

# Ursodeoxycholic Acid Can Suppress Deoxycholic Acid-Induced Apoptosis by Stimulating Akt/PKB-Dependent Survival Signaling

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**Abstract:** *The nontoxic bile acid ursodeoxycholic acid (UDCA) is reported to be an anti-apoptotic agent with efficacy against a variety of death stimuli including the cytotoxic bile acid deoxycholic acid (DCA). To gain insight into this anti-apoptotic property, we tested UDCA for its ability to protect the colon carcinoma-derived cell line HCT116 against DCA-induced apoptosis. We found that UDCA could suppress DCA-induced apoptosis in a time- and dose-dependent manner and that this effect correlated with Akt phosphorylation. Importantly, UDCA lost its ability to protect cells from DCA-induced cell death when Akt activity was suppressed genetically using a dominant negative Akt mutant or when PI3K activity was inhibited pharmacologically. These results suggest that UDCA can protect HCT116 cells against DCA-induced apoptosis by stimulating Akt-dependent survival signaling.*

## Introduction

Bile acids are polar derivatives of cholesterol, essential for the absorption of dietary lipids and regulation of the transcription of genes that control cholesterol homeostasis. However, depending on the nature of the chemical structures, different bile acids exhibit distinct biological effects (1). Although some bile acids such as deoxycholic acid (DCA) and taurohyodeoxycholic acid show cytotoxicity toward colorectal carcinoma and hepatoblastoma cells, ursodeoxycholic acid (UDCA), taurochenodeoxycholic acid (TCDC), and tauroursodeoxycholic acid are cytoprotective (1,2). UDCA is a key drug in the treatment of cholestatic liver disease because of its cytoprotective effect (3). Blockade of apoptosis triggered by a variety of stimuli including bile acids suggests that UDCA is an anti-apoptotic agent (4). Moreover, increasing evidence shows that UDCA stimulates intracellular signaling and activates a survival pathway (5,6). Indeed, mitogen-activated protein kinase and phosphoinositide 3-kinase (PI3K) are activated in UDCA-induced survival signaling cascades in primary rat hepatocytes (5,6).

This raises the possibility that UDCA can activate survival signaling to protect against cytotoxic stimuli.

The serine/threonine protein kinase Akt, or protein kinase B (PKB) is a downstream effector of PI3K that is stimulated by insulin, growth factors, and cytokines. Activation of Akt results in Akt's recruitment to the plasma membrane and phosphorylation at threonine 308 and serine 473 (7–11). Akt regulates gene transcription by phosphorylation of transcription factors such as forkhead transcription factors and NF- $\kappa$ B (12,13). Two pharmacological inhibitors of PI3K, LY294002 and wortmannin, inhibit growth factor-mediated Akt activation (14,15). Moreover, Akt/PKB plays a critical role in the regulation of cell survival and cell proliferation and has been implicated in anti-apoptosis effects against a variety of death stimuli. Epidermal growth factor receptor-mediated Akt activation confers protection against oxidative stress-induced apoptosis (16).

In light of these findings, it was of interest to determine if UDCA activates survival signaling pathways and whether UDCA could protect against DCA-induced apoptosis *in vivo*. To this end, we examined the effect that UDCA had on DCA-induced apoptosis in HCT116 cells and on Akt activity. Importantly, we found that UDCA's cytoprotective effect is Akt dependent. These results provide the first evidence that UDCA activated the Akt-dependent survival pathway.

## Materials and Methods

### Plasmids and Reagents

pUSEamp (K179M mutant), a dominant negative mutant Akt (dnAkt) expression construct, was purchased from Upstate Biotechnology (Lake Placid, NY). DCA was obtained from Sigma Chemical (St. Louis, MO) and UDCA was obtained from Calbiochem (La Jolla, CA). All of the bile acids were maintained as 100-mM stock solutions in water and were stored at 4°C. LY294002 and wortmannin were obtained from Calbiochem and were maintained as a stock solution in dimethyl sulfoxide.

## Cell Culture and Treatments

HCT116 (ATCC, Rockville, MD), a cell line derived from the adenocarcinoma patient with Lynch's syndrome, was cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO/BRL, Gaithersburg, MD) with 10% fetal bovine serum, 2 mM L-glutamine, and 100 units/ml penicillin/streptomycin at 37°C in an incubator containing 5% CO<sub>2</sub>. For experiments, cells were grown to 80–95% confluency. For treatments, cells were incubated in culture medium containing bile acids for different periods of time. LY294002 and wortmannin were added to the cell culture medium 30 min prior to exposure to bile acids.

In preparation for UDCA pretreatment experiments, HCT116 cells were plated at a 100,000 per 35-mm culture plates and allowed to attach for 48 h. This concentration of cells produced 50–60% confluency at the time bile acids were added. Cells were then incubated with 500 µM UDCA for up to 12 h, the UDCA was removed, and the cells were then exposed to DCA prior to assaying for apoptosis.

For the growth arrest experiments approximately 100,000 HCT116 cells were plated onto 35-mm tissue culture dishes and allowed to attach for 48 h. Cells were then incubated with 500 µM UDCA or 10 µg/ml aphidicolin (Sigma Chemical) for 12 h. To arrest cells by double-thymidine block (17), cells were treated with 20 mM thymidine (Sigma Chemical) for 12 h. The thymidine-containing media was removed, the cell monolayer was rinsed with warm PBS and refed fresh media, and the incubation was continued for 12 h. Following this 12-h incubation period the cells were again incubated with 20 mM thymidine for 12 h. Cells that were growth arrested by these protocols were then tested for sensitivity to DCA-induced apoptosis by incubating them with 500 µM DCA for 6 h. Apoptosis was quantitated as described subsequently.

To generate cell clones that stably expressed dnAkt, HCT116 cells were plated at a confluency of 40% in 10-cm plates and allowed to grow for 48 h. Plates were then transfected with 2 µg of purified pUSEamp plasmid using lipofectamine (Invitrogen) according to the manufacturer's instructions. After replating the transfected cells were incubated in 500 µg/ml G418 to select for resistant colonies. Resistant cell colonies were expanded and tested for expression levels of Akt by Western blotting.

## Apoptosis Assay

For apoptosis assay  $1 \times 10^5$  HCT116 cells were plated onto a 35-mm tissue culture plate and left to attach for 24–48 h. The cells were treated with 500 µM bile acids for the times indicated. The cells were stained with ethidium bromide and acridine orange, and then both viable and apoptotic cells were quantified as described previously (18).

## Western Blot Analysis

Cells were lysed in 10 mM Tris-HCl (pH 7.4), 1% Triton X-100, 1 mM EDTA, 150 mM NaCl, 50 mM NaF, 0.1% bo-

vine serum albumin, aprotinin, 20 µg/ml leupeptin, 20 µg/ml pepstatin, 20 µM Na<sub>3</sub>VO<sub>4</sub>, and 0.2 mM phenylmethylsulfonyl fluoride. Of the total cellular protein 50 µg was extracted and subjected to SDS-polyacrylamide gel electrophoresis in 12.5% gel. Western blot analyses were performed using rabbit polyclonal antibodies against Akt, p-Akt, and pGSK-3β (Cell Signaling, Beverly, MA). Bound antibody was visualized using chemiluminescent substrate (SuperSignal West Pico, Pierce, Rockford, IL) and exposure to X-ray film (Kodak, Rochester, NY).

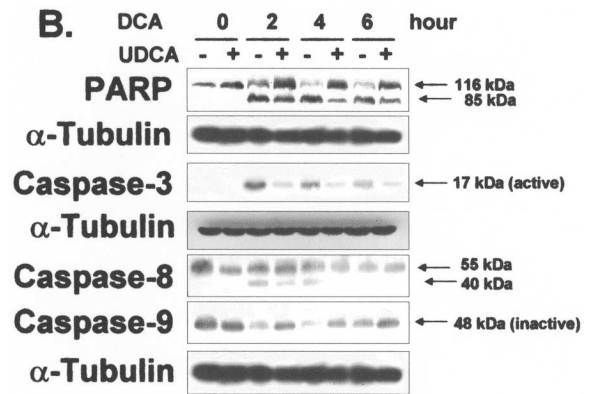
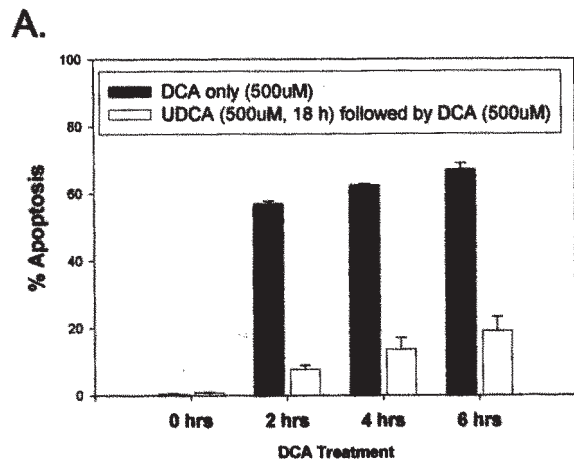
## Results

### UDCA Can Suppress DCA-Induced Apoptosis

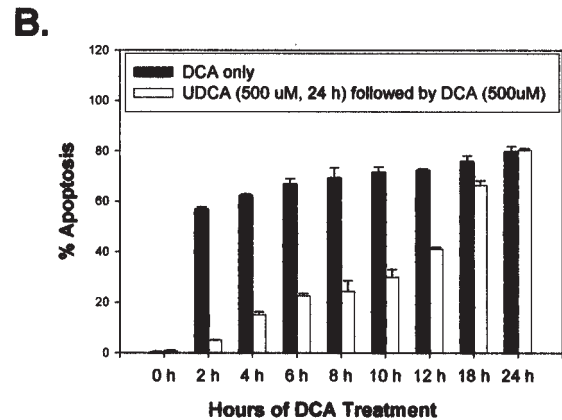
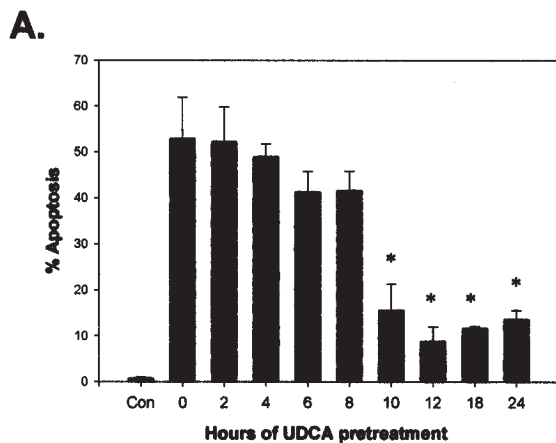
It has been shown that UDCA can suppress apoptosis that is induced by a variety of agents. Because we and others have shown that the cytotoxicity exhibited by DCA can be attributed to DCA-induced apoptosis, we sought to determine whether UDCA could counteract this. To examine this, HCT116 cells were preincubated with UDCA for 12 h and then exposed to DCA for various lengths of time as described in **Materials and Methods** (Fig. 1A). As can be seen, the preincubation with UDCA reduced DCA-induced apoptosis to approximately 10% compared with the ~60% observed when fresh HCT116 cells were exposed to the same concentrations of DCA for the same amount of time. Moreover, analysis of several biochemical markers for apoptosis by Western blotting demonstrated that poly (ADP-ribose) polymerase (PARP) and caspase cleavage was inhibited by the UDCA pretreatment (Fig. 1B). Hence, UDCA not only suppressed the appearance of apoptotic cells but also suppressed caspase activation. The concentration of DCA and UDCA used in our experiments is rational considering the DCA levels found in the fecal water of individuals consuming a high-fat diet (up to 0.78 mM) (19) and UDCA dosage used in the clinical trials for colon cancer prevention (up to 900 mg/day) (20).

To ascertain whether the protection afforded by incubation with UDCA was time dependent, we incubated with HCT116 cells UDCA for various times and then tested for protection against DCA-induced apoptosis (Fig. 2). These experiments determined that UDCA could suppress against DCA-induced apoptosis after the cells had been exposed to UDCA for 10 h or longer. Preincubating with UDCA for less than 10 h afforded very little protection, and preincubating for a longer duration did not improve the degree of protection. Moreover, UDCA-mediated protection was transient and was reversed after removal of UDCA from the cell culture media (Fig. 2B). We conclude from these experiments that the anti-apoptosis protection afforded by UDCA was a time-dependent process that was relatively slow to appear.

We previously showed that UDCA caused cells to undergo growth arrest (1). Because this growth arrest becomes prominent after 12 h of exposure to UDCA, we considered that this growth arrest could contribute to the protection



**Figure 1.** UDCA inhibits DCA-induced apoptosis. HCT116 cells were pretreated with 500  $\mu$ M UDCA for 18 h. After removing UDCA, cells were incubated with 500  $\mu$ M DCA for different time periods as indicated. HCT116 cells treated with only DCA are also shown for comparison. A: Treated cells were stained with acridine orange and ethidium bromide, and the apoptotic and viable cells were identified and counted using fluorescent microscopy. Bars represent the average of three independent experiments. Error bars indicate SE. UDCA-mediated protection in 2, 4, and 6 h of DCA treatment was found statistically significant by paired *t*-test ( $P < 0.05$ ). B: In parallel experiments, Western blot analyses were performed on extracts of treated HCT116 cells using anti-PARP, caspase-3 (Cas-3), caspase-8 (Cas-8), and caspase-9 (Cas-9) antibodies. Each blot was reprobed with polyclonal antibody against human  $\alpha$ -tubulin, which served as a loading control. The results are representative of several independent experiments.



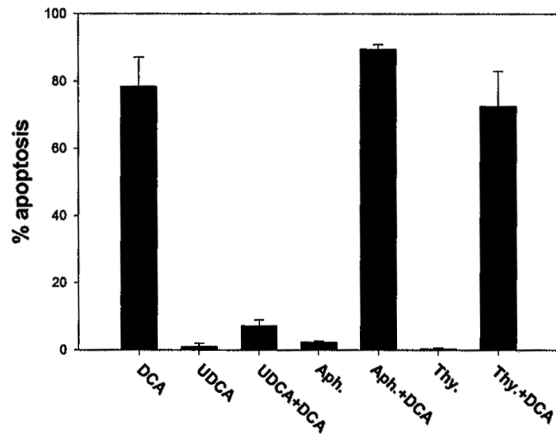
**Figure 2.** UDCA-mediated protection against DCA-induced apoptosis is time dependent and reversible. A: HCT116 cells were incubated with 500  $\mu$ M UDCA for the indicated times. The UDCA-containing media was then removed and the cells were exposed to 500  $\mu$ M DCA for 2 h. Control cells (Con) were not treated with either bile acid. Apoptotic cells were identified and quantitated using acridine orange and ethidium bromide staining. Bars represent the average values from at least three experiments  $\pm$  SD. The asterisks indicate significant reduction in apoptosis as calculated by two-sample *t*-test with unequal variance and Bonferroni adjustment for multiple comparison ( $P < 0.000125$ ). B: HCT116 cells were either untreated or incubated with 500  $\mu$ M UDCA for 24 h. UDCA was then removed from the treated cells and exposed to DCA for various times as indicated. At the appropriate time intervals cells were harvested and apoptosis was quantitated as in A. Bars represent the average values from at three experiments  $\pm$  SD. UDCA-mediated protection in 2, 4, 6, 8, 10, and 12 h of DCA treatment was found statistically significant by paired *t*-test ( $P < 0.05$ ).

against DCA-induced apoptosis exhibited by UDCA. To test this we utilized a number of unrelated compounds that also induce growth arrest. HCT116 cells were treated for 12 h with UDCA, aphidicolin, or a double-thymidine block to induce growth arrest and then were treated with 500  $\mu$ M DCA for 6 h. Growth arrest was confirmed by flow cytometry (data not shown). Quantitation of apoptosis in these cells showed that the cells growth arrested by treatment with aphidicolin or the double-thymidine block remained susceptible to DCA-induced apoptosis (Fig. 3). By comparison, however, DCA-induced apoptosis in UDCA-treated cells was reduced to about 10%. We conclude from these results that the protection from DCA-induced apoptosis was specific

to UDCA and that growth arrest, per se, did not afford protection against DCA's cytotoxic activities.

### Akt Activation by Treatment of UDCA Is Involved in Cell Survival

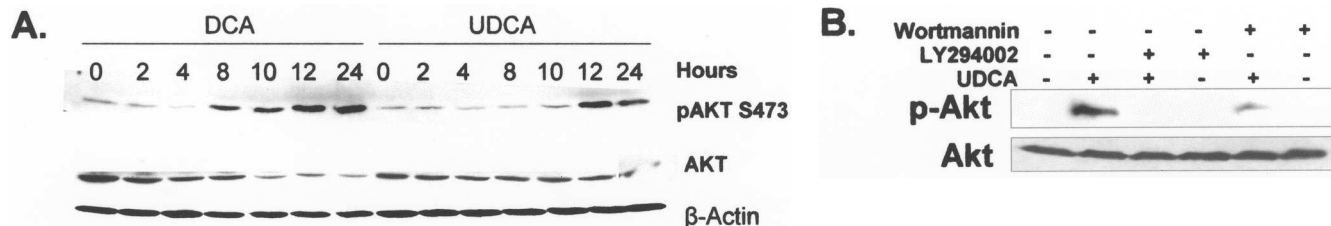
Our previous studies reported that DCA, one of the more hydrophobic bile acids, induced apoptosis, whereas less hydrophobic bile acids such as UDCA showed no cytotoxicity (1). In addition to this the recent finding that the nontoxic bile acids TCDC and taurocholate activated PI3K-dependent survival signaling, whereas cytotoxic bile acids did not, suggested to us the possibility that Akt-dependent survival path-



**Figure 3.** Growth arrest does not contribute to the UDCA-mediated protection against DCA-induced apoptosis. HCT116 cells were treated either with 500  $\mu$ M UDCA for 12 h or 100  $\mu$ g/ml aphidicolin for 12 h or blocked using the double-thymidine block protocol as described in **Materials and Methods**. After treatments the blocked cells were either released and treated with 500  $\mu$ M DCA for 6 h and apoptosis quantitated or apoptosis quantitated immediately after the release. Each bar represents the average from at least three experiments  $\pm$  SD. UDCA-mediated protection of DCA-induced apoptosis was found statistically significant by paired *t*-test ( $P < 0.05$ ) in comparison with aphidicolin or double-thymidine block.

ways might be involved in UDCA-induced cell signaling (21,22). To determine whether UDCA activated the PI3K/Akt-dependent signaling pathway, we examined Akt activity after exposure to bile acids. HCT116 cells were incubated with 500  $\mu$ M DCA or UDCA for different time periods as indicated and immunoblotting was performed. UDCA was found to induce Akt phosphorylation beginning at about 10 h (Fig. 4A). Surprisingly, DCA, the cytotoxic bile acid, also caused Akt phosphorylation, which became evident at approximately 8 h after exposure to this bile acid. Hence, both DCA and UDCA could induce Akt phosphorylation.

To confirm that UDCA induced survival signaling through the Akt pathway, the cells were pretreated with potent PI3K inhibitors, 20  $\mu$ M LY294002, and 250 nM wortmannin for 30 min and then incubated with 500  $\mu$ M UDCA for 12 h. As expected, UDCA-mediated Akt activation was either decreased or abolished (Fig. 4B). This suggests that UDCA stimulates Akt phosphorylation by activating the upstream kinase PI3K.

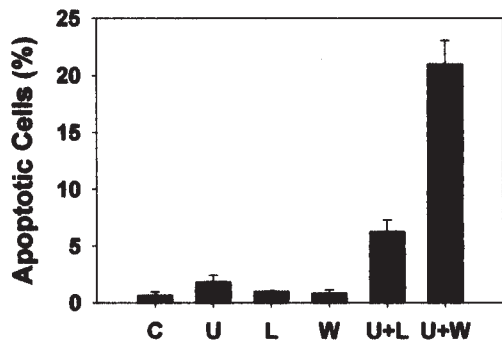


**Figure 4.** UDCA induces Akt activation that can be suppressed by PI3K inhibitors. A: HCT116 cells were treated with 500  $\mu$ M DCA or 500  $\mu$ M UDCA and harvested at the times indicated, and total cellular protein extracts were prepared. Western blot analysis was performed using an anti-serine 473 phospho Akt antibody. The filter was stripped and then reprobbed with polyclonal antibody against Akt. The blot was stripped a second time and reprobbed with anti- $\beta$ -actin, which served as a loading control. The results depicted are representative of several independent experiments. B: HCT116 cells were pretreated with 20  $\mu$ M LY294002 or 250 nM wortmannin for 30 min and then treated with 500  $\mu$ M UDCA for 12 h. Western blot analyses were performed using anti-phospho Akt antibody and reprobbed with polyclonal antibody against Akt. The results are representative of several independent experiments.

### Suppression of Akt Activity Alters the Cellular Response to UDCA

Because it has been suggested that whether bile acids can induce apoptosis is dependent on whether or not Akt is activated, we sought to determine whether suppression of Akt could alter the biological effect that UDCA had on HCT116 cells. To examine this, cells were treated with either LY294002 or wortmannin or left untreated and then exposed to UDCA. Any apoptosis that resulted was then quantitated as described in **Materials and Methods** (Fig. 5). Interestingly, inhibition of PI3K, and therefore Akt, results in conversion of UDCA into a cytotoxic agent because UDCA induced apoptosis in the drug-treated cells but not in the control cells. However, neither UDCA nor PI3K inhibitor alone caused apoptosis. This result suggests that UDCA may also activate apoptotic pathways but that the simultaneous activation of the Akt survival pathway normally overcomes this.

To establish the role of Akt in UDCA-mediated cytoprotection, we suppressed Akt activity utilizing a genetic approach. HCT116 cells were transfected with a dnAkt, and the resultant cells were pretreated with 500  $\mu$ M UDCA for 18 h and then exposed to 500  $\mu$ M DCA for 4 h. Cytoprotection of UDCA against DCA-induced cell death was abolished in the dnAkt-transfected cells, whereas in both parental cells and empty vector-transfected cells UDCA exhibited a marked protective effect on cells treated with DCA (Fig. 6A). Importantly, we found that UDCA could induce apoptosis in the dnAkt-expressing cells and is similar to that observed in cells treated with the PI3K inhibitors. An immunoblotting experiment to investigate phosphorylation of GSK-3 $\beta$ , a physiological substrate of Akt, showed that suppression of Akt activity in dnAkt-transfected cells leads to marked up-regulation of GSK-3 $\beta$  phosphorylation (Fig. 6B). Phosphorylation of GSK-3 $\beta$  is considered a pro-apoptotic event and may account for the induction of apoptosis by UDCA in cells expressing dnAkt and cells treated with the PI3K inhibitors. These findings support the concept that UDCA-induced Akt activation may play a role in UDCA-triggered cell survival signaling. Hence, the UDCA cytoprotective effect may be mediated, at least in part, through activation of Akt-dependent survival signaling cascade.



**Figure 5.** PI3K inhibitors abrogate UDCA-mediated protection against apoptosis. HCT116 cells were either untreated (C) or incubated with 500  $\mu$ M UDCA for 24 h (U), 20  $\mu$ M LY294002 for 30 min (L), 250 nM wortmannin for 30 min (W), 20  $\mu$ M LY294002 for 30 min prior to addition of 500  $\mu$ M UDCA to the culture media for 24 h (U+L), or 250 nM wortmannin for 30 min prior to addition of 500  $\mu$ M UDCA to the culture media for 24 h (U+W). Apoptosis was quantitated as described in **Materials and Methods**. Each bar represents the mean  $\pm$  SD of three independent experiments. Apoptosis induced by UDCA in combination with either LY294002 or wortmannin was found statistically significant by paired *t*-test ( $P < 0.05$ ) when compared with apoptosis induced by UDCA alone.

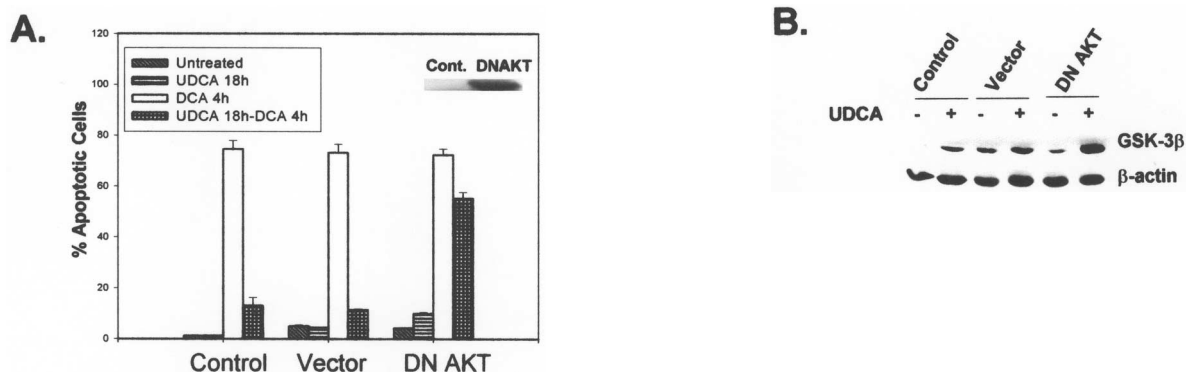
## Discussion

In this report, we demonstrate that UDCA is capable of suppressing the cytotoxic effects of DCA by stimulating survival signaling mediated through the Akt protein kinase. A large body of evidence implicates apoptosis as the mechanism that accounts for DCA's cytotoxicity (23,24). Yet UDCA, a closely related bile acid, has distinctly different effects both on cells in culture as well as in vivo (1). We demonstrate that this protective effect requires an extended exposure to UDCA and does not become measurable until after 10 h. Importantly, preincubation with UDCA suppressed the

typical markers of DCA-induced apoptosis, caspase activation, and PARP cleavage, indicating that UDCA could interfere with the biochemical pathways that become activated when HCT116 cells are treated with DCA. Rodrigues and coworkers recently demonstrated that DCA can induce membrane depolarization and cytochrome c release from purified mitochondria and that UDCA can suppress this (25).

However, experiments with radiolabeled UDCA demonstrate that this hydrophilic bile acid is slow to enter the cell, suggesting that extended incubation times may be necessary for UDCA to have a measurable protective effect on the cell. Hence, the extensive period of time required for UDCA to demonstrate a protective effect may be a consequence of the relatively hydrophilic nature of this bile acid and the slow kinetics with which it may cross the cell's membrane (18). In any case, it is unlikely that UDCA-mediated cell growth arrest accounts for the protective effect because cells growth arrested by treating with aphidicolin or double-thymidine block are still susceptible to DCA-induced apoptosis. Nevertheless, it is still a formal possibility that the growth arrest induced by UDCA is uniquely suited to interfering with DCA-induced apoptosis. However, this would be inconsistent with data showing that DCA can induce apoptosis in all stages of the cell cycle (unpublished data). Instead, a more likely possibility is that activation of some signaling pathway led to changes in the cell's susceptibility to apoptosis induced by DCA.

With regard to UDCA-activated signaling we found that UDCA induced Akt phosphorylation, suggesting that survival signaling mediated by this protein kinase may play a role in the protection against DCA-induced apoptosis. In support of this we found that UDCA-induced Akt phosphorylation occurred with the same kinetics as did the protective effect. Moreover, the genetic suppression of Akt using a dominant negative construct or by suppressing PI3K-mediated phosphorylation of Akt abrogated the protec-



**Figure 6.** Dominant negative Akt (dnAkt) abrogates protection against apoptosis mediated by UDCA. A: Parental HCT116 cells (control), HCT116 stably transfected with empty vector (vector), or a vector driving the expression of dnAkt were either untreated (slanted hatched bars), incubated with 500  $\mu$ M UDCA for 18 h (horizontal hatched bars) or 500  $\mu$ M DCA for 4 h (white bars), or preincubated with 500  $\mu$ M UDCA for 18 h before incubating with 500  $\mu$ M DCA for 4 h (grid bars). The number of apoptotic cells in the treated cultures was determined as described in **Materials and Methods**. Each bar represents the mean  $\pm$  SD of five independent experiments. The increment in apoptosis induced by DCA in UDCA-pretreated cells in dnAkt-transfected cells compared with control/vector-transfected HCT116 cells is statistically significant ( $P < 0.05$ ). The inset at the upper right depicts an immunoblot analysis of proteins extracted from control cells and from cells stably transfected with the dnAkt and shows that dnAkt is abundantly expressed in the stably transfected cells and not in the control cells. B: Western blotting using pGSK-3 $\beta$  antibody and  $\beta$ -actin antibody (loading control) was performed as described in **Materials and Methods** with 100- $\mu$ l protein extracts from UDCA-treated/untreated parental HCT116 cells (control), HCT116 stably transfected parental empty vector (vector), or a vector driving the expression of dnAkt. The result is representative of several independent experiments.

tive effect. Interestingly, inhibition of Akt resulted in phosphorylation of GSK-3  $\beta$  and induction of apoptosis in cells treated with UDCA. Hence, it seems that UDCA, although normally cytoprotective, also has the potential to activate apoptotic pathways but that UDCA's ability to activate Akt normally overcomes this.

Surprisingly, we found that DCA could also induce Akt phosphorylation and with somewhat more rapid kinetics than that seen in cells treated with UDCA. Why then are not cells treated with DCA protected from apoptosis? The reason for this may simply be a matter of timing. The onset of DCA-induced apoptosis occurs very rapidly, usually within minutes after exposure to this bile acid (26). Indeed, biochemical evidence of the onset of apoptosis is evident within 2 h after exposure to DCA (see Fig. 1B), which is well before this bile acid can induce Akt phosphorylation, which occurs only after about 8 h. Hence, Akt phosphorylation occurs after DCA-treated cells are committed to cell death at a point when the apoptotic process is irreversible. However, by preincubation with UDCA Akt is phosphorylated before exposure to DCA and before activation of the apoptotic program. These observations support the notion that these two bile acids exhibit overlapping activities but that their biological activities are a consequence of subtle differences in their actions on cellular signaling.

It has been suggested that UDCA's ability to function as an anti-apoptotic agent and to activate anti-apoptotic signaling may be important for its ability to ameliorate liver damage associated with cholestatic liver disease (4). However, the role that Akt activation may have in colon cancer chemoprevention is less clear. Indeed, activation of survival signaling is counterintuitive to accepted views of chemoprevention agent actions and colon cancer etiology. Recent evidence suggests that Akt up-regulation is an early event in human colon carcinogenesis and in azoxymethane (AOM)-treated rats (27). Hence, colon tumors may already have aberrant Akt activity. Therefore, any further up-regulation of Akt by UDCA may be irrelevant in this setting, a notion that is supported by the finding that UDCA can suppress tumor formation in AOM-treated rats (28). This would suggest that other UDCA-mediated activities may be more significant for colon cancer chemoprevention by UDCA. Ongoing experiments in our laboratory are currently addressing this issue.

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