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Induction of Apoptosis in Renal Cell Carcinoma by Reactive Oxygen Species: Involvement of Extracellular Signal-Regulated Kinase 1/2, p38δ/γ, Cyclooxygenase-2 Down-Regulation, and Translocation of Apoptosis-Inducing Factor

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ABSTRACT

Renal cell carcinoma (RCC) is the most common malignancy of the kidney. Unfortunately, RCCs are highly refractory to conventional chemotherapy, radiation therapy, and even immunotherapy. Thus, novel therapeutic targets need to be sought for the successful treatment of RCCs. We now report that 6-anilino-5,8-quinolinequinone (LY83583), an inhibitor of cyclic GMP production, induced growth arrest and apoptosis of the RCC cell line 786-0. It did not prove deleterious to normal renal epithelial cells, an important aspect of chemotherapy. To address the cellular mechanism(s), we used both genetic and pharmacological approaches. LY83583 induced a time- and dose-dependent increase in RCC apoptosis through dephosphorylation of mitogen-activated protein kinase kinase 1/2 and its downstream extracellular signal-regulated kinases (ERK) 1 and -2. In addition, we observed a decrease in Elk-1 phosphorylation and cyclooxygenase-2 (COX-2) down-regulation. We were surprised that we failed to observe an increase in either c-Jun NH_2-terminal kinase or p38α and -β mitogen-activated protein kinase activation. In contradiction, reintroduction of p38δ by stable transfection or overexpression of p38γ dominant negative abrogated the apoptotic effect. Cell death was associated with a decrease and increase in Bcl-x_L and Bax expression, respectively, as well as release of cytochrome *c* and translocation of apoptosis-inducing factor. These events were associated with an increase in reactive oxygen species formation. The antioxidant *N*-acetyl L-cysteine, however, opposed LY83583-mediated mitochondrial dysfunction, ERK1/2 inactivation, COX-2 down-regulation, and apoptosis. In conclusion, our results suggest that LY83583 may represent a novel therapeutic agent for the treatment of RCC, which remains highly refractory to antineoplastic agents. Our data provide a molecular basis for the anticancer activity of LY83583.

Renal cell carcinoma (RCC) is the sixth leading cause of cancer death; the worldwide incidence is increasing at an annual rate of 2% (Boring et al., 1994). By the time metastatic disease is diagnosed, it is present in approximately 30% of patients, and an additional 30 to 40% develop metastases within a period of months or years after nephrectomy (Tourani et al., 2003). RCC has a poor prognosis owing to its late presentation and resistance to hormonal therapy (Motzer and Russo, 2000), chemotherapy (Amato, 2000), and radiotherapy (Bukowski, 1997). Cytokine treatments of patients with RCC have produced insufficient response rates,

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ABBREVIATIONS: RCC, renal cell carcinoma; LY83583 , 6-anilino-5,8-quinolinequinone; GC, guanylate cyclase; ROS, reactive oxygen species; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase; FCS, fetal calf serum; MAb, monoclonal antibody; MKK, mitogen-activated protein kinase kinase kinase; MEK, mitogen-activated protein kinase/extracellular-regulated kinase; COX, cyclooxygenase; PAGE, polyacrylamide gel electrophoresis; siRNA, small interfering RNA; HRE, human renal epithelial; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; DN, dominant negative; wt, wild type; RT, room temperature; DHR, 1′,2′,3′-dihydrorhodamine; DMSO, dimethyl sulfoxide; AIF, apoptosis-inducing factor; NAC, *N*-acetyl L-cysteine; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; PI, propidium iodide; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene; 8-Br-cGMP, 8-bromo-cGMP; z-VAD-fmk, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone; HA14-1, ethyl 2-amino-6-bromo-4-(1-cyano-2-ethoxy-2-oxo-ethyl)-4*H*-chromene-3-carboxylate; Smac/DIABLO, second mitochondria-derived activator of caspases/direct IAP-binding protein with low pI; Ac-DEVD-CHO, *N*-acetyl-Asp-Glu-Val-Asp-aldehyde; MPT, mitochondrial permeability transition; MBB, monobromobimane; DCFH, 2′,7′-dichlorofluorescein diacetate.

and complete remission is rare (Vuky and Motzer, 2000). Thus, novel therapeutic strategies are needed to improve the efficacy in treating RCC.

The naphthoquinolinedione compound LY83583 has been described as a guanylate cyclase (GC) inhibitor (Beasley et al., 1991). LY83583 competitively inhibits soluble GC, lowering the production of cyclic GMP in a wide range of tissues (Schmidt et al., 1985; O'Donnell and Owen, 1986) with the formation of reactive oxygen species (ROS) intermediates (Lee and Wurster, 1995). LY83583-induced ROS formation can be either enzymatic- or nonenzymatic-dependent (Hasegawa et al., 2004). Increased GC activity with a corresponding increase in cGMP has previously been reported in renal tumors (Braughler et al., 1982). Furthermore, a role for this important second messenger has been implicated in cellular proliferation, differentiation, and apoptosis (Chiche et al., 1998). This, together with the fact that ROS formation may be pro- or antitumorigenic (Velarde et al., 2004; Gumpricht et al., 2005), prompted us to investigate a possible role for LY83583 as a therapeutic drug for RCC.

The mitogen-activated protein kinase (MAPK) pathway is a key integration point along the signal transduction cascade that links diverse extracellular stimuli to proliferation, differentiation, and apoptosis (Cobb, 1999). Members of the MAPK cascade include the extracellular signal-regulated kinases (ERKs, of which there are now five family members), multiple c-Jun NH_2-terminal kinase (JNK), and the p38 isoforms (Cobb, 1999). In general, the ERK pathway is associated with cellular proliferation, differentiation, and survival, whereas the JNK and p38 pathways are generally, but not exclusively, associated with inflammation, apoptosis, and cellular differentiation. We have focused on the MAPK cascade in our investigation because of the reported constitutive activation of the ERKs in human malignancies including RCC (Oka et al., 1995; Barry et al., 2001); this factor is attributed to the almost complete failure of systemic drug therapy regimes for RCC (Motzer et al., 1996). In addition, numerous reports have linked alterations in MAPKs to oxidative stress (Dong et al., 2004; Kamata et al., 2005). Thus, we have looked at the possibility that LY83583 may play a role in MAPK signaling in RCC, transcription factor activation, as well as downstream gene regulation.

Using complementary genetic and pharmacological approaches, we have now explored the possible therapeutic potential of LY83583 for the treatment of RCC. We have focused our attention on elucidating the signaling pathways involved in LY83583-induced RCC apoptosis. Inhibition of RCC cell growth was found to be dependent on ERK1/2 and MEK1/2 but did not involve JNK activation. In contrast, however, although p38α and -β do not seem to be involved, disparate roles for p38γ and -δ are indicated. In addition, the dephosphorylation of ERK1/2 correlated with Elk-1 dephosphorylation and COX-2 down-regulation. Furthermore, these events are associated with changes in the expression of the Bcl-2 family members Bcl-x_L and Bax. Together these important findings suggest that LY83583 warrants attention in RCC treatment as well as perhaps other malignancies as a novel therapeutic approach.

Materials and Methods

Reagents. EDTA, EGTA, leupeptin, aprotinin, sodium orthovanadate, sodium fluoride, RPMI 1640 medium, fetal calf serum (FCS), antibiotics, anti-FLAG M2 mAb, 1′,2′,3′-dihydrorhodamine (DHR), HA 14-1 and monobromobimane (MBB) were purchased from Sigma (Dublin, Ireland). 2′,7′-Dichlorofluorescein diacetate (DCFH) was purchased from Invitrogen (Paisley, UK). LY83583, 8-bromo-cGMP; zaprinast; 8-guanosine-3′,5′-cyclic monophosphorothioate, *R*p-isomer; z-VAD-fmk; Ac-DEVD-CHO; and the Bax channel blocker were purchased from Calbiochem (Nottingham, UK). Cytochrome *c* mAb was purchased from BD Pharmingen (San Diego, CA). Phosphorus p42/p44 MAPK, ERK5, MKK3/MKK6, Akt (Ser473), Akt (Thr308), MEK1/2, JNK, p38 MAPK, Elk-1 antibody kits as well as Bcl-x_L, Bcl-2, BAD, and Bax were purchased from New England Biolabs (Beverly, MA). Rabbit polyclonal antibody p53 and mAb apoptosis inducing factor (AIF) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse mAb p38α and -δ were purchased from BD Transduction Laboratories (Oxford, UK), rabbit polyclonal p38γ antibody from Upstate (Lake Placid, NY) and mouse mAb p38β antibody from Zymed Laboratories Inc. (South San Francisco, CA). COX-1 and -2 mAb were purchased from Cayman Chemicals (Ann Arbor, MI). si*GENOME SMART*pool AIF siRNA and si*CONTROL* RISC-Free siRNA were purchased from Dharmacon RNA Technologies (Lafayette, CO).

Cell Culture. The RCC cell line 786-0 purchased from the American Type Culture Collection (Manassas, VA) was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin. Cells were washed twice with RPMI 1640 medium, and 3×10^4 cells/well were plated into six-well tissue culture plates. After 24 h, cells were cultured in medium supplemented with antibiotics and 0.5% FCS for all experiments performed unless otherwise stated. Normal human renal epithelial cells (HRE) purchased from Cambrex Bio Science Wokingham, Ltd. (Wokingham, Berkshire, UK) were grown according to the manufacturer's instructions. In brief, cells were grown in renal epithelial cell growth medium (BulletKit) that contained renal epithelial cell basal medium and a range of supplements. Cells were plated in six-well tissue culture plates as described above.

Proliferation-Cytotoxicity Assay. Cells were plated at a density of 2×10^4 cells/well into a six-well tissue culture plate. Twenty-four hours later cells were treated with various concentrations of LY83583, including 0.03, 0.1, 0.3, and 1 μM and after 24, 48, 72, and 96 h, the cell viability was assessed by trypan blue [0.4% (w/v)] exclusion assay.

MTT Assay. To determine cell viability, MTT (5 μg/ml) was added to each well of a six well plate and the reduction of MTT was assayed to calculate viable cell numbers as described previously (Barry et al., 2001).

Immunoblot Analysis. 786-0 cells were cultured in RPMI 1640 medium supplemented with antibiotics and 0.5% FCS. Cells were lysed in ice-cold lysis buffer as described previously (Barry et al., 2001). Supernatants were used for immunoblotting with specific antibodies for the phosphorylated or total p42/p44 MAPK, MEK1/2, ERK5, JNK1/2, p38, MKK3/MKK6, or Elk-1 using the experimental conditions described by the manufacturer (New England Biolabs). Likewise, membranes were incubated with antibodies for p53 (Santa Cruz Biotechnology Inc.), Bcl-2, Bcl-x_L, BAD, and Bax (New England Biolabs), cytochrome *c* (BD Pharmingen), caspase–3 (BD Transduction Laboratories, Oxford, UK), or COX-1 and -2 (Cayman Chemicals, Ann Arbor, MI). Chemiluminescent detection was performed using the SuperSignal immunodetection system (Pierce Chemical, Dublin, Ireland) to reveal positive bands that were visualized after exposure to Hyperfilm ECL (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Stable Transfection of Cells. The generation of stable 786-0 cell lines expressing FLAG-tagged wild type (wt) and dominant-negative (DN) forms of human p38α, -β, -δ, and -γ have been described previously (Pramanik et al., 2003). These plasmids were a kind gift from Professor J. Han (Scripps Research Institute, La Jolla, CA). The constitutively active MEK1 EE plasmid was a kind gift from Profes-

sor M. G. Kazanietz (University of Pennsylvania, Philadelphia, PA). In brief, electroporations were performed using a rectangle pulse EPI 2500 electroporator (Fischer, Heidelberg, Germany). A 100-μl aliquot of 786-0 cells (2×10^6) was mixed with 10 μg of DNA in a 0.2-mm gap cuvette (eight 8-ms pulses at 330 V with 1-s intervals). After a 15-min recovery period at room temperature (RT), cells were diluted 20-fold in culture medium and incubated at 37°C and 5% CO_2. Using this protocol, we routinely observed less than 10% of dead cells after electroporation. Forty-eight to 72 h after the addition of DNA, cells were washed twice in phosphate-buffered saline and split 1:3 into 35-mm diameter dishes. The transfected cells were selected in growth medium containing 600 μg/ml G418 (Geneticin; Invitrogen). After 4 to 8 weeks, individual cell colonies were transferred for clone expansion and maintained in culture medium supplemented with 600 μg/ml G418. Protein expression levels were determined by Western blot analysis of G418-selected cell foci using anti-FLAG M2 mAb (Sigma, Dublin, Ireland). All blots were visualized with the procedure outlined in the enhanced chemiluminescence kit (GE Healthcare).

Measurement of Reactive Oxygen Species. Formation of ROS was detected using the redox sensitive dye DHR as described previously (Halliwell and Whiteman, 2004). This dye is lipophilic and readily diffuses across cell membranes, where it is oxidized to the green fluorescent rhodamine 123 (Halliwell and Whiteman, 2004). In brief, 786-0 cells (3×10^6 cells/ml) in six-well plates were preincubated in the dark with DHR (30 μM) for 30 min at 37°C and subsequently treated with LY83583 (1 μM). In the absence of drug, vehicle (0.1% DMSO) was used. Fluorescence was measured using the TECAN GENios microplate reader at λ 485 nm and λ 535 nm excitation and emission, respectively.

Mitochondrial and Cytosolic Extraction. Treated and untreated 786-0 cells (6×10^7) were harvested by centrifugation at 850*g* for 2 min at RT. Mitochondrial and cytosolic extraction was carried out using the Mitochondrial Isolation Kit (Pierce Chemical) according to manufacturer's instructions.

Immunofluorescence. AIF detection was carried out by immunofluorescence. In brief, nontransfected 786-0 cells or 786-0 cells transfected with empty vector (pcDNA3), p38δ wt, p38δ DN, p38γ wt, or p38γ DN (3×10^4) were grown on coverslips and were treated or not with LY83583 (1 μM) for 96 h in the presence or absence of *N*-acetyl L-cysteine (NAC), z-VAD-fmk, or calpeptin. Cells were then washed with PBS, fixed with 1.5% paraformaldehyde for 30 min at RT, and permeabilized with 2 ml of 0.2% Triton X-100. Nonspecific binding was blocked using 10 mg/ml bovine serum albumin in PBS for 15 min followed by incubation with anti-AIF mAb (1:100) diluted in blocking buffer for 1 h at 37°C. After addition of secondary anti-mouse FITC-conjugated IgG (1:100), slides were treated with anti-Fade mounting media (Dako UK Ltd., Ely, Cambridgeshire, UK), and cells were visualized under a fluorescence microscope.

Flow Cytometry. Apoptotic cells were identified by double-supravital staining with recombinant FITC-conjugated annexin V and propidium iodide (PI) as described previously (Bowden et al., 2002). The annexin V-FITC apoptosis detection kit (BD Pharmingen) was used to visualize DNA according to the manufacturer's instructions. In brief, after treatment with LY83583 (1 μM) or U0126 (20 μM), floating and attached cells were pooled and washed twice with 4°C PBS. HRE and 786-0 cells (1×10^5) were incubated with 5 μl of Annexin V-FITC and 5 μl of PI in the dark for 45 min at RT. Cells were analyzed on a FACScalibur (BD Biosciences, San Jose, CA). The fraction of cells in each quadrant was calculated using CellQuest software (BD Biosciences).

Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling. TUNEL of fragmented DNA was performed according to the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany).

AIF siRNA. si*GENOME SMART*pool AIF siRNA and si*CONTROL* RISC-Free siRNA were purchased from Dharmacon, Inc. In brief, 786-0 (3×10^6) cells were seeded in six-well plates in RPMI 1640 medium containing 10% FCS and without antibiotics. Transfections using Dharma*FECT* 4 reagent and *SMART*pool AIF siRNA (100 nM) or si*CONTROL* RISC-Free siRNA (100 nM) were performed 24 h later according to the manufacturer's instructions. The optimal time for protein/RNA depletion after transfection was determined. 786-0 cells were treated 48 h after transfection with LY83583 (1 μM) for 48 h.

Statistical Analysis. Results are expressed as mean ± S.E. Statistical comparisons were made by using analysis of variance with subsequent application of Student's *t* test, as appropriate.

Results

LY83583 Induced a Time- and Dose-Dependent Cell Growth Inhibition in 786-0 but Not HRE Cells. To determine whether LY83583 inhibits 786-0 RCC proliferation, cells were treated with or without LY83583 (0.03, 0.1, 0.3, and 1 μM) for 0, 24, 48, 72, and 96 h. Control cells were treated with 0.1% DMSO alone. 786-0 cells demonstrated a time- and dose-dependent decrease in cell number (Fig. 1A). Similar results were also obtained when two other RCC cell lines, ACHN and Caki-1, were treated with LY83583 (data not shown). Normal HRE cells were also treated with LY83583 and counted. These cells, however, proved refractory to LY83583 treatment even at 96 h (Fig. 1B).

LY83583 Induced a Time-Dependent Increase in 786-0 Cell Apoptosis but Not HRE Cells. A previous report (Lodygin et al., 2002) identified cellular senescence of human fibroblasts upon LY83583 treatment. Using β-galactosidase staining, we failed to observe 786-0 cellular senescence in the presence of this drug (Lodygin et al., 2002; data not shown). We did observe, however, a time-dependent in-

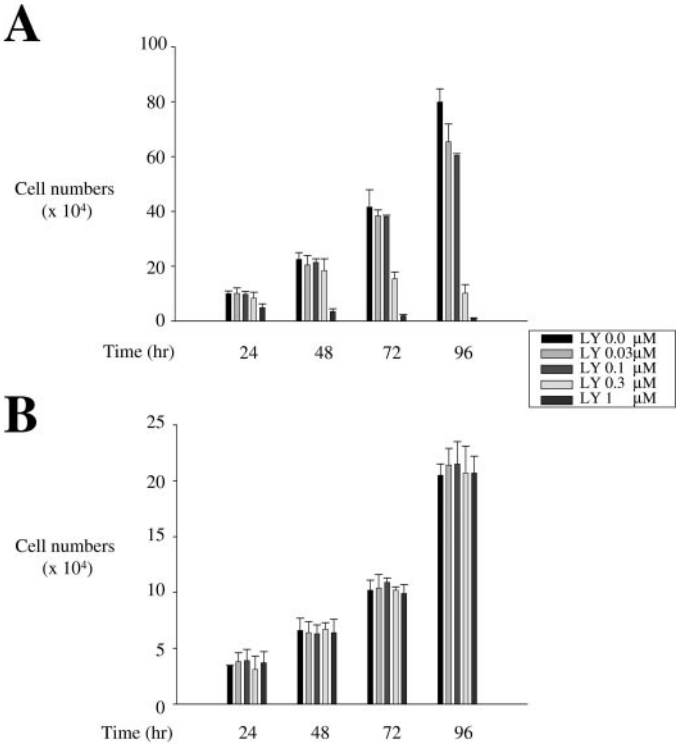

Fig. 1. LY83583 induces a time- and dose-dependent inhibition of RCC 786-0 cell proliferation but not normal human renal epithelial cells (HRE). 786-0 (A) and HRE (B) cells (2×10^4) were treated with LY83583 (0.03, 0.1, 0.3, and 1 μM) for 24 to 96 h and counted. When cells were untreated vehicle alone (0.1% DMSO) was added. The results shown are mean ± S.E. of four independent experiments.

crease in 786-0 cell apoptosis (Fig. 2). Fluorescein-conjugated annexin V and PI staining (detected by flow cytometry) were used as criteria for distinguishing cycling, early apoptotic, and late apoptotic cells. Quadrants I, II, III, and IV in Fig. 2, A and C, represent different cell groups: area I represents unlabeled viable cells (Ann-V^-/PI^-), area II contains early apoptotic cells (Ann-V^+/PI^-), area III indicates late apoptotic cells (Ann-V^+/PI^+), and area IV represents cells destroyed during experimental workup as described previously (Bowden et al., 2002). Control (0.1% DMSO-treated) 786-0 cells demonstrated 87.2, 8.2, 3.6, and 1.0% for areas I, II, III, and IV, respectively. However, after treatment with LY83583 (1 μM), there was a dramatic time-dependent increase in the number of both early and late apoptotic cells (Fig. 2A). Similar apoptotic results were obtained with ACHN and Caki-1 upon treatment with LY83583 (data not shown). TUNEL analysis was also performed to confirm the effects of LY83583 on 786-0 cell death. Consistent with the flow cytometry data control (0.1% DMSO-treated) 786-0 cells demonstrated no positive TUNEL staining (Fig. 2B). Cells treated with LY83583 (1 μM), however, demonstrated a time-dependent increase in TUNEL staining (Fig. 2B). Changes in cell morphology, such as cell cytoplasm destruction and chromatin condensation, were evident upon LY83583 treatment (Fig. 2B). By 96 h, the cell cytoplasm had been destroyed (Fig. 2B). In contrast, however, HRE cells demonstrated no increase in apoptosis as measured by either flow cytometry (Fig. 2C) or TUNEL staining (Fig. 2D) upon addition of LY83583 (1 μM).

LY83583-Induced 786-0 Apoptosis Is ROS-Dependent but GC-Independent. Previous reports have demonstrated that LY83583 competitively inhibits GC, lowering intracellular levels of cGMP (Schmidt et al., 1985; O'Donnell and Owen, 1986) with the formation of ROS intermediates (Lee and Wurster, 1995). Therefore, we decided to focus our next set of experiments on investigating the mechanism(s) of action of LY83583-induced 786-0 apoptosis. Again, using flow cytometric analysis of annexin V/PI staining as described above, 786-0 cells were treated or not with LY83583 (1 μM) in the presence or absence of two different cGMP-dependent protein kinase activators 8-bromo-cGMP (8-Br-cGMP) and 8-(4-chlorophenylthio)-cGMP. Both were added 1 h before treatment with LY83583. In addition, a previous report (O'Donnell and Owen, 1986) outlined how 8-Br-cGMP was insufficient to block the actions of LY83583 unless a phosphodiesterase inhibitor was also added. Thus, in addition to treating cells with 8-Br-cGMP, we also added the specific phosphodiesterase inhibitor zaprinast. As observed above (Fig. 2A), LY83583 induced a time-dependent decrease in cell viability (Table 1A). We did not see a change in cell viability when cells were pretreated with 8-Br-cGMP and zaprinast (1 mM and 20 μM, respectively, for 24–96 h) before the addition of LY83583 (Table 1). Likewise, 8-(4-chlorophenylthio)-cGMP (250 μM, 24–96 h) failed to reverse the apoptotic response (data not shown). To further strengthen the absence of a role for GC in the observed apoptotic response, cells were also treated with a potent and selective sGC inhibitor—1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one. Again, this specific inhibitor failed to alter the observed apoptotic response (Table 1). Thus, the apoptotic effect of LY83583 on RCC was not due to the inhibition of GC. Thus, we investigated whether ROS scavengers could reverse the apoptotic effect observed

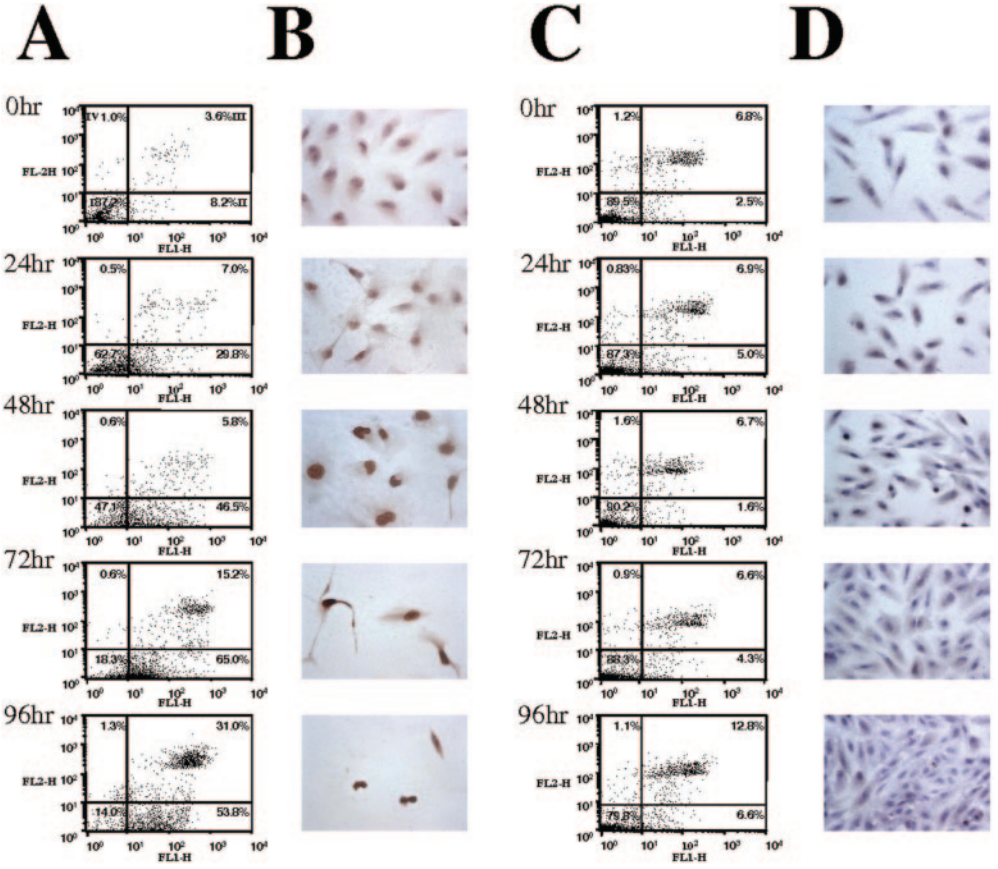

Fig. 2. LY83538 induces a time-dependent increase in apoptosis in 786-0 but not HRE cells. After treatment of 786-0 (A and B) and HRE (C and D) cells with LY83538 (1 μM, 24–96 h), apoptotic and nonapoptotic cells were detected either by flow cytometry after staining with FITC-conjugated Annexin V and PI (A and C) or by TUNEL analysis (B and D) as described under *Materials and Methods*. Ten thousand cells were counted by FACScalibur analysis to determine the distribution of cells (A and C). Quadrants I, II, III, and IV represent different cell groups; I represents unlabeled viable cells (Ann-V^-/PI^-), II represents early apoptotic cells (Ann-V^+/PI^-), III represents late apoptotic cells (Ann-V^+/PI^+), and IV represents cells destroyed during experimental workup (A and C). The figures are representative of five independent experiments.

in RCC. Cells were treated with NAC (10 mM) and LY83583 (1 μM). The decrease in cell viability was reversed at all time points (Table 1D). Likewise, treatment of 786-0 cells with four other different antioxidants—vitamins C (200 μM) and E (50 μM), N^{ω}-nitro-L-arginine methyl ester (1 mM), and melatonin (150 μM)—attenuated LY83583 (1 μM)-mediated lethality (Table 1).

LY83583 Stimulates a Dramatic Increase in ROS Production in 786-0 Cells. After the inhibitory effects of a range of antioxidants on LY83583-induced apoptosis as described above, we next turned our attention to ROS formation, employing the fluorescent dye DHR. DHR is a probe widely used to detect several reactive species (Halliwell and Whiteman, 2004). It is oxidized to rhodamine 123, which is highly lipophilic and positively charged and accumulates in mitochondria, held there by the membrane potential (Halliwell and Whiteman, 2004). We observed a time-dependent increase in ROS formation after LY83583 (1 μM) addition (Fig. 3). A significant increase in ROS formation was observed as early as 1 min after addition of LY83583 (1 μM) to 786-0 cells. This is in agreement with a previous report where LY83583-induced ROS formation in PC-12 cells was observed by electron paramagnetic resonance analysis after a 2-min incubation period (Hasegawa et al., 2004). A similar increase in ROS production was detected using a second fluorescent probe DCFH (data not shown). There was also a time-dependent increase in ROS formation upon treatment of ACHN and Caki-1 with LY83583 (1 μM) (data not shown).

Effect of LY83583 and ROS Formation on MAPK Expression. Members of the MAPK cascade have been implicated in both pro- and antiapoptotic effects (Cobb, 1999). In addition, oxidative stress is known to alter MAPK cascades (Dong et al., 2004; Kamata et al., 2005). Thus, we investigated the effect(s) of LY83583 on the different MAPKs using the phosphorylation of ERK1/2, ERK5, MEK1/2, JNK, p38, and MKK3/6 as an index of kinase activation (Barry et al., 2001). Constitutive activation of ERKs in human malignancies has been documented previously (Oka et al., 1995; Barry et al., 2001). We now report that 786-0 cells also demonstrated constitutive ERK1/2 activation (Fig. 4A). However, upon addition of LY83583, we observed dephosphorylation of ERK1/2 as early as 5 min, with complete loss of phosphorylation at 30 min (Fig. 4A). Total MAPK levels were not changed, as judged with a MAPK antibody that recognized both the phosphorylated and unphosphorylated forms of the enzyme (Barry et al., 2001). We also observed ERK1/2 inactivation when ACHN and Caki-1 were treated with LY83583 (1 μM) (data not shown). Because MEK is the upstream activator of ERKs (Oka et al., 1995), we addressed the role of MEK1/2. When 786-0 cells were treated with LY83583 (1 μM), we observed a decrease in MEK1/2 phosphorylation before ERK1/2 dephosphorylation (Fig. 4A). Another member of the mammalian MAPK pathway, ERK5 (also known as BMK1) is known to be activated by oxidant stress and has a role in many physiological processes, including carcinogenesis (Wang and Tournier, 2006). Unlike ERK1/2, we observed no constitutive ERK5 phosphorylation in 786-0 cells (Fig. 4A). Furthermore, we did not observe an increase in ERK5 phosphorylation when 786-0 cells were treated with LY83583 (1 μM) (Fig. 4A). Addition of sorbitol (20 μM) was used as a positive control to ensure phospho-ERK5 antibody functionality. The central role of the PI3K-Akt pathway in renal cancer cell survival prompted us to investigate whether LY83583-induced apoptosis involves any changes in this signaling pathway. Treatment of 786-0 cells with LY83583 (1 μM) leads to a rapid dephosphorylation of Akt as revealed by Western blotting using a phosphospecific anti-Akt antibody (phospho-Thr308) (Fig. 4A). We observed no Akt phosphory-

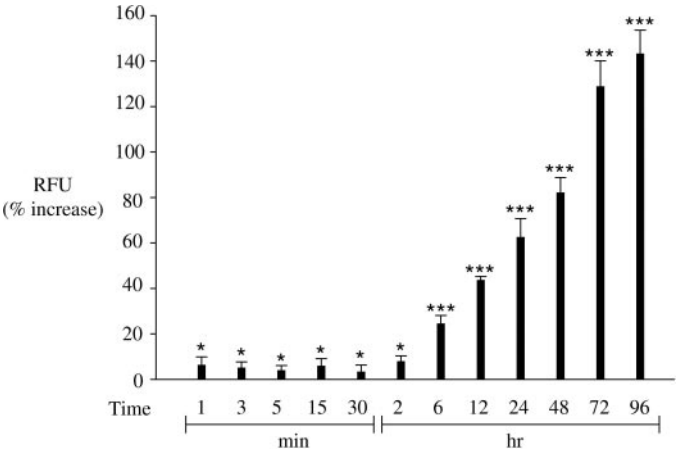

Fig. 3. Exposure of 786-0 cells to LY83583 leads to a dramatic increase in ROS production. 786-0 cells were preincubated with DHR (30 μM) for 30 min and, thereafter, stimulated with LY83583 (1 μM). Results are expressed as percentage increase in relative fluorescence units compared with control (0.1% DMSO) treated 786-0 cells. Significant (***, $p < 0.001$; *, $p < 0.05$) changes from control (0.1% DMSO) treated 786-0 cells. Data represent the mean ± S.E. of five independent experiments.

TABLE 1

Time-dependent decrease in 786−0 cell viability following LY83583 treatment is ROS-dependent but guanylate cyclase-independent

Flow cytometric analysis was performed following staining with FITC-conjugated Annexin V and PI and gated onto a fluorescent dot plot. Percentage of control (0.1% DMSO-treated) 786-0 cells are represented as viable cells (quadrant I). Cells were treated with LY83583 (1 μM) or pretreated with 8-Br-cGMP (1 mM) and zaprinast (20 μM), ODQ (20 μM), NAC (10 mM), vitamin C (200 μM), vitamin E (50 μM), L-NAME (1 mM), or melatonin (150 μM) for 1 h followed by treatment with LY83583 (1 μM) for 24–96 h. Data represent the mean ± S.E. of five independent experiments.

	Cell Viability				
	0 h	24 h	48 h	72 h	96 h
			%		
LY83583 (1 μM)	90 ± 5	65 ± 6	45 ± 10	16 ± 6	10 ± 10
8-Br-cGMP (1 mM) + zaprinast (20 μM)	95 ± 5	61 ± 7	38 ± 10	8 ± 8	3 ± 5
ODQ (20 μM)	91 ± 9	69 ± 4	40 ± 5	10 ± 6	4 ± 5
NAC (10 mM)	87 ± 5	81 ± 4	80 ± 13	89 ± 8	83 ± 7
Vitamin C (200 μM)	98 ± 6	94 ± 5	91 ± 10	89 ± 5	90 ± 3
Vitamin E (50 μM)	89 ± 5	93 ± 4	95 ± 3	91 ± 8	93 ± 9
L-NAME (1 mM)	87 ± 5	81 ± 4	88 ± 7	95 ± 8	93 ± 3
Melatonin (150 μM)	97 ± 4	91 ± 6	90 ± 3	94 ± 6	93 ± 7

ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; L-NAME, N^{ω}-nitro-L-arginine methyl ester.

lation on Ser473 in 786-0 cells (data not shown). To address the question of whether other members of the MAPK cascade could also be involved in LY83583-induced 786-0 apoptosis, we examined the phosphorylation (activation) of both JNK and p38 MAPK. Unlike ERK1/2, 786-0 cells do not express constitutive JNK and p38 MAPK activation (Fig. 4A). Upon treatment with LY83583 (1 μM), we surprisingly failed to see an increase in either JNK or p38 MAPK phosphorylation (Fig. 4A), two putative MAPKs previously linked to apoptosis (Cobb, 1999). Cells were treated with anisomycin (1 μg/ml), a JNK activator, and sorbitol (20 μM), a p38 MAPK activator, as positive controls to ensure antibody functionality (Fig. 4A). Furthermore, using antibodies against MKK3 and MKK6, we investigated whether the pan-p38 pathway MAPKKs are activated by LY83583. We observed no increase in MKK3 or MKK6 phosphorylation upon treatment with LY83583 (1 μM) for all time points studied (Fig. 4A). Sorbitol (20 μM)-treated cells were again used to ensure antibody functionality.

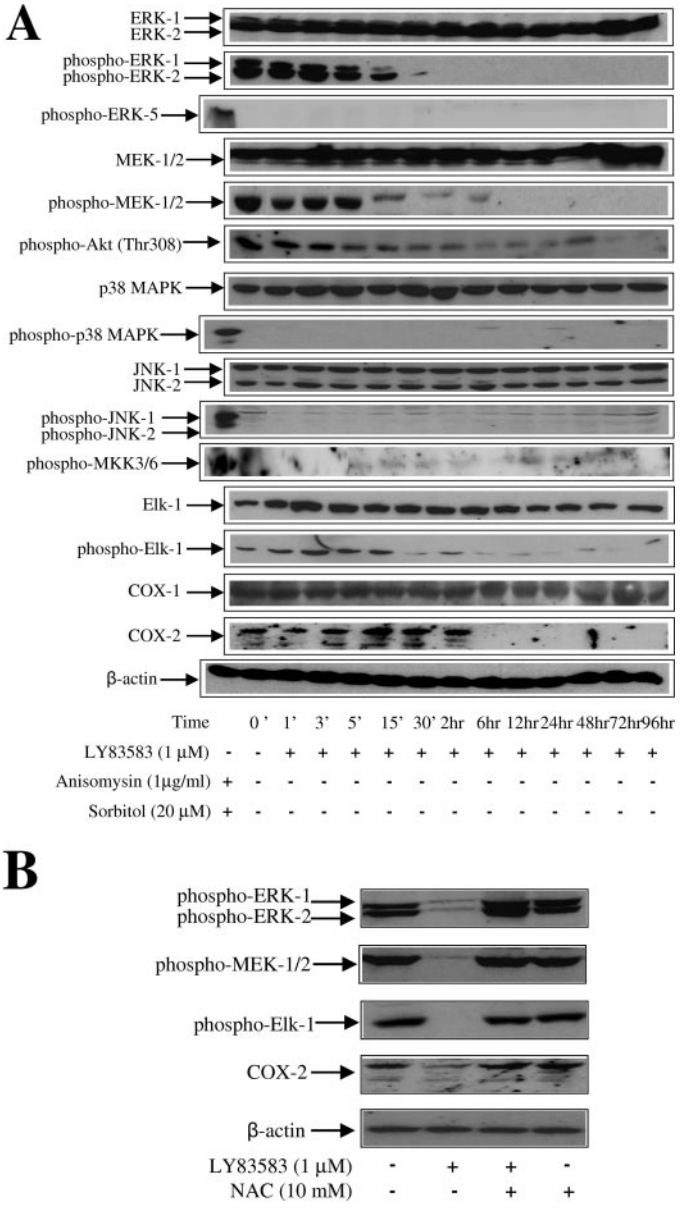

Fig. 4. Effect of LY83583 on 786-0 MAPK, Elk-1, and COX-2 expression is ROS-dependent. 786-0 cells were treated with and without LY83583 (1 μM) for the indicated times (A). Aliquots of 786-0 cells containing 30 μg of protein lysate were loaded on a 10% SDS-PAGE gel and analyzed by immunoblot using total and phospho-anti-ERK1/2 (A and B) and -5 (A), anti-MEK1/2 (A and B), anti-Akt (A), anti-p38α/β (A), anti-JNK1/2 (A), anti-MKK3/MKK6, and anti-Elk-1 (A and B) antibodies. Treatment of cells with anisomycin (1 μg/ml) was included as a positive control for phosphorylated JNK1/2, or sorbitol (20 μM) for phosphorylated-p38α/β MAPK, MKK3/MKK6 and ERK5 (A). Forty micrograms of protein lysate was used for analysis of COX-2 and 20 μg for COX-1, and 7% and 3% was used, respectively, for the separating and stacking gels (A and B). Cells were or were not treated with NAC (10 mM) and LY83583 (1 μM) for 96 h (B). All membranes were probed with β-actin as a loading control. The figures are representative of four independent experiments.

To further evaluate the effect of LY83583 on 786-0-induced apoptosis, we investigated its effect on both the transcription factor Elk-1 and its downstream target COX-2. Previous reports have documented that COX-2 is overexpressed in RCC and plays a role in the tumorigenesis of these cells (Chen et al., 2004). Thus, COX-2 may be a therapeutic target for the treatment of RCC. We observed that 786-0 cells also express COX-2 as well as constitutive Elk-1 phosphorylation (Fig. 4A). Upon treatment of 786-0 cells with LY83583 (1 μM), we observed a decrease in Elk-1 phosphorylation as early as 30 min, consistent with the inactivation of upstream ERK1/2. We were not surprised to also observe a decrease in COX-2 expression after Elk-1 dephosphorylation (Fig. 4A). No changes in COX-1 expression were observed upon addition of LY83583 (1 μM) (Fig. 4A).

Because we observed that ROS scavengers could null the LY83583-induced apoptotic effect, we investigated whether they could also have an effect on the MAPK signaling cascade. 786-0 cells were treated with NAC (10 mM) alone or NAC (10 mM) and LY83583 (1 μM). NAC (10 mM) alone does not quench ERK1/2 phosphorylation, as has been observed in some tumor cell lines. It did, however, prevent the dephosphorylation of ERK1/2, MEK1/2, and the transcription factor Elk-1 as well as the down-regulation of COX-2 expression after LY83583 (1 μM) treatment at all time points studied (i.e., 5, 15, and 30 min and 2 to 96 h; only the 96 h time point is shown) (Fig. 4B).

LY83583-Induced 786-0 Cell Death Rescued by p38δ and p38γ DN Is ROS-Dependent. The involvement of p38 MAPK in apoptosis is well documented (Cobb, 1999). In addition, previous studies have shown that ROS formation can activate p38 MAPK, depending on the cell type (Adler et al., 1999). We were surprised to observe no increase in p38 MAPK phosphorylation (activation) in LY83583-induced 786-0 apoptosis (Fig. 4A). The p38 MAPK antibodies (New England Biolabs) used in Fig. 4A, however, identify only the phosphorylation of p38α and -β and lack specificity in terms of identifying the different p38 isoforms. Therefore, to investigate the role(s) of p38 MAPK in more detail, we employed the use of antibodies specific for each of the p38 isoforms (Yang et al., 1999; Ho et al., 2004; Kuma et al., 2004). We observed that the RCC cell line 786-0 expresses the p38 isoforms- α, -β, and -γ, but not δ (Fig. 5A). In contrast, normal HRE cells express all four isoforms (Fig. 5A). This prompts the question of whether the loss of p38δ may play a role in the tumorigenicity of RCC. To address this issue, we developed 786-0 stable cell lines expressing each of the p38 MAPK isoforms and their DN mutants, confirmed by Western blot analysis of the FLAG-tagged plasmids (Fig. 5B). All p38 MAPK plasmids have been described previously in detail (Pramanik et al., 2003). pcDNA3 (empty vector) was used as

a control. Because we failed to see p38α/β MAPK phosphorylation above (Fig. 4A), it was not surprising that 786-0 cells stably transfected with p38α, p38β, or their DN mutants displayed a similar reduction in cell numbers and apoptosis compared with nontransfected cells in the presence of LY83583 (1 μM) (Fig. 5, C and D). We were surprised, however, to observe that reintroduction of p38δ could abrogate the apoptotic effect of LY83583 (Fig. 5, C and D). As expected, cells expressing the p38δ DN isoform behaved as nontransfected cells in the presence of LY83583 (because 786-0 cells do not express p38δ) (Fig. 5, C and D). In addition we observed that overexpression of the p38γ DN mutant also res-

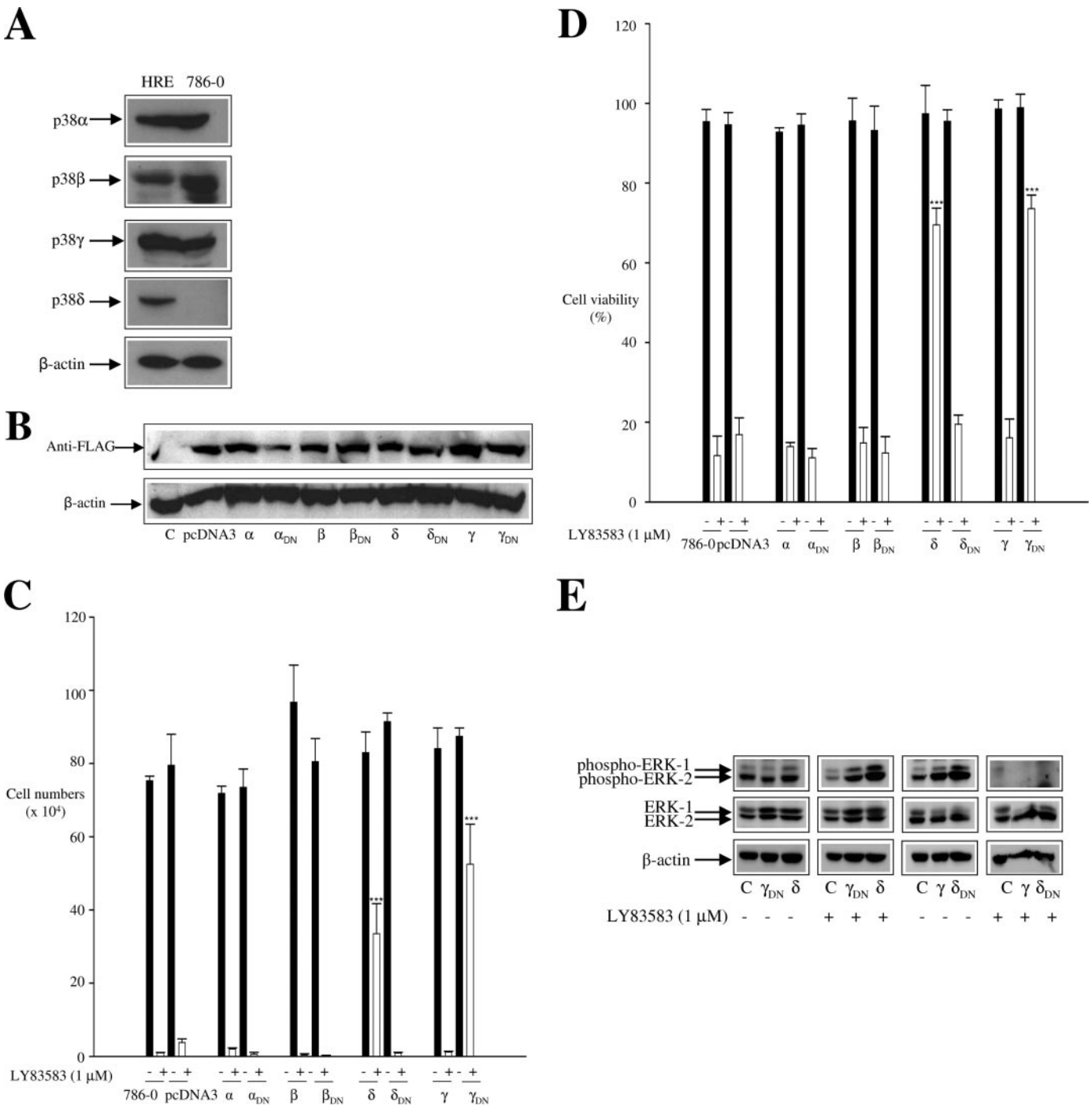

Fig. 5. p38δ and p38γ DN rescue LY83583-mediated 786-0 apoptosis in a ROS-dependent manner. Western blot analysis of p38 MAPK isoform expression in HRE and 786-0 cells (A). 786-0 cells were or were not (control C) stably transfected with pcDNA3 (empty vector), p38 MAPK wt isoforms (α, -β, -γ, and -δ), and p38 MAPK dominant-negative (DN) isoforms. Sixty micrograms of protein was probed with anti-FLAG M2 mAb, which recognizes the FLAG tag on the pcDNA3 empty vector and p38 constructs (B). Transfected and nontransfected cells were or were not treated with LY83583 (1 μM, 96 h) (C and D). Cells were counted (C) and viable cells were detected by flow cytometry after staining with FITC-conjugated Annexin V and PI (D) as described under *Materials and Methods*. Significant (***, $p < 0.001$) changes from nontransfected 786-0 cells treated with LY83583 (1 μM) (C and D). Western blot analysis of ERK1/2 and phospho-ERK1/2 in p38δ, p38δ DN, p38γ, and p38γ DN transfected and nontransfected cells in the presence or absence of LY83583 (1 μM, 96 h) (E). Membranes were probed with β-actin as a loading control (A, B, and E). The figures are representative of four independent experiments.

cued the cells from LY83583-induced apoptosis (Fig. 5, C and D). Cells overexpressing the p38γ isoform displayed a similar reduction in cell numbers and apoptosis compared with nontransfected cells in the presence of LY83583 (Fig. 5, C and D).

To further investigate the possible mechanism(s) involved in the role of p38δ and p38γ DN in reversing LY83583-induced apoptosis, we examined whether there could possibly be cross-talk between the different MAPK pathways. We examined whether the mutant cells could alter the constitutive ERK1/2 activation observed in RCC. Although RCC cells overexpressing p38δ and p38γ DN did not display an altered pattern of ERK1/2 phosphorylation, we did observe that treatment of these cells with LY83583 (1 μM) did not result in ERK1/2 dephosphorylation (inactivation) (Fig. 5E). This is in contrast to p38δ DN or p38γ wt (used as controls in this experiment), which display complete loss of ERK1/2 dephosphorylation after LY83583 (1 μM) treatment (Fig. 5E). Because we did not observe MKK3 or MKK6 phsophorylation when nontransfected 786-0 cells (Fig. 4A) or transfected 786-0δ or 786-0γ DN were treated with LY83583 (1 μM) (data not shown), then it is probable that p38δ and p38γ are altering ERK1/2 function through mass action effects. In summary, we can conclude that p38δ and p38γ have opposing roles in LY83583-induced 786-0 apoptosis. Although p38γ seems to confer sensitivity of 786-0 cells to LY83583-induced apoptosis, p38δ confers resistance.

U0126 Mimics the Effect of LY83583. To correlate the above data with the mechanisms of RCC-induced apoptosis, we investigated whether MEK1/2 inactivation can bring about the observed effects of LY83583-induced apoptosis. Using a specific and potent MEK1/2 inhibitor, U0126, we observed a time-dependent decrease in 786-0 cell viability (Fig. 6). This is in agreement with a previous report using colorectal cancer cell lines with constitutively active MEK/MAPK, where cell death by U0126 was found only in p53 wild-type cells, as is the case with 786-0 cells (Wang et al., 2004). We also examined the effect of U0126 on cells overexpressing p38δ and p38γDN. As we observed for LY83583, we again observed a reduction in U0126-induced loss of cell viability upon treatment with U0126, albeit not completely. This is obvious at 72 and 96 h (Fig. 6). In contrast to inactivating MEK (using U0126 and observing a loss in cell viability), stable 786-0 cells expressing active MEK (kindly donated by Prof. M. G. Kazanietz, University of Pennsylvania) mimicked the effect of p38δ and p38γ DN. No loss in cell viability was observed when 786-0 MEK cells were treated with LY83583 (1 μM) (Fig. 6).

Effect of LY83583 on Apoptotic-Related Gene Expression Is ROS-Dependent. In view of the role of the expression and subcellular localization of Bcl-2 family proteins in apoptosis regulation, expression of these proteins was investigated in LY83583-treated 786-0 cells. No major changes in expression was observed for Bcl-2 or BAD. We did, however, observe a decrease in the expression of the anti-apoptotic member Bcl-x_L and an increase in the expression of the pro-apoptotic member Bax after treatment with LY83583 (1 μM), albeit at the later time points of 48, 72, and 96 h (Fig. 7A). These changes in expression were abrogated in the presence of NAC (10 mM) (Fig. 7B). To investigate whether the observed effect of LY83538 on the down-regulation of Bcl-x_L expression is a secondary effect of caspase activation, cells were treated with the pan-caspase inhibitor z-VAD-fmk (50 μM). Because caspase inhibitors are unstable, the drug was resupplemented every 24 h. We observed no change in Bcl-x_L down-regulation or in Bax up-regulation in the presence of the caspase inhibitor (Fig. 7B). We also analyzed p53 wild-type expression but observed no change in its expression after LY83583 treatment (Fig. 7A). In agreement with a previous report, we did observe $p21^{waf1/cip1}$ up-regulation when 786-0 cells were treated with this drug, albeit in a p53-independent manner (data not shown) (Lodygin et al., 2002). To examine further the role(s) of Bcl-2 family members and Bax in the apoptotic response, we used two novel pharmacological inhibitors, HA 14-1 (An et al., 2004) and a Bax channel blocker (Bombrun et al., 2003), respectively. HA14-1 is a synthetic cell-permeable molecule that specifically competes with Bak BH3 domain-derived peptide in binding Bcl-2 family members (An et al., 2004). The Bax channel blocker (3,6-dibromocarbazole piperazine derivatives of 2-propanol) inhibits Bax channel-forming activity (Bombrun et al., 2003). Thus, if members of the antiapoptotic Bcl-2 family are involved in the apoptotic response, then addition of an inhibitor should further increase the apoptosis induced by LY83583. Conversely, if the pro-apoptotic Bax is involved, then the use of a Bax channel blocker should decrease the observed apoptosis. We used both inhibitors at concentrations that alone do not induce apoptosis, as reported previously (Bombrun et al., 2003). We observed a time-dependent increase and decrease, respectively, in the presence of HA-1 and the Bax channel blocker (Table 2). We did not, however, observe complete suppression of apoptosis in the presence of the latter

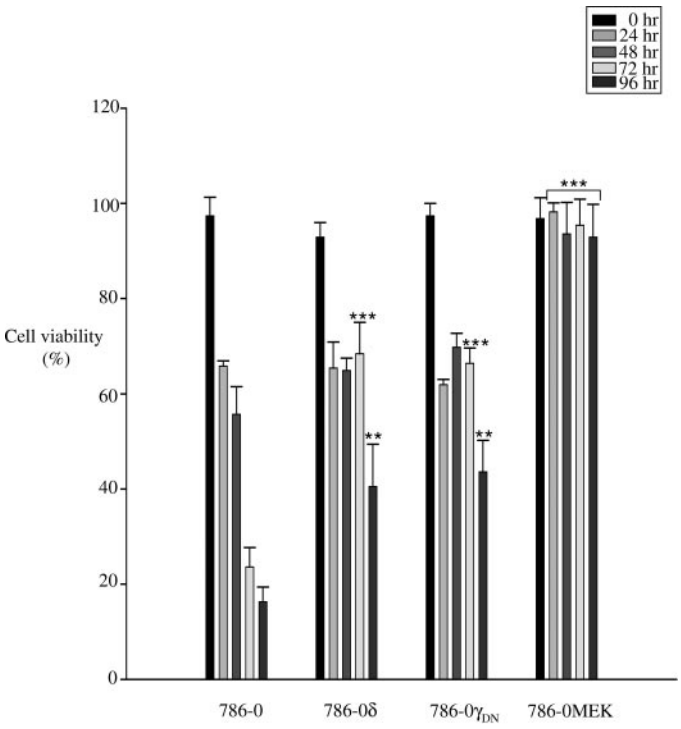

Fig. 6. The MEK inhibitor U0126 mimics the effect of LY83583 whereas active MEK opposes its effect. 786-0, 786-0δ, and 786-0γ DN cells were treated with and without U0126 (20 μM) for 24 to 96 h. 786-0 MEK cells were treated with and without LY83583 (1 μM) for 24–96 h. Cell viability was detected by flow cytometry after staining with FITC-conjugated Annexin V and PI as described in *Materials and Methods*. Significant (***, $p < 0.001$; **, $p < 0.01$) changes from nontransfected 786-0 cells treated with U0126 (20 μM). Data represent the mean ± S.E. of three independent experiments.

drug (Table 2). This is not surprising because Bax can bind to pre-existing mitochondrial membrane pores (i.e., the permeability transition pore), whereas the inhibitor is operational during Bax pore formation.

LY83583 Induced Cytochrome *c* Release, Caspase-3 Activation, and AIF Translocation in a ROS-Dependent Manner. Mitochondria play a crucial role in apoptosis by releasing apoptogenic factors. The proapoptotic family member Bax plays a role in the release of such factors as cytochrome *c*, AIF, and Smac/DIABLO. Because we observed an increase in Bax expression, we examined whether these apoptosis-associated factors could also play a role in LY83583-induced RCC apoptosis. Upon subcellular fractionation, we observed a release into the cytosol of cytochrome *c* after treatment with LY83583 (1 μM) (Fig. 8A). This translocation was evident at 24 h and continued to 96 h. Only results for 96 h are shown (Fig. 8A). This effect was blocked in the presence of NAC (10 mM) (Fig. 8A). We consistently did not observe Smac/DIABLO in the 786-0 cell line (data not shown). This is agreement with a recent report demonstrating down-regulation of Smac/DIABLO in RCC (Mizutani et al., 2005). Cytochrome *c* released into the cytosol forms part of the apoptosome, which leads ultimately to the activation of downstream procaspases such as procaspase-3. We observed processing of procaspase 3 by Western blotting with the formation of the p20 form of the large subunit after addition of LY83583 (Fig. 8B). This was ROS-dependent because addition of NAC (10 mM) prevented the cleavage of this caspase (Fig. 8B). To investigate the role of caspase-3 in 786-0-induced apoptosis, cells were treated with the pan-caspase inhibitor z-VAD-fmk (50 μM) and the specific caspase-3 inhibitor Ac-DEVD-CHO (50 μM). We observed that both inhibitors were able to prevent processing of procaspase-3 (data not shown) but surprisingly failed to abrogate LY83583-induced apoptotic cell death (Fig. 8C). Both caspase inhibitors have been shown to prevent 786-0-induced apoptosis (G. C. O'Sullivan and O. P. Barry, manuscript in preparation; data not shown). We decided not to look further at other caspases because the pan-caspase inhibitor was without effect (Fig. 8C). Thus, LY83583-induced 786-0 apoptotic cell death seems to occur in a caspase-independent fashion. Cellular apoptosis, however, can proceed without the involvement of caspases. LY83583-induced cell death may progress by an alternative route originating from mitochondrial permeability transition (MPT) mediated by oxidative changes in critical redox-sensing mitochondrial membrane proteins. To investigate this alternative pathway, we used MBB, a protein that prevents changes in mitochondrial membrane proteins that are required to initiate MPT. We observed, however, that MBB (1 μM) did not alter the apoptotic response of 786-0 cells in the presence of LY83583 (Fig. 8C). Similar results were observed when MBB was also used at 10 μM (data not shown). AIF is also reportedly released during the apoptotic response. AIF normally resides in the mitochondrial intermembrane space, whereby it translocates to the nucleus during apoptosis. The release of AIF from the mitochondria can be mediated by a caspase-dependent or -independent pathway. Using immunofluorescence, we observed that treatment of 786-0 cells with LY83583 (1 μM) causes translocation of AIF from the cytoplasm to the nucleus in a caspase-independent manner (Fig. 8D). AIF translocation has also been reported to be calpain-dependent. However, upon treatment of our cells with calpeptin (20 μM), we still observed AIF in the nucleus (Fig. 8D). In addition, we found that calpeptin administration to 786-0 cells before the addition of LY83583 failed to reverse the apoptotic effect (Fig. 8C). We did observe, however, that treatment of 786-0 cells with NAC (10 mM) prevented both the apoptotic effect and the translocation of AIF to the nucleus (Fig. 8, C and D). A role for p38 MAPK in AIF translocation has recently been reported (Yu et al., 2004). Thus, we sought to investigate whether similar control exists in 786-0 cells treated with LY83583 (1 μM). This was indeed the case with 786-0 cells transfected with p38δ and p38γ DN opposing AIF translocation to the nucleus,

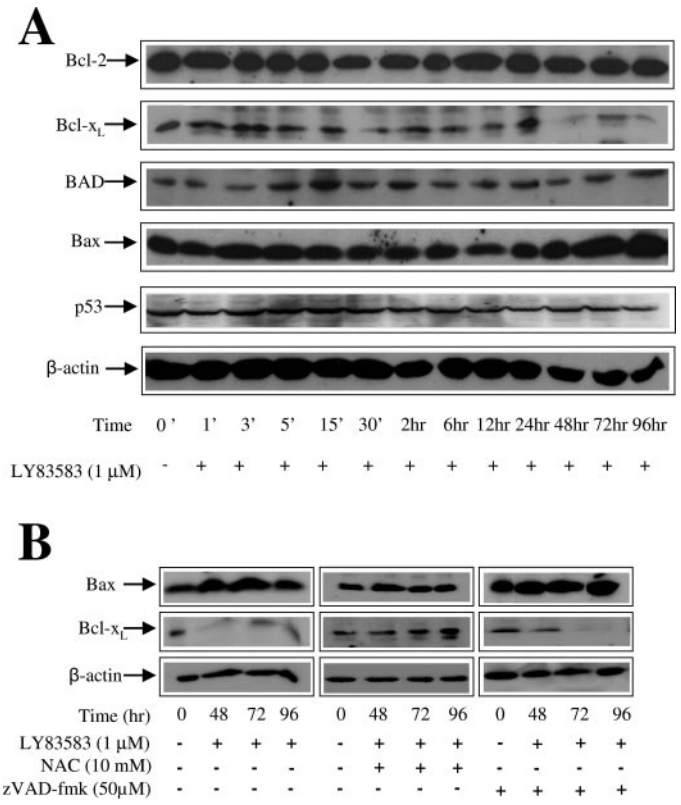

Fig. 7. Effect of LY83583 on 786-0 apoptosis-related gene expression is ROS-dependent. 786-0 cells were treated with and without LY83583 (1 μM) for the indicated time points. Equal amounts of cellular protein (30 μg) were fractionated on 15% SDS-PAGE gels and immunoblotted with Bcl-2, Bcl-x_L, BAD, Bax, and p53 monoclonal antibodies (A). Cells were or were not treated with NAC (10 mM), z-VAD-fmk (50 μM), and LY83583 (1 μM) (B). Membranes were probed with β-actin as a loading control. The figures are representative of four independent experiments.

TABLE 2

LY83583-induced 786-0 apoptosis is Bcl-2- and Bax-dependent

Flow cytometric analysis was performed after staining with FITC-conjugated Annexin V and PI and gated onto a fluorescent dot plot. Percentage of control (0.1% DMSO-treated) 786-0 cells are represented as viable cells (quadrant I). Cells were treated with LY83583 (1 μM) alone or pretreated with the Bcl-2 inhibitor HA 14-1 (1 μM) or a Bax channel blocker (5 μM) for 1 h followed by treatment with LY83583 (1 μM) for 24 to 96 h. Data represent the mean ± S.E. of three independent experiments.

	Cell Viability				
	0 h	24 h	48 h	72 h	96 h
			%		
LY83583 (1 μM)	85 ± 7	66 ± 4	48 ± 11	15 ± 10	11 ± 10
HA 14-1 (1 μM)	93 ± 6	41 ± 9	28 ± 13	6 ± 8	3 ± 5
Bax channel blocker (5 μM)	94 ± 9	89 ± 10	84 ± 5	64 ± 10	50 ± 7

whereas 786-0 p38δ DN and p38γ wt cells behaved as nontransfected cells with a corresponding migration of AIF to the nucleus after LY83583 treatment (Fig. 8D). Finally, to further strengthen a role for AIF in LY83583-induced RCC apoptosis, we used an siRNA approach. AIF levels were reduced by ~90% (by densitometric analysis) in 786-0 cells upon transfection with *SMART*pool AIF siRNA for 48 h (Fig. 8E). si*CONTROL* RISC-Free siRNA used as a control did not alter the levels of AIF protein expression in 786-0 cells. AIF siRNA transfected and nontransfected 786-0 cells were then treated with LY83583 (1 μM) for 48 h [levels of AIF were still suppressed at this time point (i.e., 96 h after the start of transfection)]. Using flow cytometric analysis, we observed an increase in cell viability in AIF depleted cells after LY83583 (1 μM) addition albeit not to the level of untreated cells (Fig. 8F). Nontransfected 786-0 cells displayed a 53.2 ± 4.3% loss in cell viability after treatment with LY83583 (1 μM) for 48 h, whereas *SMART*pool AIF siRNA transfected cells displayed a loss of 24.2 ± 1.9% (Fig. 8F).

Discussion

The lack of effective therapy for the treatment of RCC has recently led to the investigation and development of various immunological treatment strategies (Vuky and Motzer, 2000). Despite this, however, overall response rates of 6 to 20% remain inadequate (Vuky and Motzer, 2000). These poor clinical responses, together with the toxicity associated with such treatment, have meant that identification of other less toxic biological molecules is imperative. Unfortunately, few antineoplastic compounds have been identified in a recent specific search for agents active in this disease (Mertins et

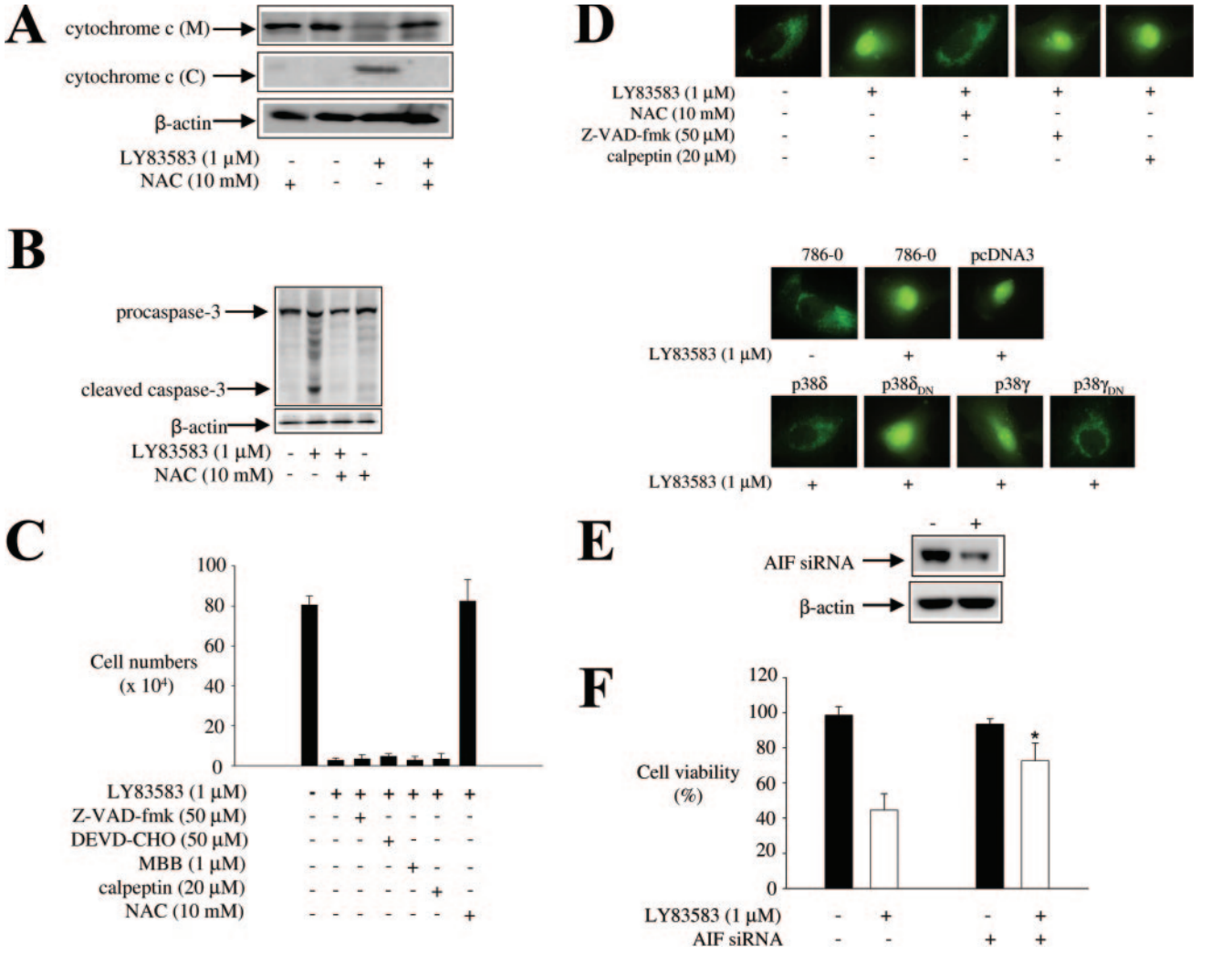

Fig. 8. LY83583-induced cytochrome *c* release, caspase-3 activation, and AIF translocation are ROS-dependent. Cytochrome *c* was measured in the mitochondrial (M) and cytosolic (C) fractions (A) and proteolytic processing of procaspase-3 was evident (B) when 786-0 cells were treated with LY83583 (1 μM) for 96 h. Forty micrograms of protein was fractionated by 10% and 15% SDS-PAGE gels for cytochrome *c* and caspase-3, respectively. Cells were treated with NAC (10 mM) and LY83583 (1 μM) for 96 h (A–D). The specific caspase-3 inhibitor Ac-DEVD-CHO (50 μM), the pan-caspase inhibitor z-VAD-fmk (50 μM), an inhibitor of MPT formation, MBB (1 μM), or the calpain I inhibitor calpeptin (20 μM), were added as indicated 1 h before the addition of LY83583 (1 μM) (C and D). Immunostaining for AIF in transfected or nontransfected 786-0 cells in the presence or absence of LY83583 (1 μM) (D). Western blot analysis shows depletion of AIF after transfection of 786-0 cells with AIF siRNA for 48 h (E). siRNA AIF-transfected and nontransfected 786-0 cells were treated with LY83583 (1 μM) for 48 h. Flow cytometric analysis was performed after staining with FITC-conjugated Annexin V and PI and gated onto a fluorescent dot plot. Percentage of control (0.1% DMSO-treated) 786-0 cells are represented as viable cells (quadrant I) (F). Significant (*, $p < 0.05$) changes from nontransfected 786-0 cells treated with LY83583 (1 μM) (F). The figures are representative of five independent experiments.

al., 2001). In the present study, however, we have identified an antitumorigenic agent, the naphthoquinolinedione compound LY83583, for RCC. Normal HRE cells proved to be refractory to this compound. Despite some observations (Lee and Wurster, 1995; Lodygin et al., 2002), work pertaining to this drug as a useful therapeutic agent is lacking. We now report on the molecular mechanisms that underlie the sensitivity of renal tumor cells to this possible future therapeutic agent.

Proliferation and programmed cell death are key features of oncogenesis and tumor progression. Despite the fact that ROS can act as mediators of proliferation or act as key players in apoptosis, cell cycle arrest and cellular senescence (Velarde et al., 2004; Gumpricht et al., 2005), a defined mechanistic model of how ROS contribute to the cascade of apoptotic events is still lacking. In this report, we document the mechanisms involved in LY83583-induced 786-0 apoptosis through ROS formation. Both DCFH and DHR have been used as indicators of respiratory burst in different cell types (Halliwell and Whiteman, 2004). However, their fluorescence is an assay of generalized oxidative stress rather than of any particular reactive species. We observed an increase in the oxidation of both DHR and DCFH in a time-dependent fashion when 786-0 cells were treated with LY83583 (Fig. 3 and data not shown). As outlined in a recent review by Halliwell and Whiteman (2004) this reflects an increase in the reactive species $OH^{\cdot}$, $NO_2^{\cdot}$, and $ONOO^-$ rather than H_2O_2 or O_2.

In contrast to previous studies, in which the elimination of ROS by chemical or enzymic antioxidants decreased the tumorigenicity of various types of tumor cells, we now demonstrate that excessive production of ROS in RCC can have an antitumorigenic effect. Depending on the cell type, different MAPK family members, such as ERK1/2, p38 MAPK, and JNK, have been shown to possess ROS-sensitive kinase activity (Dong et al., 2004; Kamata et al., 2005). In addition, constitutive activation of the Ras/ERK/MAPK pathway has been implicated in the progression of and often the de-differentiated phenotype of RCC (Oka et al., 1995), which contributes to the refractory nature of these tumors to conventional treatment strategies (Motzer et al., 1996). We have now shown that members of the MAPK cascade act as targets for ROS-induced apoptosis in RCC. Early inactivation of MEK1/2 and ERK1/2 was observed on treatment of 786-0 cells with LY83583 with a corresponding inactivation of the downstream transcription factor Elk-1 as well as down-regulation of COX-2. In addition, we observed a role for the serine threonine kinases Akt and Pak1 (which was found to be constitutively activated in these cells, data not shown) in mediating LY83583-induced apoptosis. In the presence of ROS scavengers, however, we failed to observe apoptosis as well as kinase inactivation.

The roles played by the p38 subfamily of MAPKs in apoptosis are more complex than previously thought; distinct members seem to have different roles. Although a role for p38α and p38β was not apparent in our studies, we did observe, however, that p38δ and p38γ seem to have disparate roles in LY83583-induced apoptosis. Cells expressing the former isoform or a dominant-negative mutant of the latter isoform conferred resistance to LY83583 with a concomitant decrease in AIF translocation. This resistance, however, was not related to their activation and may be due to their mass action effects on altering ERK1/2 function. It is not clear how p38δ and p38γ possess different functions in the apoptosis observed in our system. One obvious possibility is that the two isoforms have different downstream substrates. Further studies are presently under way to address this issue in an attempt to delineate their role(s) in LY83583-induced apoptosis.

There are at present incongruent results with regard to the relevance of the Bcl-2 gene family, apoptotic induction, and cellular proliferation in the development and progression of RCC (Kallio et al., 2004). In general, both Bcl-2 and Bcl-x_L have been shown to antgonize ROS production in apoptosis and to protect cells from exogenous oxidant-induced apoptosis. In agreement with published data for RCC, we have observed high levels of expression of antiapoptotic Bcl-2 as well as proapoptotic Bax in 786-0 (and ACHN and Caki-1 cells; data not shown). Despite this, however, Bax expression was further increased upon addition of LY83583, with a corresponding decrease in Bcl-x_L expression. We observed a change in Bax and Bcl-x_L expression at 48, 72, and 96 h (Fig. 7A), yet apoptosis occurred at 24 h (Fig. 2, A and B). Their role in the apoptotic response at the earlier time of 24 h may not be evident by Western blotting because early small changes in their expression may not be discernible against high basal background expression. The additional experiments, however, using the Bax channel blocker as well as the Bcl-2 inhibitor (HA14-1) (Table 2), confirm the role of both Bax and Bcl-x_L at 24 to 96 h in LY83583-induced RCC apoptosis.

One of the most puzzling paradigms related to cell death at present is the release of AIF. In agreement with recent reports involving p38 MAPK and ERK in AIF translocation (Yu et al., 2004), we have demonstrated that ERK1/2 inactivation as well as the specific isoforms p38δ and p38γ are involved in AIF translocation. A review of the literature has demonstrated conflicting reports regarding the role of caspases in its release. Both caspase-dependent and -independent effects have been ascribed to the release of AIF. To make the process somewhat more complicated, a role for calpain I-mediated Bid cleavage has been associated with AIF release. In our studies, however, we have found that neither caspases nor calpain I seems to affect AIF translocation to the nucleus during apoptosis. This does not, however, preclude the involvement of other endogenous proteases present in the mitochondrial intermembrane space that may be required for proteolysis of AIF before its translocation. We are presently investigating this intriguing mechanism.

RCC is one of the most difficult malignancies to treat, mainly because it is reported to express (except for adrenocortical cancer) the highest levels of P-glycoprotein among tumors (Goldstein et al., 1989). We now report on a therapeutic drug that does not seem to be influenced by the high levels of this protein (O. P. Barry, unpublished observations). In addition, the lack of cytotoxicity of LY83583 in normal human renal epithelial cells as outlined in this study is an obvious advantage. One explanation may be the lack of constitutive ERK1/2 activation in HRE cells (O. P. Barry, unpublished observations), which seems to play a central role in mediating the apoptotic process of LY83583 in RCC. In addition, the presence of p38δ, which we found to confer resistance to LY83583-induced apoptosis in 786-0 cells, may be another factor contributing to the resistance of HRE cells to this drug. Present studies are now focusing on the effect of

this novel compound on RCC growth in vivo using animal models. It is our hope that this body of work may pave the way to the future usage of this compound as a therapeutic agent in a tumor that presently has few effective therapies.

Acknowledgments

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References

Adler V, Yin Z, Tew KD, and Ronai Z (1999) Role of redox potential and reactive oxygen species in stress signaling. *Oncogene* **18:**6104–6111.

Amato RJ (2000) Chemotherapy for renal cell carcinoma. *Semin Oncol* **27:**177–186.

An J, Chen Y, and Huang Z (2004) Critical upstream signals of cytochrome *c* release induced by a novel Bcl-2 inhibitor. *J Biol Chem* **279:**19133–19140.

Barry OP, Mullan B, Sheehan D, Kazanietz MG, Shanahan F, Collins JK, and O'Sullivan G (2001) Constitutive ERK1/2 activation in esophagogastric rib bone marrow micrometastatic cells is MEK-independent. *J Biol Chem* **276:**15537–15546.

Beasley D, Schwartz JH, and Brenner BM (1991) Interleukin 1 induces prolonged L-arginine-dependent cyclic guanosine monophosphate and nitrite production in rat vascular smooth muscle cells. *J Clin Investig* **87:**602–608.

Bombrun A, Gerber P, Casi G, Terradillos O, Antonsson B, and Halazy S (2003) 3,6-Dibromocarbazole piperazine derivatives of 2-propanol as first inhibitors of cytochrome *c* release via Bax channel modulation. *J Med Chem* **46:**4365–4368.

Boring CC, Squires TS, Tong T, and Montgomery S (1994) Cancer statistics, 1994. *CA Cancer J Clin* **44:**7–26.

Bowden ET, Stoica GE, and Wellstein A (2002) Anti-apoptotic signaling of pleiotrophin through its receptor, anaplastic lymphoma kinase. *J Biol Chem* **277:**35862–35868.

Braughler JM, Gilloteaux J, and Steggles AW (1982) Alterations in the subcellular distribution of guanylate cyclase and its responsiveness to nitric oxide in diethylstilbestrol-induced renal tumors. *Cancer* **50:**78–84.

Bukowski RM (1997) Natural history and therapy of metastatic renal cell carcinoma: the role of interleukin-2. *Cancer* **80:**1198–1220.

Chen Q, Shinohara N, Abe T, Wanabe T, Nonomura K, and Koyanagi T (2004) Significance of COX-2 expression in human renal cell carcinoma cell lines. *Int J Cancer* **108:**825–832.

Chiche JD, Schlutsmeyer SM, Bloch DB, de la Monte SM, Roberts JD Jr, Filippov G, Janssens SP, Rosenzweig A and Bloch KD (1998) Adenovirus-mediated gene transfer of cGMP-dependent protein kinase increases the sensitivity of cultured vascular smooth muscle cells to the antiproliferative and pro-apoptotic effects of nitric oxide/cGMP. *J Biol Chem* **273:**34263–34271.

Cobb MH (1999) MAP kinase pathways. *Prog Biophys Mol Biol* **71:**479–500.

Dong J, Ramachandiran S, Tikoo K, Jia Z, Lau SS, and Monks TJ (2004) EGFR-independent activation of p38 MAPK and EGFR-dependent activation of ERK1/2 are required for ROS-induced renal cell death. *Am J Physiol* **287:**F1049–F1058.

Goldstein LJ, Galski H, Fojo A, Willingham M, Lai SL, Gazdar A, Pirker R, Green A, Crist W, Grodeur GM, et al. (1989) Expression of a multidrug resistance gene in human cancers. *J Natl Cancer Inst* **81:**116–124.

Gumpricht E, Dahl R, Devereaux MW, and Sokol RJ (2005) Licorice compounds glycyrrhizin and 18beta-glycyrrhetinic acid are potent modulators of bile acid-induced cytotoxicity in rat hepatocytes. *J Biol Chem* **280:**10556–10563.

Halliwell B and Whiteman M (2004) Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean?. *Br J Pharmacol* **142:**231–255.

Hasegawa T, Bando A, Tsuchiya K, Abe S, Okamoto M, Kirima K, Ueno S, Yoshizumi M, Houchi H, and Tamaki T (2004) Enzymatic and nonenzymatic formation of reactive oxygen species from 6-anilino-5,8-quinolinequinone. *Biochim Biophys Acta* **1670:**19–27.

Ho RC, Alcazar O, Fujii N, Hirschman MF, and Goodyear LJ (2004) p38gamma MAPK regulation of glucose transporter expression and glucose uptake in L6 myotubes and mouse skeletal muscle. *Am J Physiol* **286:**R342–R349.

Kallio JP, Hirvikoski P, Helin H, Luukkaala T, Tammela TL, Kellokumpu-Lehtinen P, and Martikainen PM (2004) Renal cell carcinoma Mib-1, BAX and Bcl-2 expression and prognosis. *J Urol* **172:**2158–2161.

Kamata H, Honda S, Maeda S, Chang L, Hirata H, and Karin M (2005) Reactive oxygen species promote TNF alpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. *Cell* **120:**649–661.

Kuma Y, Campbell DG, and Cuenda A (2004) Identification of glycogen synthase as a new substrate for stress-activated protein kinase 2b/p38beta. *Biochem J* **379:**133–139.

Lee YS and Wurster RD (1995) Mechanism of potentiation of LY83583-induced growth inhibition by sodium nitroprusside in human brain tumor cells. *Cancer Chemother Pharmacol* **36:**341–344.

Lodygin D, Menssen A, and Hermeking H (2002) Induction of the Cdk inhibitor p21 by LY83583 inhibits tumor cell proliferation in a p53-independent manner. *J Clin Investig* **110:**1717–1727.

Mertins SD, Myers TG, Hollingshead M, Dykes D, Bodde E, Tsai P, Jefferis CA, Gupta R, Linehan WM, Alley M, et al. (2001) Screening for and identification of novel agents directed at renal cell carcinoma. *Clin Cancer Res* **7:**620–633.

Mizutani Y, Nakanishi H, Yamamoto K, Li YN, Matsubara H, Mikami K, Okihara K, Kawauchi A, Bonavida B, and Miki T (2005) Downregulation of Smac/DIABLO expression in renal cell carcinoma and its prognostic significance. *J Clin Oncol* **23:**448–454.

Motzer RJ, Bander NH, and Nanus DN (1996) Renal-cell carcinoma. *N Engl J Med* **335:**865–875.

Motzer RJ and Russo P (2000) Systemic therapy for renal cell carcinoma. *J Urol* **163:**408–417.

O'Donnell ME and Owen NE (1986) Role of cyclic GMP in atrial natriuretic factor stimulation of Na^+, K^+, Cl^- cotransport in vascular smooth muscle cells. *J Biol Chem* **261:**15461–15466.

Oka H, Chatani Y, Hoshino R, Ogawa O, Kakehi Y, Terachi T, Okada Y, Kawaichi M, Kohno M, and Yoshida O (1995) Constitutive activation of mitogen-activated protein (MAP) kinases in human renal cell carcinoma. *Cancer Res* **55:**4182–4187.

Pramanik R, Qi X, Borowicz S, Choubey D, Schultz RM, Han J, and Chen G (2003) p38 isoforms have opposite effects on AP-1-dependent transcription through regulation of c-Jun. The determinant roles of the isoforms in the p38 MAPK signal specificity. *J Biol Chem* **278:**4831–4839.

Schmidt MJ, Sawyer BD, Truex LL, Marshall WS, and Fleisch JH (1985) LY83583: an agent that lowers intracellular levels of cyclic guanosine 3′,5′-monophosphate. *J Pharmacol Exp Ther* **232:**764–769.

Tourani J-M, Pfister C, Tubiani N, Ouldkaci M, Prevot G, Lucas V, Oudard S, Malet M, Cottu P, Ferrero J-M, et al. (2003) Subcutaneous interleukin-2 and interferon alfa administration in patients with metastatic renal cell carcinoma: final results of SCAPP III, a large, multicenter, phase II, nonrandomized study with sequential analysis design-the Subcutaneous Administration Propeukin Program Cooperative Group. *J Clin Oncol* **21:**3987–3994.

Velarde V, de la Cerda PM, Duarte C, Arancibia F, Abbott E, Gonzalez A, Moreno F, and Jaffa AA (2004) Role of reactive oxygen species in bradykinin-induced proliferation of vascular smooth muscle cells. *Biol Res* **37:**419–430.

Vuky J and Motzer R (2000) Cytokine therapy in renal cell cancer. *J Urol Oncol* **5:**249–257.

Wang Z, Li Y, Liu ET, and Yu Q (2004) Susceptibility to cell death induced by blockade of MAPK pathway in human colorectal cancer cells carrying Ras mutations is dependent on p53 status. *Biochem Biophys Res Commun* **322:**609–613.

Wang X and Tournier C (2006) Regulation of cellular functions by the ERK5 signalling pathway *Cell Signal* **18:**753–760.

Yang SH, Galanis A, and Sharrocks AD (1999) Targeting of p38 mitogen-activated protein kinases to MEF2 transcription factors. *Mol Cell Biol* **19:**4028–4038.

Yu C, Rahmani M, Almenara J, Sausville EA, Dent P, and Grant S (2004) Induction of apoptosis in human leukemia cells by the tyrosine kinase inhibitor adaphostin proceeds through a RAF-1/MEK/ERK- and AKT-dependent process. *Oncogene* **23:**1364–1376.

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