Carcinogenesis vol.22 no.1 pp.35–41, 2001

Activation and role of mitogen-activated protein kinases in deoxycholic acid-induced apoptosis

Dianhua Qiao, Elias D.Stratagouleas and Jesse D.Martinez[1]

Arizona Cancer Center, Department of Radiation Oncology, University of Arizona, 1501 North Campbell Avenue, Tucson, AZ 85724, USA

[1]To whom correspondence should be addressed
Email: jmartinez@azcc.arizona.edu

The bile acid deoxycholic acid (DCA) is a known tumor promoter and it has been suggested that DCA-induced apoptosis plays an important role in colon tumor development. In this study we have characterized the capacity of DCA to stimulate mitogen-activated protein kinase (MAPK) activity and examined the effect that MAPK activity had on DCA-induced apoptosis. Analysis of MAPK activity in DCA-treated HCT116 cells using phosphorylation-specific antibodies and *in vitro* kinase assays indicated that both the extracellular signal-regulated kinase (ERK) and p38 MAPK (p38), but not the c-Jun N-terminal kinase (JNK), were activated. Using pharmacological inhibitors we determined that only ERK could influence DCA cytotoxicity and that elevated ERK activity could suppress DCA-induced apoptosis. This observation was confirmed genetically. Suppressing ERK activity by overexpressing a dominant negative form of the ERK MAP kinase resulted in increased sensitivity to DCA-induced apoptosis whereas elevated ERK activity artificially produced by overexpression of the wild-type ERK kinase blunted DCA-induced apoptosis. Taken together, our results suggest that DCA can stimulate pro-apoptotic and anti-apoptotic signaling pathways and that sensitivity to DCA-induced apoptosis can be modulated by the ERK MAP kinase.

Introduction

Epidemiological studies have consistently shown that elevated fecal bile acids are associated with an increased risk of colon cancer, suggesting that bile acids may foster colon tumor development (1–3). The notion that elevated fecal bile acids are linked to increased risk for colorectal cancer is also supported by the observation that patients with colorectal adenomas have elevated concentrations of bile acids in their blood and stool (1,4). However, the most compelling evidence for a causal link between certain bile acids and increased colonic carcinogenesis comes from animal model studies. For example, rats treated with azoxymethane (AOM) and fed a diet supplemented with deoxycholic acid (DCA) develop more tumors than do animals treated with AOM alone (5–7). Furthermore, in an elegant series of experiments Morvay and co-workers demonstrated directly that the presence or absence of bile acids is a strong determinant of tumor incidence in the colon by surgically altering the flow of bile acids in rats (8). These surgically altered rats were treated with AOM and the distribution of tumors within the gut determined. Significantly, that portion of the colon that was excluded from exposure to bile acids developed no or fewer tumors when compared with the section that continued to be exposed to bile acids. Hence, bile acids, and DCA specifically, appear to be tumor promoters that have an important role in the pathogenesis of colon cancer.

The mechanism by which DCA or other bile acids function is not clear. However, increasing evidence suggests that they may exert their tumor promoting activity by affecting intracellular signaling and gene expression, which ultimately alters cell death and proliferation. It has been reported that exposure to DCA can lead to the induction of a variety of growth regulatory genes, including cyclooxygenase-2 (9) and GADD153 (10), as well as stimulate the activity of the AP-1 and NF-κB transcription factors (11,12). In addition, bile acids can regulate the levels of several enzymes involved in their own biosynthesis, including Cyp7α and Cyp27 in cells of hepatic origin (13,14). The Cyp7α enzyme is regulated at the level of transcription by a negative feedback loop in which bile acids suppress transcription from the *Cyp7* gene by interfering with an as yet unidentified transcription factor that binds to a bile acid-responsive element in the 5′ promoter region of the gene (15,16). Importantly, this regulation of *Cyp7* may involve protein kinase C (PKC) (16). This is consistent with pervious studies suggesting that bile acids can influence the activity of these protein kinases and suggests that bile acids may alter the activity of intracellular signaling pathways (17–19).

In a previous study we demonstrated that DCA could cause activation of AP-1 and that this required both PKC and mitogen-activated protein kinase (MAPK) activities (11). MAPKs constitute an important group of signaling mediators that transduce diverse extracellular stimuli and that govern cellular processes such as proliferation, differentiation and cell death. Three MAPK cascades appear to transduce the majority of signals from the cell membrane to the nucleus. These are the extracellular signal-regulated kinase (ERK) cascade, the c-Jun N-terminal kinase (JNK) cascade and the p38 MAP kinase (p38) cascade (20). All of the MAPKs are activated through dual phosphorylation and their substrates include diverse cellular molecules, including protein kinases (21), mitochondrial proteins (22), phospholipases (23), cytoskeletal proteins (24) and transcription factors (25). In this study we have focused on the effect of DCA on MAPKs and the role of MAPKs in DCA-induced apoptosis. Bile acid-induced apoptosis has been thought to play important roles in the tumor promotion process of bile acids in the colon (26). Our results suggest that the ERK MAP kinase, but not the JNK or p38 pathways, is involved in DCA-induced apoptosis. Paradoxically, we found that elevated ERK activity can actually suppress DCA-induced apoptosis. This result can be explained by a model in which DCA can stimulate both apoptotic and anti-

Abbreviations: AOM, azoxymethane; DCA, deoxycholic acid; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; NGF, neuron growth factor; p38, p38 MAP kinase; PKC, protein kinase C.

apoptotic signaling, but that the apoptotic pathway predominates in cells treated with elevated levels of DCA.

Materials and methods

Plasmids and reagents

pCMV-p41, a constitutive expression construct of ERK2, and pCMV-p41(Ala^{54}Ala^{55})^{mapk}, a dominant negative mutant ERK2 expression construct, were kindly provided by Dr Roger Davis (27). Lipofectamine was from Gibco BRL (Gaithersburg, MD). The sodium salt of DCA was from Sigma Chemical Co. (St Louis, MO) and maintained as a 100 mM stock solution in water. PD98059 (2′-amino-3′-methoxyflavone) and SB202190 [4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1*H*-imidazole] were from Calbiochem (La Jolla, CA) and maintained as stock solutions in dimethyl sulfoxide.

Cell culture, treatment and transfection

HCT116, a cell line derived from an adenocarcinoma patient with Lynch's syndrome, was cultured in Dulbecco's modified Eagle's medium (Gibco BRL) with 10% fetal bovine serum, 2 mM L-glutamine and 100 U/ml penicillin/streptomycin at 37°C in an incubator containing 5% CO_2. All experiments were conducted with cells grown to 80–90% confluency. Treatment of the cells with MAPK inhibitors was performed by pre-treating the cells with the inhibitors for 30 min prior to addition of bile acids.

Transient transfection was conducted by cationic lipid-mediated DNA transfection using Lipofectamine as previously described (11). After transfection, the lipid–DNA complex was replaced with fresh culture medium and the cells were cultured for an additional 12 h. The cells were then treated with DCA and harvested.

Stable transfection of HCT116 cells with pCMV-p41(Ala^{54}Ala^{55})^{mapk} or the vector plasmid pCMV-Bam-neo was performed by the calcium phosphate precipitation method (28). The stably transfected cells were selected in the presence of 500 μg/ml G418 and individual clones were subcultured.

Western blot analyses for expression and activation of MAPKs

Cells were lysed in 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM sodium vanadate and 10 mM sodium fluoride. An aliquot of 50 μg total cellular protein was subjected to SDS–PAGE in 10% gels. Western blot analyses for expression and activation of MAPKs were performed using rabbit polyclonal antibodies against ERK, p38 and JNK total proteins or the corresponding phospho-specific antibodies which recognize activated phosphorylated MAPK isoforms (Santa Cruz Biotechnology, Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (Santa Cruz Biotechnology) and Luminol ECL reagents (Amersham Life Science, Little Chalfont, UK) were used to visualize the specific immunocomplexes.

Protein kinase assay

Cells were lysed in 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM sodium vanadate, 1 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride. Aliquots of 100 μg protein were incubated with phospho-specific ERK antibody or phospho-specific p38 MAPK antibody (New England Biolabs, Beverley, MA) with gentle rocking overnight at 4°C. Protein A–Sepharose beads were added and the mixture was rotated at 4°C for 3 h and washed twice in lysis buffer and twice in kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycerol phosphate, 2 mM DTT, 0.1 mM sodium vanadate and 10 mM $MgCl_2$). Beads were suspended in 25 μl of kinase buffer with 100 μM ATP and 0.5 μg Elk-1 or ATF-2 fusion proteins (New England Biolabs) and incubated at 30°C for 30 min. For JNK kinase assay 250 μg total protein were incubated with 2 μg c-Jun fusion protein–Sepharose beads (New England Biolabs) with gentle rocking overnight at 4°C. After washing twice in lysis buffer and twice in kinase buffer, the beads were suspended in 50 μl of kinase buffer with 100 μM ATP and incubated for 30 min at 30°C. Samples were boiled in SDS sample buffer and resolved on 12% SDS–PAGE gels. Phosphorylated Elk-1, ATF-2 and c-Jun proteins were analyzed by western blotting using corresponding phospho-specific antibodies (New England Biolabs).

Apoptosis assay

DCA-induced apoptosis was examined with microscopic analysis as described previously (26). In brief, treated and untreated cells were trypsinized and combined with the floating cells. Cells were then applied to glass slides using a cytospin centrifuge. After fixing with 100% methanol, the slides were stained with Accustain Giemsa (Sigma Chemical Co.) and observed by light microscopy. Cells undergoing apoptosis were identified visually by a characteristic morphology that included chromatin condensation, an increase in cytoplasmic vacuolization, cell shrinkage, change in tinctorial appearance of the cytoplasm, nuclear fragmentation and apoptotic body formation (26).

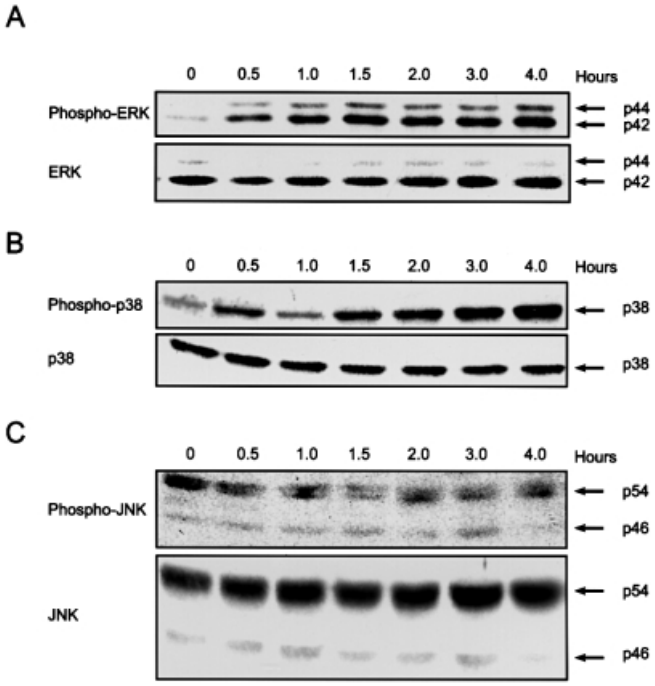

Fig. 1. Effect of DCA on MAPK phosphorylation. HCT116 cells were treated with 250 μM DCA for different time periods as indicated, total cellular protein extracts prepared and western blots performed using 50 μg protein. The blots were probed with antibodies specific for total proteins or activated phosphorylated forms of (**A**) ERK, (**B**) p38 and (**C**) JNK, respectively.

A minimum of 200 cells were counted and the percentage of apoptotic cells was calculated from the total number of viable-appearing and apoptotic cells.

Results

DCA stimulates ERK and p38 MAPKs

We previously showed that DCA can activate AP-1 and that this required both PKC and ERK/MAPK activities (11). To better understand the significance of MAPK activation to DCA-induced apoptosis, HCT116 cells were treated with DCA (250 μM) and the phosphorylation status of three MAPK subfamilies, including ERK, p38 and JNK, analyzed at regular intervals by immunoblotting with phospho-specific antibodies. As shown in Figure 1, both ERK and p38 showed a marked increase in phosphorylation after cells were treated with DCA, which occurred within 30 min after exposure. The quantity of phosphorylated ERK continued to increase smoothly over time. Interestingly, there was a transient and reproducible reduction in p38 phosphorylation at 1 h, although overall p38 phosphorylation increased with extended exposure to the bile acid. No increase in JNK phosphorylation was observed, suggesting that JNK is not activated by DCA. Western blots using antibodies to detect total MAPK proteins showed that DCA treatment did not alter the levels of total cellular MAPK proteins. Hence, these results indicated that DCA induced phosphorylation of ERK and p38, but not JNK. To confirm the relationship between phosphorylation and enzymatic activation of the MAPKs, HCT116 cells were exposed to DCA (250 μM) for 4 h and the activities of ERK, p38 and JNK were detected by testing MAPK-induced phosphorylation of an Elk-1, an ATF-2 and a c-Jun fusion protein, respectively. The results showed increased enzymatic activity of both ERK and p38 in DCA-treated cells, as demonstrated by increased

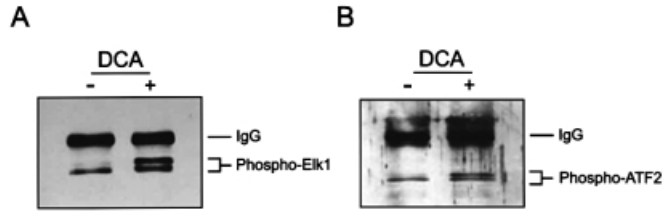

Fig. 2. Assay for ERK and p38 activities. HCT116 cells were treated with 250 μM DCA for 4 h. Treated and untreated cells were lysed and 100 μg total protein immunoprecipitated with phospho-specific antibodies for (**A**) ERK and (**B**) p38 MAPKs. *In vitro* kinase assays were then performed with recombinant Elk-1 (ERK) and ATF-2 (p38) proteins as substrates and the phosphorylated substrates were detected by western blot analyses using phospho-specific antibodies.

phosphorylation of Elk-1 and ATF-2 (Figure 2). We found no JNK activation in DCA-treated cells, although JNK activity could be stimulated using UV light (data not shown), indicating that the cellular signaling for JNK activation is intact but is not stimulated by DCA. Consequently, the phosphorylation of MAPKs is indicative of their enzymatic activation and exposure to DCA caused selective up-regulation of both ERK and p38 kinase activities.

Inhibition of ERK activation by PD98059 augments DCA-induced apoptosis

Previous studies suggested that bile acid-induced apoptosis may play an important role in bile acid promotion of colon tumorigenesis (26). In addition, because there is evidence that both ERK and p38 can mediate extracellular stimulus-induced apoptosis (29,30), we sought to determine whether ERK or p38 could modulate DCA-induced apoptosis. For this purpose, HCT116 cells were pretreated for 30 min with a specific inhibitor for MAPK/ERK kinase (MEK), PD98059, or a potent specific inhibitor for p38 MAPK, SB202190. Subsequently, the inhibitor-treated cells were exposed to DCA and apoptosis quantitated. Western blot analyses showed that DCA-induced ERK activation was effectively inhibited by PD98059 without affecting p38 phosphorylation (Figure 3A and B). Unexpectedly, inhibition of ERK activation by PD98059 resulted in an increase of ~14% over and above the apoptosis induced by DCA alone. In contrast, SB202190 resulted in a 7% decrease in apoptosis relative to DCA-treated cells (Figure 3C). When p38 activity was blocked with SB202190, DCA caused super-phosphorylation of both ERK and p38 that was well above that observed in cells treated with DCA only or with DCA and PD98059 (Figure 3A and B). This effect has been previously described by others and is thought to be due to feedback between the two MAPK pathways (31,32).

To clarify whether p38 activity played a role in modulating DCA-induced apoptosis, HCT116 cells were pretreated with both 80 μM PD98059 and 10 μM SB202190 and DCA-induced apoptosis examined. As shown in Figure 4A, at 80 μM PD98059 could completely abolish both DCA-induced ERK phosphorylation as well as the phosphorylation that resulted from incubation with inhibitor SB202190. Moreover, when DCA-induced apoptosis was examined, we found that the level of apoptosis was comparable with that found in cells treated with the PD98059 inhibitor only (Figure 4B). Hence, suppression of p38 activity had no effect on DCA-induced apoptosis, suggesting that ERK, but not p38, could influence DCA-induced apoptosis.

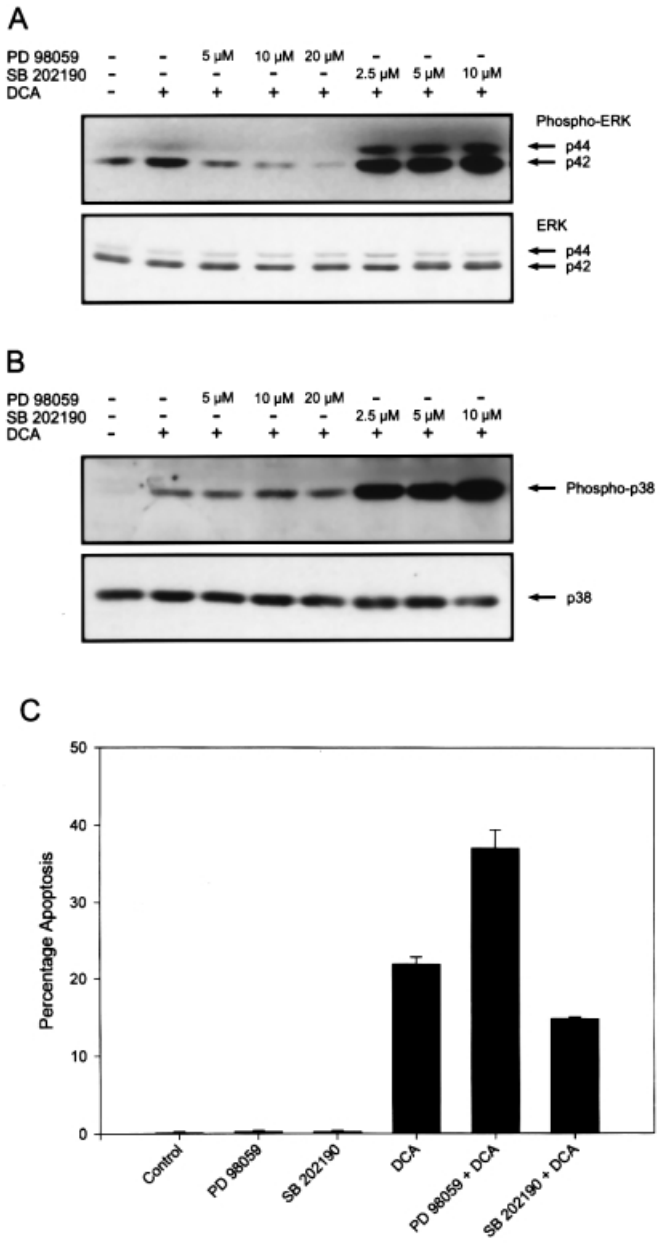

Fig. 3. Effects of PD98059 and SB202190 on DCA-induced ERK and p38 phosphorylation and DCA-induced apoptosis. (**A** and **B**) HCT116 cells were treated with 250 μM DCA for 4 h after preincubation for 30 min with different concentrations of PD98059 or SB202190 as indicated. Total cellular protein extracts were prepared and western blot analyses performed with 50 μg protein using antibodies specific for the total proteins or activated phosphorylated forms of (**A**) ERK and (**B**) p38, respectively. (**C**) HCT116 cells were treated with 500 μM DCA for 3 h after preincubation with 20 μM PD98059 or 10 μM SB202190 for 30 min. The fraction of apoptotic cells was determined as described in Materials and methods. Each bar represents the mean ± SE of two independent experiments.

Overexpression of ERK2 down-regulates DCA-induced apoptosis

To further investigate the role of ERK activation in DCA-induced apoptosis, we introduced wild-type ERK protein into cells by transient transfection. As a control, transient transfection of HCT116 cells with the vector plasmid, pCMV-Bam-neo, was also performed. The transfected cells were treated with DCA and both the level of phospho-ERK in the cells and DCA-induced apoptosis were determined. ERK2

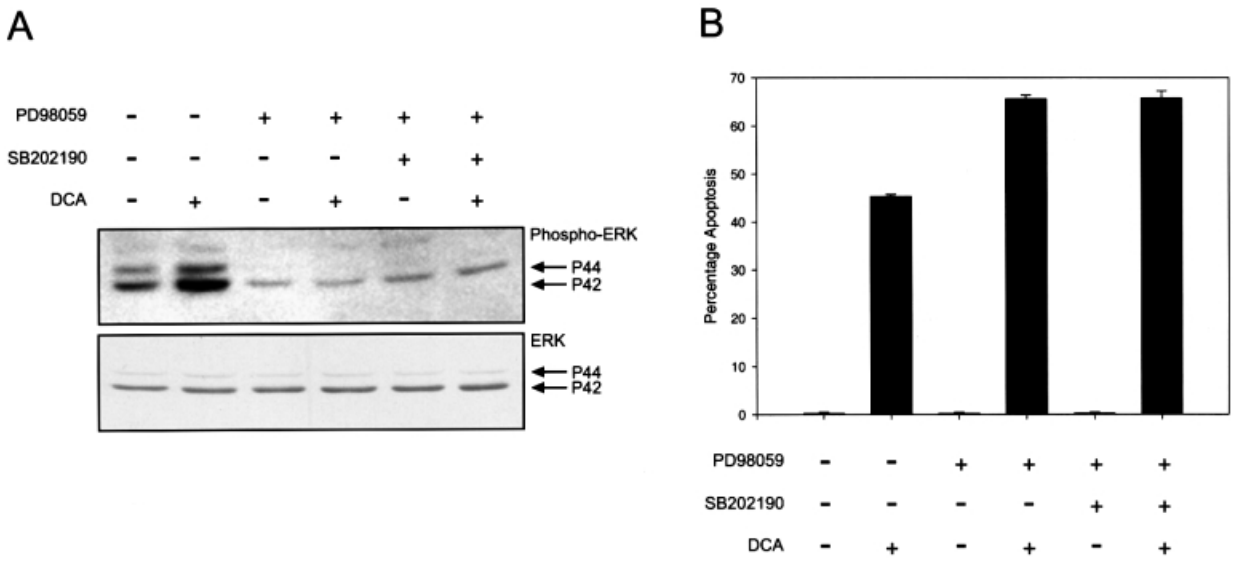

Fig. 4. Effect of simultaneous inhibition of ERK and p38 on DCA-induced apoptosis. HCT116 cells were pretreated with 80 μM PD98059 alone or both 80 μM PD98059 and 10 μM SB202190 for 30 min as indicated before DCA was added. (**A**) The cells were incubated with 250 μM DCA for 4 h, total cellular protein extracts prepared and western blot analyses performed with 50 μg protein using antibodies specific for total ERK or phospho-ERK. (**B**) The cells were incubated with 500 μM DCA for 3 h and DCA-induced apoptosis was examined. Each bar represents the mean ± SE of two independent experiments.

protein was overexpressed in the cells transfected with pCMV-p41 and this resulted in elevated levels of phosphorylated ERK upon stimulation with DCA when compared with cells transfected with the control plasmid (Figure 5A and B). Apoptosis assays showed that DCA-induced apoptosis in the cells transfected with pCMV-p41 was attenuated when compared with cells transfected with the control plasmid (Figure 5C). These results indicated that DCA-induced ERK activity acted to suppress DCA-induced apoptosis.

Expression of dominant negative mutant ERK2 increases DCA-induced apoptosis

To avoid the possible non-specific effect of chemical inhibitors and further confirm the role of ERK in DCA-induced apoptosis, HCT116 cells were stably transfected with the dominant negative mutant ERK2 expression construct, pCMV-p41($Ala^{54}Ala^{55}$)^{mapk} (27). It has been reported that constitutive expression of the mutant ERK protein could effectively interfere with activation and function of ERK in the cells (27,33). DCA induction of phospho-ERK and apoptosis were investigated in the stable transfectants. Compared with parental HCT116 cells and cells stably transfected with control plasmid, DCA-mediated induction of phospho-ERK was suppressed in B6 and C6, two clones from a stable transfection with dominant negative mutant ERK2 (Figure 6A). Apoptosis assays showed that DCA-induced apoptosis in both B6 and C6 was markedly increased compared with parental HCT116 cells and the vector control (Figure 6B). These results combined with the observations using MAPK inhibitors (Figures 3 and 4) indicated that ERK functioned to suppress DCA-induced apoptosis.

Discussion

The three major MAP kinase signaling cascades mediated by ERK, JNK and p38 transduce signals elicited by a wide variety of extracellular stimuli which in turn produce a variety of cellular responses, including proliferation, differentiation and apoptosis (20). Our finding that DCA can influence the activity of at least two of these MAPK signaling pathways suggests that bile acids may act, at least in part, by modifying intracellular signaling.

In this study we have shown that both ERK and p38 MAPKs were markedly stimulated by DCA and that ERK, but not p38, could influence DCA-induced apoptosis. Paradoxically, we found that inhibition of ERK with the inhibitor PD98059 resulted in stimulation of DCA-induced apoptosis. Evidence suggesting that an activated ERK acted in opposition to DCA-induced apoptosis was also seen when p38 MAP kinase signaling was inhibited with SB202190. Incubating HCT116 cells with the p38 inhibitor resulted in marked up-regulation of ERK activity and a concomitant decrease in DCA-induced apoptosis (see Figure 3). The elevation of ERK activity observed when cells were treated with the p38 inhibitor is likely due to cross-talk between ERK and p38 signaling and has been reported previously, although the mechanism by which this occurs is not known (31,32). Given that inhibition of ERK with PD98059 stimulated DCA-induced apoptosis it seems likely that the decreased apoptosis in SB202190-treated cells seen in Figure 3 could be attributed to the increased ERK activity. This notion was supported by experiments in which both ERK and p38 activity was suppressed by treating with both inhibitors, which resulted in a level of apoptosis that was similar to that seen in cells where only ERK activity was suppressed. Consequently, the same elevated level of apoptosis was observed when ERK was suppressed regardless of the level of p38 activity, suggesting that p38 signaling could not alter DCA-induced signaling (Figures 3 and 4).

Importantly, we observed the same effect on DCA-induced apoptosis in cells where ERK activity had been modified genetically. Overexpression of ERK, which resulted in higher levels of phospho-ERK than in control cells during DCA treatment, led to a decrease in DCA-induced apoptosis. Also, in cells constitutively expressing a dominant negative mutant ERK DCA-induced apoptotic response was enhanced. Hence, in HCT116 cells ERK, but not p38, MAPK activity suppresses DCA-induced apoptosis. However, our experiments do not

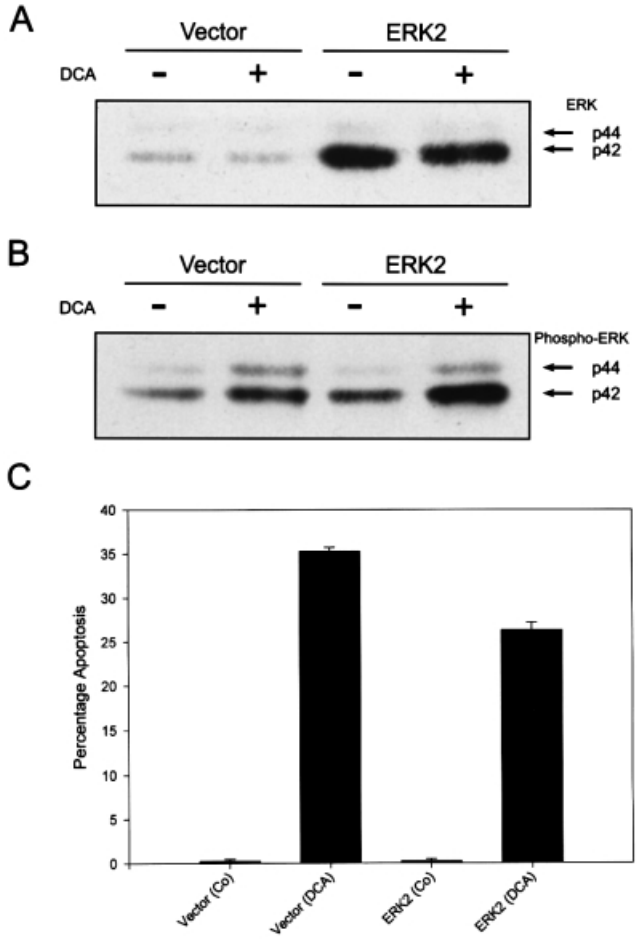

Fig. 5. Overexpression of ERK2 inhibits DCA-induced apoptosis. HCT116 cells were transiently transfected with a constitutive expression construct of ERK2, pCMV-p41 or control vector pCMV-Bam-neo. After transfection the cells were cultured in growth medium for 12 h before further treatments. (**A**,**B**) The transfected cells were treated with 250 μM DCA for 4 h, total cellular protein extracts prepared and western blot analyses performed with 50 μg protein using antibodies specific for (**A**) total protein or (**B**) the activated phosphorylated form of ERK. (**C**) The transfected cells were treated with 500 μM DCA for 3 h and the percentage of DCA-induced apoptosis was examined. Each bar represents the mean ± SE of two independent experiments.

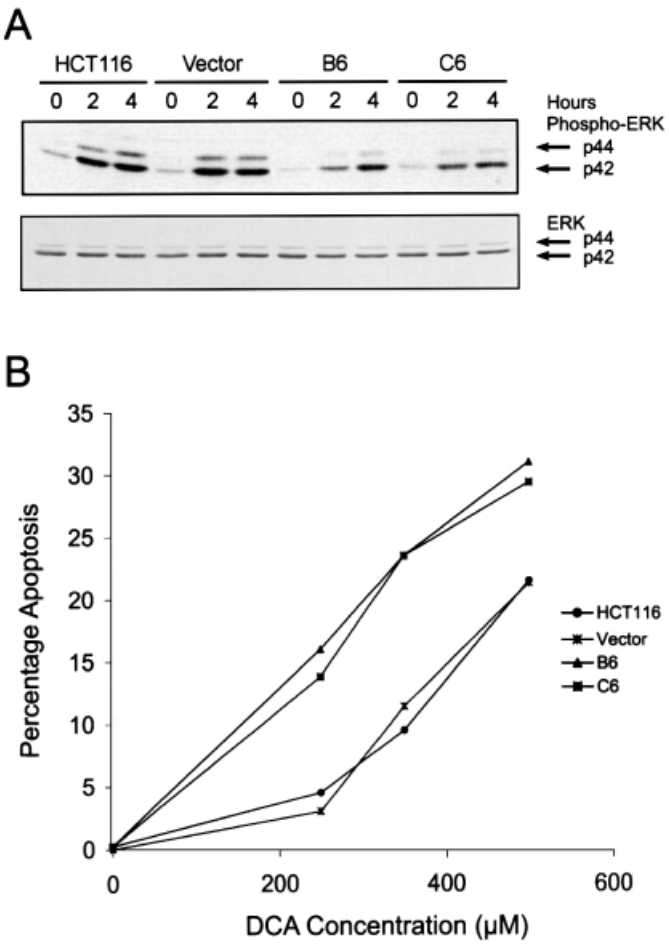

Fig. 6. Expression of dominant negative mutant ERK2 increases DCA-induced apoptosis. HCT116 cells were stably transfected with a dominant negative mutant ERK2 expression construct, pCMV-p41(Ala^{54}Ala^{55})^{mapk}, or the control plasmid pCMV-Bam-neo. (**A**) The cells were treated with 250 μM DCA for different time periods as indicated. Total cellular protein extracts were prepared and western blot analysis performed with 50 μg protein using antibodies specific for the total protein or activated phosphorylated form of ERK. (**B**) Parental HCT116 cells and the transfected cells were treated with 250, 350 or 500 μM DCA for 3 h and the percentage of DCA-induced apoptosis was determined. Values are means from two independent experiments. B6 and C6 are two clones from the stable transfection with pCMV-p41(Ala^{54}Ala^{55})^{mapk}.

rule out the possibility that p38 may play a role in other effects that have been attributed to DCA.

There is a precedent for ERK functioning as an anti-apoptotic intermediate and increasing evidence supports a role for ERK in cell survival. In PC-12 cells withdrawal of neuron growth factor (NGF) led to inhibition of ERK activity and increased cell death (30) whereas constitutive activation of the ERK pathway in these cells inhibited the apoptosis induced by NGF withdrawal (30). In Jurkat T cells activation of ERK by TPA inhibited Fas-induced apoptosis (34). Moreover, in cardiomyocytes apoptosis induced by H_2O_2 resulted in up-regulation of ERK activity, but, as with DCA-induced apoptosis in HCT116 cells, elevated ERK activity blunted the apoptotic response (35). A similar situation was also observed in satratoxin-induced apoptosis, where ERK was activated and acted to suppress apoptosis (36). Consequently, elevated ERK activity may be indicative of reduced sensitivity to apoptogenic stimuli.

Intriguingly, recent studies of MAPK activity in colorectal adenomas and carcinomas from carcinogen-treated rats indicate that ERK activity was elevated, on average, 29-fold above that found in the normal mucosa (37). Given that recent data has emerged showing that human colon tumors have a reduced sensitivity to DCA-induced apoptosis (38), it is tempting to speculate that the evolution of colon tumors may involve development of reduced sensitivity to DCA-induced apoptosis and that this may be accompanied by elevated ERK activity. This notion is consistent with the observations that oncogenic activation of Ras widely occurs in the early stage of colon tumor development and that the protein and activity levels of PKC are decreased in human colon tumors (39,40). Ras activation can cause up-regulation of the ERK pathway through the classical Ras/Raf/MEK/ERK signaling cascade (41) while PKC activity seems to be required for DCA-induced apoptosis according to the data from human colon cancer cell lines (26).

The concurrence of induction of apoptosis and stimulation of anti-apoptotic signaling by DCA may have important significance in bile acid promotion of colon cancer. It has been suggested that cell death induced by bile acids may cause compensatory hyperproliferation of colonic epithelial cells and

provide selection for subpopulations of cells resistant to bile acid- and/or other stimulus-induced apoptosis (26). Hyperproliferation may attenuate the ability of cells to maintain genomic integrity and is consistently related to increased cell transformation (42). Moreover, because apoptosis is an important mechanism for clearing DNA-damaged cells, modulation of the apoptotic response by anti-apoptotic signaling and selection for apoptosis-resistant cells may result in accumulation of DNA-damaged cells and consequently increased cancer risk. Hence, it is likely that bile acid-induced apoptosis accounts for at least part of the pathological effects of bile acids that promote colon tumor development. This notion can be supported by the observation that the tumor promoting activity of different bile acids correlates well with their capacity to induce apoptosis *in vitro* (26).

In conclusion, we have shown in this study that ERK is activated by DCA and that DCA-induced ERK activity functions to suppress DCA-induced apoptosis. More significantly, our results suggest that DCA stimulates both apoptotic and anti-apoptotic signaling. This may represent an important mechanism by which bile acids promote colonic carcinogenesis. Further studies will be needed to determine the downstream effectors of ERK that execute the anti-apoptotic effect of DCA-stimulated ERK activity.

Acknowledgements

We thank Dr Roger Davis for providing the pCMV-p41 and pCMV-p41(Ala^{54}Ala^{55})^{mapk} plasmids. This work was supported by National Institutes of Health Grant PO1-CA-72008 and Cancer Center Core Grant CA-23074. E.D.S. was supported in part by Howard Hughes Medical Institute Grant 71195-521303 (to the Undergraduate Biological Research Program at the University of Arizona).

References

1. Bayerdorffer,E., Mannes,G.A., Richter,W.O., Ochsenkuhn,T., Wiebecke,B., Kopcke,W. and Paumgartner,G. (1993) Increased serum deoxycholic acid levels in men with colorectal adenomas [see comments]. *Gastroenterology*, **104**, 145–151.
2. Reddy,B.S. and Wynder,E.L. (1973) Large-bowel carcinogenesis: fecal constituents of populations with diverse incidence rates of colon cancer. *J. Natl Cancer Inst.*, **50**, 1437–1442.
3. Reddy,B.S. and Wynder,E.L. (1977) Metabolic epidemiology of colon cancer. Fecal bile acids and neutral sterols in colon cancer patients and patients with adenomatous polyps. *Cancer*, **39**, 2533–2539.
4. Mastromarino,A.J., Reddy,B.S. and Wynder,E.L. (1978) Fecal profiles of anaerobic microflora of large bowel cancer patients and patients with nonhereditary large bowel polyps. *Cancer Res.*, **38**, 4458–4462.
5. Reddy,B.S., Watanabe,K., Weisburger,J.H. and Wynder,E.L. (1977) Promoting effect of bile acids in colon carcinogenesis in germ-free and conventional F344 rats. *Cancer Res.*, **37**, 3238–3242.
6. Sutherland,L.A. and Bird,R.P. (1994) The effect of chenodeoxycholic acid on the development of aberrant crypt foci in the rat colon. *Cancer Lett.*, **76**, 101–107.
7. Narisawa,T., Magadia,N.E., Weisburger,J.H. and Wynder,E.L. (1974) Promoting effect of bile acids on colon carcinogenesis after intrarectal instillation of N-methyl-N′-nitro-N-nitrosoguanidine in rats. *J. Natl Cancer Inst.*, **53**, 1093–1097.
8. Morvay,K., Szentleleki,K., Torok,G., Pinter,A., Borzsonyi,M. and Nawroth,R. (1989) Effect of change of fecal bile acid excretion achieved by operative procedures on 1,2-dimethylhydrazine-induced colon cancer in rats. *Dis. Colon Rectum*, **32**, 860–863.
9. Zhang,F., Subbaramaiah,K., Altorki,N. and Dannenberg,A.J. (1998) Dihydroxy bile acids activate the transcription of cyclooxygenase-2. *J. Biol. Chem.*, **273**, 2424–2428.
10. Zheng,Z.Y., Bernstein,H., Bernstein,C., Payne,C.M., Martinez,J.D. and Gerner,E.W. (1995) Bile acid activation of the GADD153 promoter and of p53-independent apoptosis: relevance to colon cancer. *Cell Death Differ.*, **3**, 407–414.
11. Qiao,D., Chen,W., Stratagoules,E.D. and Martinez,J.D. (2000) Bile acid-induced activation of activator protein-1 requires both extracellular signal-regulated kinase and protein kinase C signaling. *J. Biol. Chem.*, **275**, 15090–15098.
12. Payne,C.M., Crowley,C., Washo-Stultz,D., Briehl,M., Bernstein,H., Bernstein,C., Beard,S., Holubec,H. and Warneke,J. (1998) The stress-response proteins poly(ADP-ribose) polymerase and NF-κB protect against bile salt-induced apoptosis. *Cell Death Differ.*, **5**, 623–636.
13. Pandak,W.M., Vlahcevic,Z.R., Heuman,D.M., Redford,K.S., Chiang,J.Y. and Hylemon,P.B. (1994) Effects of different bile salts on steady-state mRNA levels and transcriptional activity of cholesterol 7 alpha-hydroxylase. *Hepatology*, **19**, 941–947.
14. Twisk,J., de Wit,E.C. and Princen,H.M. (1995) Suppression of sterol 27-hydroxylase mRNA and transcriptional activity by bile acids in cultured rat hepatocytes. *Biochem. J.*, **305**, 505–511.
15. Chiang,J.Y. and Stroup,D. (1994) Identification and characterization of a putative bile acid-responsive element in cholesterol 7 alpha-hydroxylase gene promoter. *J. Biol. Chem.*, **269**, 17502–17507.
16. Stravitz,R.T., Vlahcevic,Z.R., Gurley,E.C. and Hylemon,P.B. (1995) Repression of cholesterol 7 alpha-hydroxylase transcription by bile acids is mediated through protein kinase C in primary cultures of rat hepatocytes. *J. Lipid Res.*, **36**, 1359–1369.
17. Rao,Y.P., Stravitz,R.T., Vlahcevic,Z.R., Gurley,E.C., Sando,J.J. and Hylemon,P.B. (1997) Activation of protein kinase C alpha and delta by bile acids: correlation with bile acid structure and diacylglycerol formation. *J. Lipid Res.*, **38**, 2446–2454.
18. Fitzer,C.J., O'Brian,C.A., Guillem,J.G. and Weinstein,I.B. (1987) The regulation of protein kinase C by chenodeoxycholate, deoxycholate and several structurally related bile acids. *Carcinogenesis*, **8**, 217–220.
19. Jones,B.A., Rao,Y.P., Stravitz,R.T. and Gores,G.J. (1997) Bile salt-induced apoptosis of hepatocytes involves activation of protein kinase C. *Am J. Physiol.*, **272G**, 1109–1115.
20. Widmann,C., Gibson,S., Jarpe,M.B. and Johnson,G.L. (1999) Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiol. Rev.*, **79**, 143–180.
21. Seger,R. and Krebs,E.G. (1995) The MAPK signaling cascade. *FASEB J.*, **9**, 726–735.
22. Scheid,M.P., Schubert,K.M. and Duronio,V. (1999) Regulation of bad phosphorylation and association with Bcl-x (L) by the MAPK/Erk kinase. *J. Biol. Chem.*, **274**, 31108–31113.
23. Mitchell,R.A., Metz,C.N., Peng,T. and Bucala,R. (1999) Sustained mitogen-activated protein kinase (MAPK) and cytoplasmic phospholipase A2 activation by macrophage migration inhibitory factor (MIF). Regulatory role in cell proliferation and glucocorticoid action. *J. Biol. Chem.*, **274**, 18100–18106.
24. Veeranna,G.J., Shetty,K.T., Takahashi,M., Grant,P. and Pant,H.C. (2000) Cdk5 and MAPK are associated with complexes of cytoskeletal proteins in rat brain. *Brain Res. Mol. Brain Res.*, **76**, 229–236.
25. Treisman,R. (1996) Regulation of transcription by MAP kinase cascades. *Curr. Opin. Cell Biol.*, **8**, 205–215.
26. Martinez,J.D., Stratagoules,E.D., LaRue,J.M., Powell,A.A., Gause,P.R., Craven,M.T., Payne,C.M., Powell,M.B., Gerner,E.W. and Earnest,D.L. (1998) Different bile acids exhibit distinct biological effects: the tumor promoter deoxycholic acid induces apoptosis and the chemopreventive agent ursodeoxycholic acid inhibits cell proliferation. *Nutr. Cancer*, **31**, 111–118.
27. Seth,A., Gonzalez,F.A., Gupta,S., Raden,D.L. and Davis,R.J. (1992) Signal transduction within the nucleus by mitogen-activated protein kinase. *J. Biol. Chem.*, **267**, 24796–24804.
28. Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K. (1987) *Current Protocols in Molecular Biology*. Green Publishing Associates, New York, NY.
29. Petrache,I., Choi,M.E., Otterbein,L.E., Chin,B.Y., Mantell,L.L., Horowitz,S. and Choi,A.M. (1999) Mitogen-activated protein kinase pathway mediates hyperoxia-induced apoptosis in cultured macrophage cells. *Am. J. Physiol.*, **277L**, 589–595.
30. Xia,Z., Dickens,M., Raingeaud,J., Davis,R.J. and Greenberg,M.E. (1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science*, **270**, 1326–1331.
31. Rosenberger,S.F., Gupta,A. and Bowden,G.T. (1999) Inhibition of p38 MAP kinase increases okadaic acid mediated AP-1 expression and DNA binding but has no effect on TRE dependent transcription. *Oncogene*, **18**, 3626–3632.
32. Singh,R.P., Dhawan,P., Golden,C., Kapoor,G.S. and Mehta,K.D. (1999) One-way cross-talk between p38(MAPK) and p42/44(MAPK). Inhibition of p38(MAPK) induces low density lipoprotein receptor expression through activation of the p42/44(MAPK) cascade. *J. Biol. Chem.*, **274**, 19593–19600.

33. Beno,D.W., Brady,L.M., Bissonnette,M. and Davis,B.H. (1995) Protein kinase C and mitogen-activated protein kinase are required for 1,25-dihydroxyvitamin D3-stimulated Egr induction. *J. Biol. Chem.*, **270**, 3642–3647.
34. Wilson,D.J., Alessandrini,A. and Budd,R.C. (1999) MEK1 activation rescues Jurkat T cells from Fas-induced apoptosis. *Cell Immunol.*, **194**, 67–77.
35. Adderley,S.R. and Fitzgerald,D.J. (1999) Oxidative damage of cardiomyocytes is limited by extracellular regulated kinases $\frac{1}{2}$-mediated induction of cyclooxygenase-2. *J. Biol. Chem.*, **274**, 5038–5046.
36. Yang,G.H., Jarvis,B.B., Chung,Y.J. and Pestka,J.J. (2000) Apoptosis induction by the satratoxins and other trichothecene mycotoxins: relationship to ERK, p38 MAPK and SAPK/JNK activation. *Toxicol. Appl. Pharmacol.*, **164**, 149–160.
37. Licato,L.L., Keku,T.O., Wurzelmann,J.I., Murray,S.C., Woosley,J.T., Sandler,R.S. and Brenner,D.A. (1997) *In vivo* activation of mitogen-activated protein kinases in rat intestinal neoplasia. *Gastroenterology*, **113**, 1589–1598.
38. Payne,C.M., Bernstein,H., Bernstein,C. and Garewal,H. (1995) Role of apoptosis in biology and pathology: resistance to apoptosis in colon carcinogenesis. *Ultrastruct. Pathol.*, **19**, 221–248.
39. Fearon,E.R. and Vogelstein,B. (1990) A genetic model for colorectal tumorigenesis. *Cell*, **61**, 759–767.
40. Kopp,R., Noelke,B., Sauter,G., Schildberg,F.W., Paumgartner,G. and Pfeiffer,A. (1991) Altered protein kinase C activity in biopsies of human colonic adenomas and carcinomas. *Cancer Res.*, **51**, 205–210.
41. Marshall,M.S. (1995) Ras target proteins in eukaryotic cells. *FASEB J.*, **9**, 1311–1318.
42. Wang,X.J., Greenhalgh,D.A. and Roop,D.R. (2000) Transgenic coexpression of v-Ha-ras and transforming growth factor alpha increases epidermal hyperproliferation and tumorigenesis and predisposes to malignant conversion via endogenous c-Ha-ras activation. *Mol. Carcinog.*, **27**, 200–209.

Received July 19, 2000; revised September 15, 2000; accepted September 25, 2000