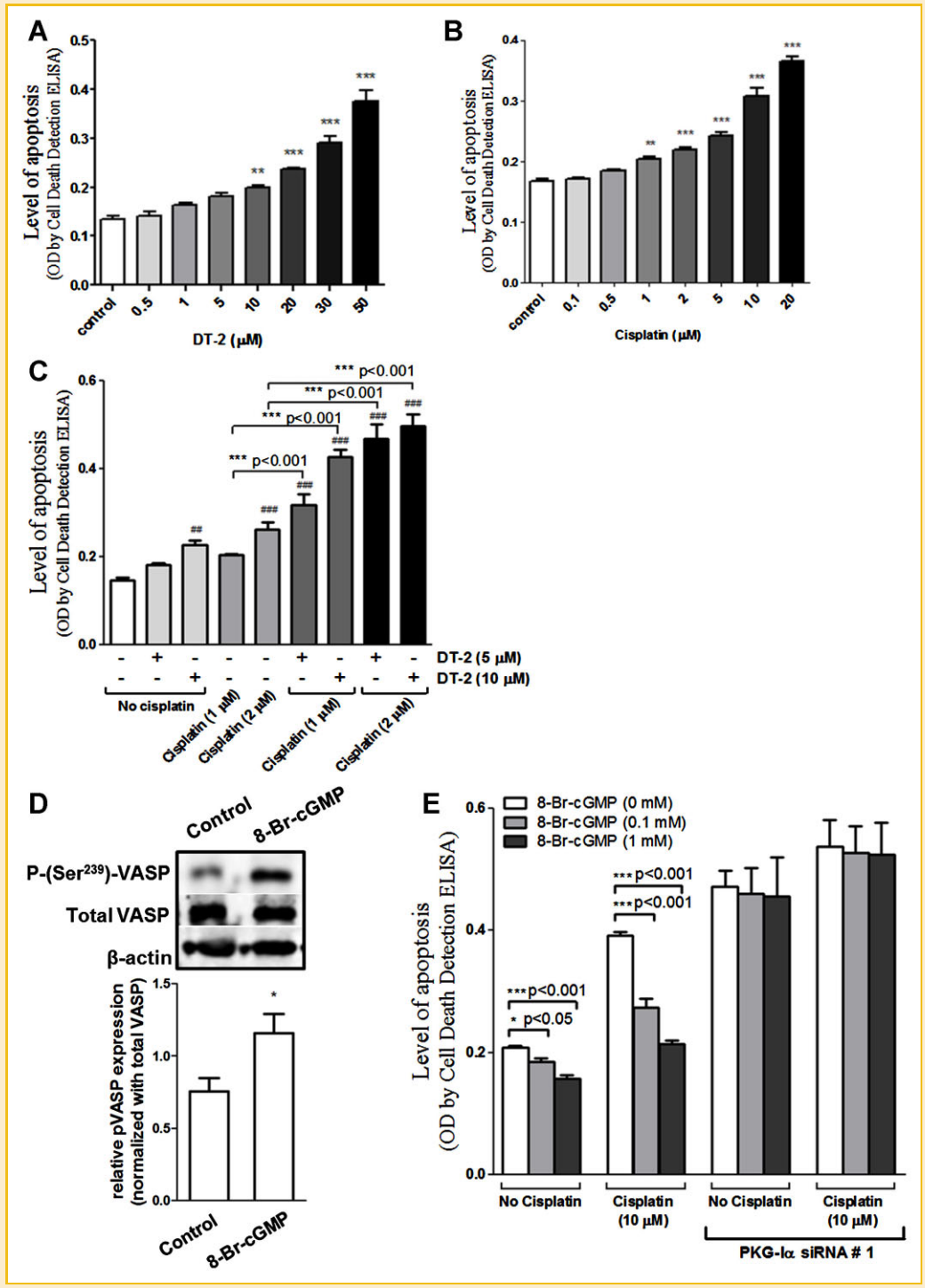

Fig. 7. Data of Cell Death Detection ELISA showing concentration-dependent induction of apoptosis by DT-2 (A) and cisplatin (B) in A549 cells. C: Synergistic effect of DT-2 with cisplatin in A549 cells. Combined treatment of DT-2 (5 or 10 μM) and cisplatin (1 or 2 μM) significantly ($^{***}P < 0.001$) increased apoptosis, compared with cisplatin (1 or 2 μM) alone. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared with no treatment control. D: Western blot analysis showing basal PKG-Iα activity was stimulated by 8-Br-cGMP (1 mM), as reflected by increased VASP phosphorylation at ser239, an indication of intracellular PKG kinase activity. E: Pretreatment of A549 cells with 8-Br-cGMP with 0.1 and 1 mM prior to cisplatin significantly protect the cells against cisplatin-induced apoptosis ($^{***}P < 0.001$). However, the cytoprotective effects of 8-Br-cGMP against cisplatin was abolished in the absence of PKG-Iα (siRNA gene knockdown using PKG-Iα siRNA #1), indicating that the 8-Br-cGMP-induced cytoprotection (chemoresistance) was mediated by PKG-Iα.

(5 or 10 μM) with cisplatin (2 μM) significantly ($^{***}P < 0.001$) increased apoptosis by twofold compared with cisplatin (2 μM) alone. Thus, DT-2 and cisplatin have a synergistic effect on induction of apoptosis.

To further test whether prior activation of PKG-Iα has cytoprotective effects against cisplatin, 8-bromo-cGMP (8-Br-GMP), which is a cell-permeable cGMP analog that directly activates PKG [Fiscus, 2002; Fiscus et al., 2002], was used to pretreat the cells 3 h prior to addition of cisplatin for 24 h. Figure 7D shows that basal PKG-Iα activity was stimulated by 8-Br-cGMP, as reflected by increased VASP phosphorylation at ser239, an indication of intracellular PKG kinase activity. Figure 7E shows that pretreatment of A549 cells with 8-Br-cGMP with 0.1 and 1 mM, both caused significant protections ($^{***}P < 0.001$) against cisplatin-induced apoptosis. Interestingly, when the same treatments were used on PKG-Iα knockdown cells (using PKG-Iα siRNA #1), the cytoprotective effect of 8-Br-cGMP against cisplatin-induced apoptosis was abolished, confirming that the cytoprotection (chemoresistance) was mediated by PKG-Iα.

DISCUSSION

CREB is a 43 kDa basic/leucine zipper transcription factor that is expressed in most tissues and is activated by phosphorylation on ser133 by serine–threonine kinases, which then induces its transcriptional activity by recruitment of the transcriptional co-activator CREB-binding protein [Mayr et al., 2001]. CREB has been implicated as a potential molecular target for treatment of cancer [Sakamoto and Frank, 2009], and it has been shown that expression levels of mRNA and protein of CREB and phosphorylated CREB are significantly higher in most of the NSCLC cell lines and tumor specimens [Seo et al., 2008]. CREB in turn regulates the expression of the Bcl-2 anti-apoptotic family proteins (Bcl-2, Bcl-xL, and Mcl-1). For example, it has been shown that Akt up regulates Bcl-2 expression through CREB in PC-12 cells [Pugazhenthi et al., 2000]. CREB is also shown to be involved in IL-3 induced up-regulation of Mcl-1 gene expression through a PI3K/Akt-dependent pathway in erythroleukemia cells [Wang et al., 1999].

In the present study, we observed that basal PKG-Iα kinase activity is necessary for CREB phosphorylation at ser133 and maintaining Mcl-1 protein expression. The important role of PKG-Iα in promoting CREB ser133 phosphorylation and Mcl-1 expression was confirmed using siRNA gene knockdown of PKG-Iα expression. We also observed a decrease in Bcl-2 expression using PKG-Iα siRNA gene knockdown in some experiments (Fig. 5C), however, Bcl-2 expression was not observed to decrease in response to PKG-Iα kinase inhibition with DT-2 (Fig. 5B). The difference between kinase inhibition (over 24 h) and siRNA knockdown (over 3 days) may related to the different turnover rates of the proteins. Mcl-1 has an extended amino-terminal PEST region, which is responsible for its relatively short half-life [Yang et al., 1995], whereas Bcl-2 and Bcl-xL have much slower turnover rates. Bcl-2 and Bcl-xL may be regulated by other mechanisms, such as proteasomal degradation. For example, in a study using NCI-H460 cells, Bcl-2 is shown to be regulated through *S*-nitrosylation and inhibition of ubiquitination in NO-impaired cisplatin-induced apoptosis [Chanvorachote et al., 2006]. Although Bcl-2 has also been reported to be enzymatically cleaved by caspase-3 in leukemic cells [Zhang et al., 1999], Chanvorachote et al. showed lack of caspase-mediated cleavage of Bcl-2 at the early time period and that proteasomal degradation is a key event in controlling Bcl-2 stability.

The present study suggests that PKG-Iα kinase activity promotes CREB phosphorylation at ser133 and maintains higher levels of protein expression of the three anti-apoptotic proteins, c-IAP1, livin, and survivin. Whether these IAPs are regulated by PKG-Iα in a CREB-dependent manner, and whether CREB directly regulates the expression of the IAPs in NSCLC cells, remains to be investigated in future studies.

In the present study, we also found that inhibition of PKG-Iα kinase activity using DT-2 sensitized A549 cells to the pro-apoptotic effects of cisplatin and that the combined treatment of DT-2 and cisplatin have a synergistic effect on induction of apoptosis. Our future studies will include further investigations of the mechanisms of the cGMP/PKG-Iα signaling pathway in the regulation of apoptosis in cisplatin-resistant cells. These studies could provide new information to add to our knowledge of the mechanisms of drug resistance and for identifying novel potential targets in treatment of cancer.

In summary, data from the present study suggest that CREB at ser133 is a downstream target phosphorylated by PKG-Iα, and the cGMP/PKG-Iα signaling pathway maintains the expression of c-IAP1, livin, survivin, and Mcl-1 to promote cell survival in NSCLC cells. The combined treatment of DT-2 and cisplatin have a synergistic effect on induction of apoptosis in NSCLC cells.

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