Advances in Brief

Exisulind Induction of Apoptosis Involves Guanosine 3′,5′-Cyclic Monophosphate Phosphodiesterase Inhibition, Protein Kinase G Activation, and Attenuated β-Catenin

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Abstract

Sulindac sulfone (exisulind), although a nonsteroidal anti-inflammatory drug derivative, induces apoptosis in tumor cells by a mechanism that does not involve cyclooxygenase inhibition. SW480 colon tumor cells contain guanosine 3′,5′-monophosphate (cGMP) phosphodiesterase (PDE) isoforms of the *PDE5* and *PDE2* gene families that are inhibited by exisulind and new synthetic analogues. The analogues maintain rank order of potency for PDE inhibition, apoptosis induction, and growth inhibition. A novel mechanism for exisulind to induce apoptosis is studied involving sustained increases in cGMP levels and cGMP-dependent protein kinase (PKG) induction not found with selective PDE5 or most other PDE inhibitors. Accumulated β-catenin, shown to be a substrate for PKG, is decreased by exisulind, suggesting a mechanism to explain apoptosis induction in neoplastic cells harboring adenomatous polyposis coli gene mutations.

Introduction

Chemotherapeutic and chemopreventive agents such as sulindac and similar NSAIDs[2] induce apoptosis (1, 2). Exisulind, the oxidative metabolite of sulindac, induces apoptosis and inhibits growth of tumor cell lines of diverse origins (3–5), suggesting that an important survival pathway is modified by the drug. Exisulind is not an NSAID because it lacks the hallmark cyclooxygenase inhibitory activities of NSAIDs (6). The mechanism of exisulind-induced apoptosis is independent of p53, Bcl-2, and cell cycle arrest (4, 5). Exisulind inhibits tumor growth in rodent models of colon, mammary, prostate, bladder, and lung carcinogenesis (3, 6–8). We report here studies indicating that exisulind induces apoptosis in colon tumor cells by inhibiting cGMP PDE, causing a persistent increase in cellular cGMP, and inducing PKG. This approach has led to the development of a novel new class of chemopreventive and antineoplastic drugs that lack NSAID-induced gastrointestinal, renal, and hematological toxicities. However, exisulind did show dose-limiting toxicity of mild to moderate hepatic enzyme elevations in some FAP patients that was reversible on dose reduction.

Cyclic nucleotide PDEs consist of 10 gene families, each having one or more isoforms. These enzymes are being used as pharmaceutical targets for new drugs designed to manipulate cellular processes modulated by cAMP and/or cGMP (9–12). PDE inhibitors influence many pathologies, but their use as anticancer agents has not been developed (13, 14). The majority of PDE isozyme inhibitors are not proapoptotic in epithelial-derived tumor cells, although inhibitors of PDE1 and PDE4

isoforms induce apoptosis in lymphoid cells (14, 15). We found that like exisulind, nonselective PDE5 inhibitors MY5445 and dipyridamole induced apoptosis in HT29, SW480, and T84 human colon tumor cell lines used for these studies. Therefore, the hypothesis that exisulind may induce apoptosis via cGMP PDE inhibition was tested.

Materials and Methods

Cell Growth. SW480 and HT29 cells were grown in RPMI 1640, 2 mM glutamine, 1% antibiotic/antimycotic solution, and 5% FBS in 150-cm^2 flasks or dishes. SW480 cells were also grown in Corning 850-cm^2 roller bottles with the addition of 25 mM HEPES for the fast protein liquid chromatography profile. T84 cells were grown in 47% ATCC Ham's F-12 media, 47% Sigma DMEM, 1% antibiotic/antimycotic solution, 8.4 mM sodium bicarbonate, and 5% serum, pH 7.25. Cell lines were grown using serum from Sigma in 5% CO$_2$ at 37°C. Cells were harvested at 70–100% confluence with either Trypsin/EDTA (Life Technologies, Inc.) or Pancreatin (Life Technologies, Inc.) and either used fresh or were frozen on liquid nitrogen and stored at −70°C.

Protein Purification. SW480 cells were grown in roller bottles at 0.5 rpm. Approximately 600 million cells were manually homogenized in 5 mM Tris-acetate, 5 mM magnesium acetate, 0.1 mM EDTA, 0.8% Triton X-100, 10 μM benzamidine, 10 μM N-α-p-tosyl-L-lysine chloromethyl ketone (TLCK), 2000 units/ml aprotinin, 2 μM leupeptin, and 2 μM pepstatin A (pH 7.5). After ultracentrifugation at 100,000 × g at 4°C for 1 h, supernatants were diluted 5-fold with the buffer minus Triton and loaded at 1 ml/min onto an 18-ml DEAE Trisacryl M column (BioSepra) using Pharmacia AKTA/fast protein liquid chromatography. The column was washed with 8 mM TRIS-acetate, 5 mM magnesium acetate, and 0.1 mM EDTA (pH 7.5). Enzymes were eluted with a gradient of 0–1 M sodium acetate at a flow rate of 1 ml/min into 1.5-ml fractions.

Apoptosis and Cell Growth Inhibition. DNA fragmentation in SW480 cells at 10,000 cells/well in 96-well plates was measured using a double antibody ELISA kit (Boehringer Mannheim) that detects DNA/histone complexes. After 24 h, cells were redosed and grown for an additional 48 h. Growth inhibition was determined by plating cells at 1000 cells/well in 96-well plates. Cells were dosed after 24 h and incubated for 6 days. Cells were fixed with 10% trichloroacetic acid at 4°C for 1 h, rinsed five times with deionized H$_2$O, and incubated for 10 min with 0.4% sulforhodamine B in 1% acetic acid. Plates were rinsed four times with 1% acetic acid, dried 30 min, and solubilized in 10 mM Tris. Absorbance was determined at 540 nM using a Molecular Devices Spectra Max 340 plate reader.

cGMP and cAMP RIA. cGMP and cAMP levels were measured by RIA. Approximately 5 × 10^6 cells were used for each assay. After drug treatment, cells were washed with cold PBS. Cyclic nucleotides were extracted with 0.2 N HCl/50% methanol and dried. The dried samples were reconstituted in water and acetylated before RIA with anti-cGMP and anti-cAMP antibodies. The results were expressed in fmol of cGMP/cAMP generated per mg protein of the cells.

PKG Activation. SW480 cells were treated with compounds for 48 h, and PKG activity was measured using a substrate of cloned GST fusion protein of a fragment of human PDE5 bound to GSH-Sepharose affinity beads. The PDE5 fragment contains its phosphorylation site (Ser-92) and cGMP binding domains (residue 35–530, relative to bovine PDE5). Cell lysate (100 μg), substrate (20 μg), 0.25 μM protein kinase inhibitor, 4.5 mM magnesium, and [γ-^{32}P]ATP (10 μCi; 190 μM) with or without added cGMP (8 μM) were

3338

mixed and incubated at 30°C for 30 min. The phosphorylated GST-cGB-PDE5 was resolved on 7.5% SDS-PAGE and exposed to X-ray film or quantitated by phosphorimaging (Packard Cyclone).

Western Blotting. SW480 cells were treated for 48 h and lysed with modified RIPA buffer. Fifty μg of lysate were loaded to each lane of 10% precast Novex gels. The transferred membrane was probed with the primary antibody and then with the corresponding horseradish peroxidase-conjugated secondary antibodies. Western blotting results were quantitated using an AlphaImager 2000 (Alpha Innotech). Anti-PKG 1β, anti-β-catenin, and anti-cyclin D1 antibodies were purchased from StressGen Biotechnologies Corp. (British Columbia, Canada), Upstate Biotechnology (Lake Placid, NY), and NeoMarkers, Inc. (Fremont, CA), respectively.

Results and Discussion

Cyclic nucleotide PDE isoforms in SW480 colon cell lysates fractionated by anion-exchange chromatography showed expression of isoforms that were cGMP specific (peak 1), cAMP specific (peak 3), and cAMP/cGMP hydrolyzing (peak 2; Fig. 1A). These PDEs showed no calcium/calmodulin stimulation or cGMP inhibition, indicating little or no PDE1 or PDE3 isoforms. The cGMP activity of peak 1, but not of peak 2 (Fig. 1B), was inhibited by 100 nM E4021 (IC$_{50}$, 3 nM), a specific inhibitor of

PDE5, and by sildenafil (IC$_{50}$, 0.3 nM), dipyridamole (IC$_{50}$, 0.6 μM), and zaprinast (IC$_{50}$, 1.5 μM), also PDE5 inhibitors (11). The cAMP activity of peak 2 was stimulated by cGMP (Fig. 1a), cGMP activity of peak 2 showed positive cooperativity (5 μM $versus$ 0.25 μM cGMP substrate; $inset$), and peak 2 was inhibited by trequinsin and EHNA (IC$_{50}$s, 1.0 and 3.7 μM), characteristic of PDE2 (16). In contrast to the more selective PDE inhibitors, exisulind inhibited both cGMP PDE activities [Fig. 1B; IC$_{50}$s, 128 $\pm$ 26 μM and 335 $\pm$ 67 μM (n = 6), respectively, at 0.25 μM cGMP substrate]. HT29 and T84 cells showed various expressions of the same isoforms with comparable inhibitory responses to exisulind. Most of the cAMP activity of peak 3 was inhibited by rolipram and confirmed by reverse transcription-PCR as $PDE4$ (A-D) genes (data not shown). Despite these enzyme expression patterns, reverse transcription-PCR analysis of SW480, T84, and HT29 colon tumor cell lines showed mRNA for all PDE1–10 families, suggesting important posttranslational regulation of PDE expression (primers available on request).

Inhibition of SW480 PDE5 and PDE2 by exisulind occurred at concentrations below those required to inhibit tumor cell growth at 6 days of treatment (IC$_{50}$, 165 $\pm$ 25 μM) and induce apoptosis after 2 days of treatment (EC$_{50}$, 557 $\pm$ 45 μM; Fig. 2). Because exisulind blood levels above these IC$_{50}$s have been achieved in $vivo$ (6), inhibition of one or both of these enzymes could account for the antineoplastic activity of exisulind. This possibility was strongly supported by finding that derivatives of exisulind screened by structure/PDE5/2 inhibitory activity analyses led to the identification of a trimethoxy acid (CP78) and benzylamide (CP461 and CP248) analogues that show a >1000-fold range of inhibitory activity. The compounds maintained similar rank orders of potency for apoptosis induction and PDE5 and growth inhibitions as did exisulind (Fig. 2). Isoform selectivity for PDE5 and PDE2 increased in parallel for exisulind and analogues that were without COX1 or COX2 inhibitory activity up to 1 mM, indicating that potent proapoptotic drugs can be identified independently of cyclooxygenase activity.

Exisulind has been shown to cause regression of colorectal polyps in patients with FAP by a mechanism involving apoptosis (17). In addition, mucus differentiation was stimulated by the drug in cells of adenomatous glands in biopsies of regressing polyps. Germ-line mutations in the APC tumor suppressor gene are known in FAP, and somatic APC mutations occur commonly in sporadic adenomas (18–20). APC mutations are thought to be carcinogenic in part because of β-catenin/Tcf4/Lef transcriptional activation. Normal APC protein mediates phosphorylation by GSK3-β and ubiquitin/proteosomal degradation of β-catenin (21–23). APC mutations result in cytoplasmic and nuclear β-catenin accumulations, leading to transcription factor complex deregulation and activation of antiapoptotic and proliferation genes such as $cyclin$ $D1$ and c-myc (24, 25). The efficacy of exisulind in FAP patients and reports that transfection of a wild-type APC gene into cells with mutations could induce apoptosis (26) and β-catenin degradation (27) suggested the possibility that exisulind may induce apoptosis by circumventing the β-catenin accumulations. We tested the hypothesis that exisulind inhibition of cGMP PDE increases cGMP levels to down-regulate β-catenin, possibly via PKG phosphorylation to initiate apoptosis.

Because cGMP PDE inhibition by exisulind and analogues correlated with apoptosis, cellular cGMP changes after drug treatment were determined. Representative, selective PDE5 inhibitors, E4021 and zaprinast, that do not induce apoptosis were also studied. Exisulind and CP461 treatments require 24–48 h to initiate apoptosis measured by DNA fragmentation. In the short-term (<60 min), cGMP was increased by exisulind (Fig. 3) and CP461 (139 $\pm$ 17 to 316 $\pm$ 60 fmol/mg), but only E4021 (139 $\pm$ 17 to 327 $\pm$ 60) and not zaprinast of the more selective PDE5 inhibitors was effective. Furthermore, exisulind and CP461, but not E4021, increases in cGMP persisted to 72 h at doses required for apoptosis (Fig. 3, A and B). Cyclic AMP
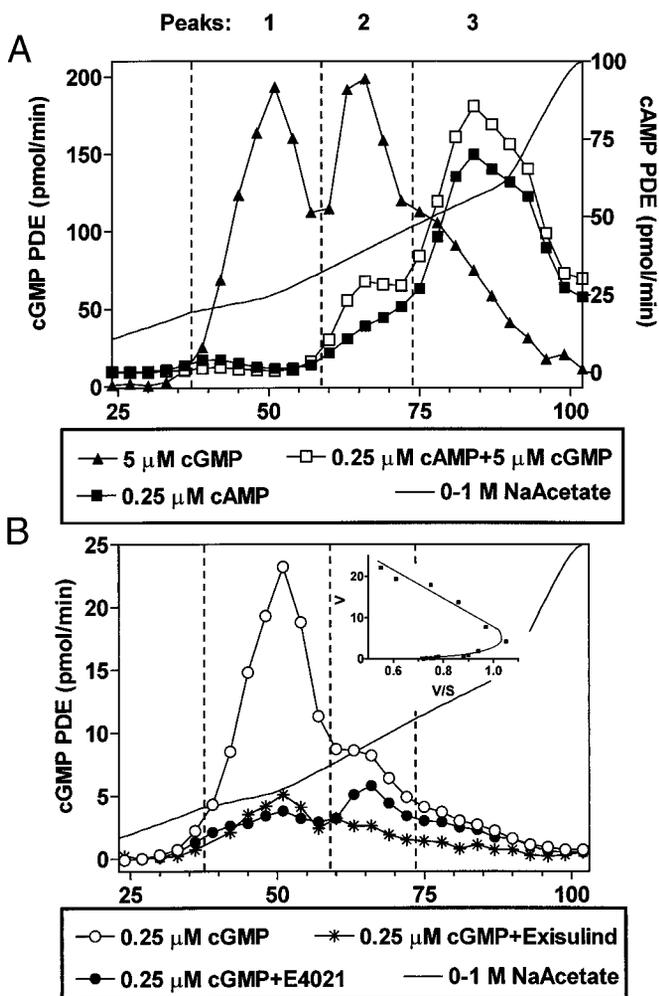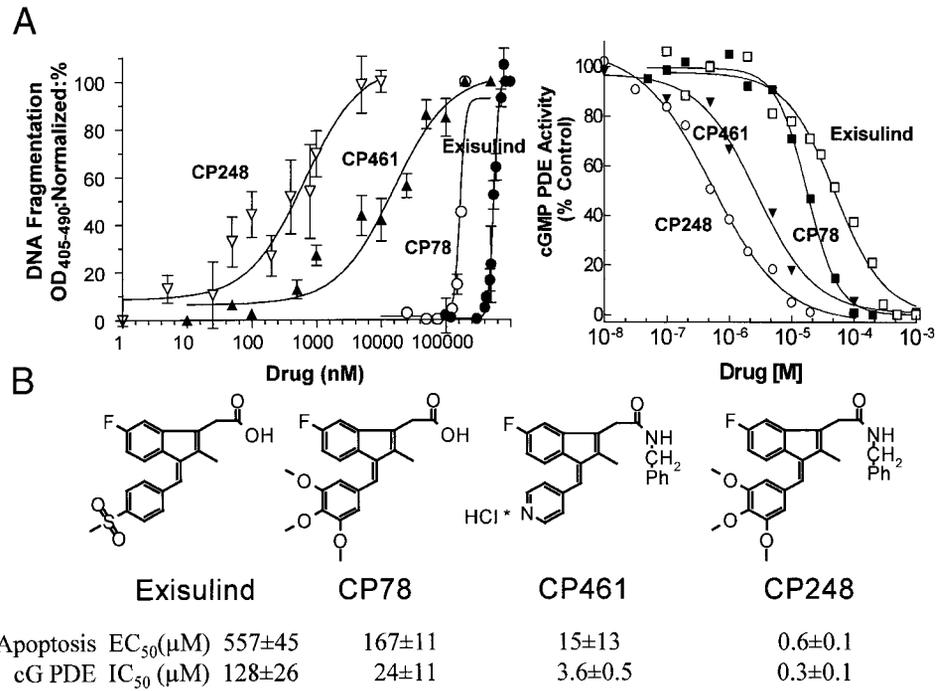
Fig. 1. DEAE-Trisacryl M chromatography of SW480 cGMP and cAMP phosphodiesterases. Enzymes were eluted from DEAE with a gradient of 0–1 M sodium acetate at a flow rate of 1 ml/min into 1.5-ml fractions. [^{3}H]cAMP or [^{3}H]cGMP substrate was used to determine PDE activity according to Thompson et $al.$ (38). Two peaks of activity were present at 0.25 μM (B) or 5 μM cGMP (A) with peak 2 displaying cGMP activation, determined to be positive cooperativity ($inset$ B). A, peak 2 also had cAMP activity that was activated by 5 μM cGMP, indicative of PDE2. B, peak 1, not peak 2, cGMP PDE was inhibited by 100 nM E4021, indicative of PDE5. Both cGMP peaks were inhibited by exisulind.

3339

Fig. 2. Correlation between rank order potency of cGMP PDE inhibition and apoptosis induction. DNA fragmentation in SW480 cells at 10,000 cells/well in 96-well plates was measured using a double antibody ELISA kit. *Upper left,* cells were treated for 48 h with increasing concentrations of exisulind, a trimethoxy acid (CP78), or benzylamide analogues (CP461 and CP248). Apoptosis data are normalized from 4 to 15 full curves with triplicates of each dose. *Upper right,* cyclic GMP PDE inhibition data from 2 to 6 full curves using different batches of DEAE-purified PDE5. $EC_{50}s$ and $IC_{50}s$ were calculated using sigmoidal dose-response, variable slope, nonlinear regression in Prizm (Graph-Pad); *bars,* SE.

|	Exisulind	CP78	CP461	CP248
Apoptosis $EC_{50}(\mu M)$	557 ± 45	167 ± 11	15 ± 13	0.6 ± 0.1
cG PDE $IC_{50}(\mu M)$	128 ± 26	24 ± 11	3.6 ± 0.5	0.3 ± 0.1

levels remained constant throughout treatment with exisulind and CP461, indicating a minimal effect on PDE4 and selectivity for cGMP PDEs in the intact cell (Fig. 3*B*). E4021 and zaprinast showed no significant cGMP or cAMP changes (Fig. 3*C*), except for an increase in cAMP at 72 h at doses well above their enzyme inhibition constants. T84 colon tumor cells also responded to exisulind and CP461 with increased cGMP, absent cAMP changes at concentrations needed to effect apoptosis and growth. The data may reflect unknown metabolic changes in E4021 or zaprinast but suggest that exisulind and higher affinity analogues, unlike the more selective PDE5 drugs,

inhibit PDE5/2 to sustain increased cGMP levels in colon cancer cells to trigger apoptosis.

To determine whether persistent rather than transient cGMP increases are sufficient for induction of apoptosis, cells were treated with the GC activator, YC-1 (28) or cGMP analogues. YC-1 treatment of SW480 cells inhibited growth and induced apoptosis at doses required to activate guanylate cyclase (Fig. 3*D*). Furthermore, the analogue 8-bromo-cGMP also induced apoptosis measured by morphology assays after 7 days of treatment (data not shown). Possible cGMP-mediated apoptosis is supported by previous reports in rat myocytes (29), pancreatic B-cells (30),

Fig. 3. RIA of exisulind-treated colon cancer cells detected elevated cGMP but not cAMP. SW480 cells (1×10^6) were plated on 100-mm dishes; drugs were added after 2 days of growth for the times and doses indicated, followed by a rapid wash, 0.2 N HCl/50% methanol extraction, and drying. Cyclic nucleotides were acetylated and measured by RIA (39). *A,* time course shows exisulind and CP461 elevated cGMP at 1–72 h. Data are means from three to five experiments with four replicates in each; *bars,* SE. *B,* dose response of CP461 at 72 h shows increased cGMP but not cAMP. *C,* E4021 and zaprinast dose responses at 72 h do not show elevated cGMP or cAMP. *B* and *C* are representative experiments with four replicates at each dose with the ★ indicating $P < 0.05$ by unpaired, two-tailed t tests. *D,* guanylate cyclase activator, YC-1, induces apoptosis and growth inhibition with similar doses ($D_{0.5}$, 1.4 and 1.9). Experiments were determined as indicated in the legend to Fig. 2.
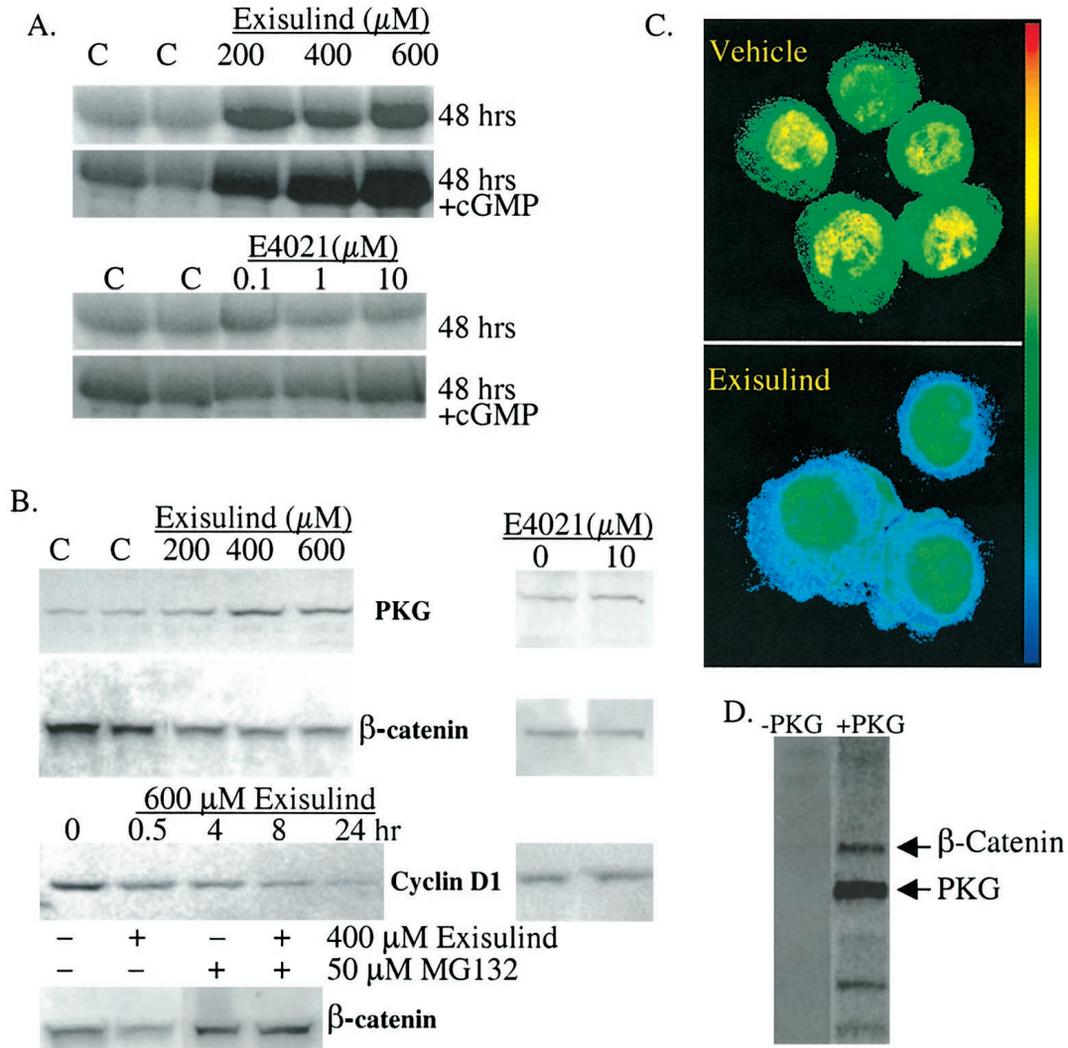
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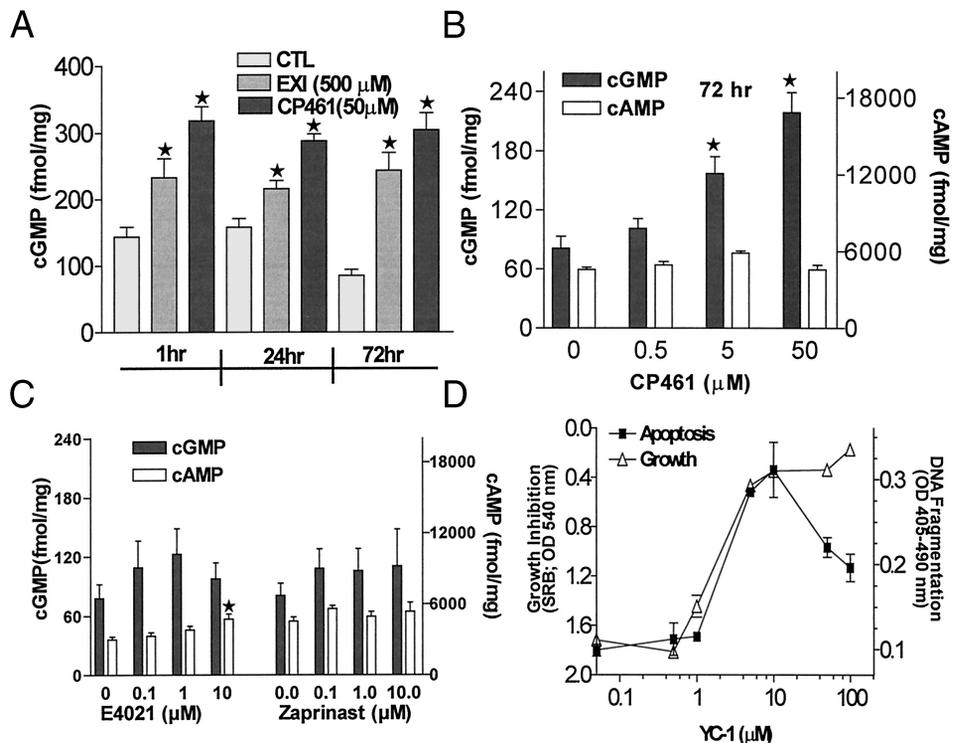
Fig. 4. PKG, β-catenin, and cyclin D1 regulation. *A,* PKG is activated by exisulind but not E4021. SW480 cells were treated with DMSO (0.03%), exisulind, or E4021 for 48 h, and PKG activity was measured using a substrate of cloned bovine PDE5 GST fusion protein bound to GSH-Sepharose affinity beads. Cell lysate (100 μg), substrate (20 μg), 0.25 μM protein kinase inhibitor, 4.5 mM magnesium, and [γ-^{32}P]ATP (10 μCi; 190 μM), with or without added cGMP (8 μM), were mixed and incubated for 30 min. The phosphorylated GST-cGB-PDE5 was resolved on 7.5% SDS-PAGE and phosphorimaging. *B,* Western blots of exisulind increased PKG, decreased β-catenin, and decreased cyclin D1. SW480 cells were treated for 48 h, lysed with modified RIPA buffer, and analyzed by Western blots (50 μg lysate/lane; 10% precast Novex gels) with quantitation by AlphaImager 2000 (Alpha Innotech). *C,* confocal microscopy images of exisulind-induced decreases in β-catenin in SW480 cells. SW480 cells were treated with exisulind (600 μM) for 48 h, and cytospin preps were fixed in 3.7% formaldehyde. Anti-β-catenin fluorescent images were obtained by confocal microscopy (LCS Ultraview). *D, in vitro* PKG phosphorylation of β-catenin. Anti-β-catenin immunoprecipitates from SW480 cells were incubated with purified PKG (Iα, 49 ng, 200 units) in phosphate buffer containing Mg^{2+} and [γ-^{32}P]ATP (10 μCi; 190 μM) for 10 min and resolved using 7.5% SDS-PAGE and autoradiography.

and endothelial cells (31) and data showing PKG transfection increased cell sensitivity to apoptosis inducers (32).

The effect of exisulind-induced cGMP on SW480 cell PKG activity was studied using an affinity bead-bound-specific substrate assay (Fig. 4, *A* and *B*) of supernatants from exisulind and vehicle-treated cells with or without cGMP added *in vitro*. Exisulind (600 μM), but not E4021 (10 μM), increased PKG activity 5-fold, as determined by phosphorimaging. Exisulind had no effect on activity *in vitro* when added directly to purified PKG or cell supernatants, indicating a mechanism requiring the intact cell. cGMP added *in vitro* increased substrate phosphorylation, confirming that PKG, and not another kinase, was increased by exisulind. The increased intensity of substrate phosphorylation in the absence of added cGMP attributable to exisulind treatment (Fig. 4*A*) was attributable to increased expression of PKG protein because Western blots using antibodies to PKG-1β showed dose-dependent induction of PKG immunoreactivity by 200% (Fig. 4*B*). Time courses with exisulind showed earliest detectable induction of PKG between 8 and 24 h of drug treatment (data not shown).

To explore potential substrates of the sustained induction of PKG by exisulind that might be relevant to the effect of the drug in patients with APC mutations, we determined whether PKG induces phosphorylation of β-catenin *in vitro*. SDS-PAGE gels of β-catenin immunoprecipitates treated with purified PKG for 10 min show phosphorylation at M_r 97,000, indicating that the oncogene can serve as substrate for PKG (Fig. 4*D*). Because phosphorylation of β-catenin leads to degradation and exisulind-induced PKG and apoptosis, it is possible that exisulind regulates apoptosis via PKG-mediated β-catenin phosphorylation. The effect appears to require nonselective rather than selective PDE5 inhibitors. Other effects of exisulind-induced PKG phosphorylation may also contribute to growth inhibition and coordinate with apoptosis induction, such as decreased raf kinase (33) or IκB kinase β inhibition (34).

Because exisulind increased PKG in SW480 cells and PKG can phosphorylate β-catenin *in vitro*, the effect of the drug on β-catenin expression and function through cyclin D1 were determined in the intact cell. Western blots of lysates from exisulind-treated SW480 cells showed reductions in β-catenin and cyclin D1 up to 50–80% of control values

3341

(Fig. 4*B*) at doses that induce apoptosis and PKG induction, whereas the nonapoptotic E4021 was inactive (20, 35, 36). Time courses with exisulind (600 μM) showed that like PKG induction, β-catenin degradation could be seen between 8 and 24 h of drug treatment (data not shown) or before apoptosis was detected. MG-132, a blocker of ubiquitin-conjugated protein degradation, effectively inhibited exisulind-induced β-catenin decreases (Fig. 4*B*) without affecting PKG induction by the drug. Confocal fluorescence microscopy of SW480 cells labeled with anti-β-catenin antibodies demonstrated that exisulind reduced both the cytoplasmic and nuclear pools of β-catenin (Fig. 4*C*). These data suggest that exisulind, like wild-type APC protein, causes proteosomal degradation of β-catenin via phosphorylation in APC-deficient cells.

These studies have identified cGMP PDEs of SW480 cells as biochemical targets of the chemopreventive agent exisulind. The drug and its analogues are novel PDE5/2 inhibitors that cause sustained cellular cGMP, activation of PKG, proteosomal degradation of β-catenin, and induce apoptosis. Direct phosphorylation of β-catenin by PKG could be the mechanism of its proteosomal degradation. Recent studies have suggested that β-catenin-regulated peroxisome proliferator-activated receptor δ may be a noncyclooxygenase NSAID target (37). The applicability of a cGMP regulatory mechanism to non-colon cancer cells and its integration to this other potential target of exisulind remains to be established, but screening with cGMP PDE inhibition and apoptosis induction has been used to produce a new class of proapoptotic drugs to prevent and treat cancer.

Acknowledgments

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