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A novel, protective role of ursodeoxycholate in bile-induced pancreatic ductal injury

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Katona M, Hegyi P, Kui B, Balla Z, Rakonczay Jr Z, Rázga Z, Tiszlavicz L, Maléth J, Venglovecz V. A novel, protective role of ursodeoxycholate in bile-induced pancreatic ductal injury. *Am J Physiol Gastrointest Liver Physiol* 310: G193–G204, 2016. First published November 25, 2015; doi:10.1152/ajpgi.00317.2015.—We have previously shown that chenodeoxycholic acid (CDCA) strongly inhibits pancreatic ductal HCO_3^- secretion through the destruction of mitochondrial function, which may have significance in the pathomechanism of acute pancreatitis (AP). Ursodeoxycholic acid (UDCA) is known to protect the mitochondria against hydrophobic bile acids and has an ameliorating effect on cell death. Therefore, our aim was to investigate the effect of UDCA pretreatment on CDCA-induced pancreatic ductal injury. Guinea pig intrainterlobular pancreatic ducts were isolated by collagenase digestion. Ducts were treated with UDCA for 5 and 24 h, and the effect of CDCA on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), intracellular pH (pH_i), morphological and functional changes of mitochondria, and the rate of apoptosis were investigated. AP was induced in rat by retrograde intraductal injection of CDCA (0.5%), and the disease severity of pancreatitis was assessed by measuring standard laboratory and histological parameters. Twenty-four-hour pretreatment of pancreatic ducts with 0.5 mM UDCA significantly reduced the rate of ATP depletion, mitochondrial injury, and cell death induced by 1 mM CDCA and completely prevented the inhibitory effect of CDCA on acid-base transporters. UDCA pretreatment had no effect on CDCA-induced Ca^{2+} signaling. Oral administration of UDCA (250 mg/kg) markedly reduced the severity of CDCA-induced AP. Our results clearly demonstrate that UDCA *1*) suppresses the CDCA-induced pancreatic ductal injury by reducing apoptosis and mitochondrial damage and *2*) reduces the severity of CDCA-induced AP. The protective effect of UDCA against hydrophobic bile acids may represent a novel therapeutic target in the treatment of biliary AP.

acute pancreatitis; chenodeoxycholic acid; ursodeoxycholic acid; epithelial cells; mitochondria

ACUTE PANCREATITIS (AP) IS a sudden and severe disease in which no specific therapy or medication is currently available. Obstruction of the common biliopancreatic duct by a gallstone is a frequent cause of AP however, the exact mechanism is not completely known (1, 34). A number of theories have been proposed to explain how gallstones cause pancreatitis. One of the most accepted views is that bile reflux into the pancreatic ductal system leads to AP. This "bile reflux" theory is largely based on animal studies where retrograde infusion of bile acids into the pancreatic duct triggers pancreatitis (33, 35, 64). Therefore, the cytotoxic effects of bile acids have been widely investigated in the pancreas (15, 23, 59, 61). Initial studies, in which the main pancreatic duct was perfused with various bile acids, demonstrated that in the millimolar range, hydrophobic bile acids cause mucosal damage and increase the permeability of the pancreatic ducts to different ions (12, 39).

The cytotoxic effect of chenodeoxycholic acid (CDCA) is basically attributed to its detergent characteristic, which is responsible for the disruption of the membrane integrity and consequently the release of intracellular constituents. However, an increasing number of studies suggest that nondetergent effects of bile acids are also involved in the bile-induced cellular injury. The monohydroxy bile acid, taurolithocholic acid-3-sulfate (TLC-S) causes acinar injury through the induction of long-term elevation of intracellular calcium concentration ($[Ca^{2+}]_i$), mitochondrial membrane depolarization, and consequently intracellular ATP (ATP_i) depletion.(15, 61, 63).

The specific effects of bile acids have been also demonstrated in the pancreatic ductal epithelial cells (PDECs). Recent studies from our laboratory showed that the dihydroxy bile acid, CDCA, dose dependently decreased the intracellular pH (pH_i) and caused elevation of $[Ca^{2+}]_i$ in guinea pig pancreatic ducts (59). In addition, we showed that high concentration of this bile acid (1 mM) strongly inhibited both oxidative and glycolytic metabolism of the ductal cells and caused an irreversible depletion of ATP_i (29) Moreover, it has been recently demonstrated that CDCA induces ATP release from both ductal and acinar cells, which probably play a role in the CDCA-induced $[Ca^{2+}]_i$ elevation by the activation of P2 receptors (24). In the absence of ATP_i, the acid/base transporters fail to function properly, which finally causes decreased fluid and HCO_3^- secretion (29). Impaired fluid secretion can lead to pancreatic injury and likely contributes to the development of pancreatitis (11, 33, 49). We speculate that restoration of pancreatic ductal fluid and HCO_3^- secretion could be beneficial in the early phase of biliary pancreatitis.

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Ursodeoxycholic acid (UDCA) is a secondary, hydrophilic bile acid, which is currently used for gallstone dissolution and considered as first-choice therapy for various liver diseases, such as cholelithiasis, primary biliary cirrhosis or sclerosing cholangitis (10, 14, 36, 47). The mechanism by which UDCA increases liver function is not completely understood. Basically, there are three concepts for the action of UDCA: *1*) stimulation of hepatobiliary secretion, *2*) displacement of the hydrophobic, toxic bile acids from the liver, and *3*) direct cytoprotection against toxic bile acids. The cytoprotective effects of UDCA or its taurin-conjugated form, tauroursodeoxycholic acid (TUDCA), have been widely investigated in the liver. Studies on hepatocytes have shown that UDCA pretreatment significantly reduces bile acid-induced opening of the mitochondrial permeability transition pore (mPTP) and consequently apoptosis (8, 37, 41–43, 50), indicating that stabilization of the mitochondrial membrane, at least in part, plays an important role in the cytoprotective action of UDCA. Because the CDCA-induced failure in pancreatic ductal function is also strongly associated with mitochondrial damage, we wondered whether UDCA pretreatment is able to prevent the CDCA-induced ductal injury. Therefore, we tested whether the toxic effect of CDCA on pancreatic ducts can be attenuated by the hydrophilic bile acid, UDCA.

Using in vitro and in vivo approaches, we demonstrated for the first time that UDCA *1*) completely prevented the inhibitory effect of CDCA on ductal acid-base transporters, *2*) decreased the rate of CDCA-induced mitochondrial injury, *3*) and decreased the rate of CDCA-induced cell death, and *4*) reduced the severity of experimental AP induced by CDCA.

MATERIALS AND METHODS

Ethics. All experiments were conducted in compliance with the *Guide for the Care and Use of Laboratory Animals* (National Academies Press, 8th ed., 2011), with the 2010/63/EU guideline and the Hungarian 40/2013 (II.14.) government decree. The experiments were approved by the Committee on Investigations Involving Animals at the University of Szeged and also by independent committees assembled by local authorities (XII./3773/2012.).

Solutions and chemicals. The standard Na-HEPES solution contained (in mM): 130 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose, and 10 Na-HEPES. NH_4Cl-HEPES solution was supplemented with 20 mM NH_4Cl, while NaCl concentration was lowered to 110 mM. HEPES-buffered solutions were gassed with 100% O_2, and their pH was set to 7.4 with HCl. The standard HCO_3^-/CO_2-buffered solution contained (in mM): 115 NaCl, 25 $NaHCO_3$, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, and 10 D-glucose. NH_4Cl-HCO_3^-/CO_2 solution was supplemented with 20 mM NH_4Cl, while NaCl concentration was lowered to 95 mM. HCO_3^-/CO_2-buffered solutions were gased with 95% O_2-5% CO_2, and their pH was set to 7.4 with NaOH.

Chromatographically pure collagenase was purchased from Worthington (Lakewood, NJ). 2,7-Bis-(2-carboxyethyl)-5-(and-6-)carboxyfluorescein, acetoxymethyl ester (BCECF AM), 5-oxazolecarboxylic, 2-[6-[bis(carboxymethyl) amino]-5-[2-[2-[bis(carboxymethyl)amino]-5-methylphenoxy]-ethoxy]-2-benzofuranyl]-5-oxazolecarboxylic acetoxymethyl ester (fura-2 AM), calcein acetoxymethyl ester (calcein AM), tetramethylrhodamine methyl ester (TMRM) and magnesium green acetoxymethyl ester (MgGreen AM) were from Life Technologies (Grand Island, NY). Apoptotic cells were quantified by using an in situ cell death detection kit from Roche Diagnostics (Mannheim, Germany). Bile acids and all other chemicals were obtained from Sigma-Aldrich (Budapest, Hungary).

Isolation and culture of the ducts. Small intrainterlobular ducts were isolated from the pancreas of guinea pigs weighing 150–250 g. The guinea pig was humanely killed by cervical dislocation, the pancreas was removed and intrainterlobular ducts were isolated, as described previously (3). The ducts were cultured overnight in a 37°C incubator gased with 5% CO_2-95% air.

Bile acid treatments. Isolated pancreatic ducts were treated with bile acids as follows: no treatment (control group), 5-min CDCA (1 mM) treatment (CDCA group), 24-h UDCA (0.5 mM) treatment (UDCA group), and 24 h preincubation with 0.5 mM UDCA, and then parallel incubation for further 5 min with 1 mM CDCA (UDCA+CDCA group).

Measurement of intracellular Ca^{2+} concentration, pH, and ATP level. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), intracellular pH (pH_i), and intracellular ATP level (ATP_i) were measured by loading the pancreatic ducts with the Ca^{2+}-sensitive fluorescent dye, fura-2 AM (5 μM, 60 min, in the presence of 0.05% pluronic F-127), the pH-sensitive fluorescent dye, BCECF AM (2 μM, 30 min), and the Mg^{2+}-sensitive fluorescent dye, MgGreen AM (5 μM, 60 min, in the presence of 0.05% pluronic F-127), respectively. Ducts were attached to a poly-L-lysine-coated coverslip (24 mm) forming the base of a perfusion chamber and were mounted on the stage of an inverted fluorescence microscope linked to an Xcellence imaging system (Olympus, Budapest, Hungary). Ducts were then bathed with different solutions at 37°C at the perfusion rate of 5 or 6 ml/min. Six or seven region of interests (ROIs) were examined in each experiments, and one measurement per second was obtained.

To determine the changes of $[Ca^{2+}]_i$, cells were excited with 340- and 380-nm wavelength, and the changes in $[Ca^{2+}]_i$ were calculated from the 340/380 fluorescence ratio measured at 510 nm. To estimate pH_i, cells were excited with 490- and 440-nm wavelength, and the 490/440 fluorescence emission ratio was measured at 535 nm. The calibration of the fluorescent emission ratio to pH_i was performed with the high-K^+-nigericin technique, as previously described (19, 56). Changes in $[ATP]_i$ was determined by exciting the cells at a wavelength of 490 nm, with emitted light monitored at 535 nm. Fluorescence signals were normalized to initial fluorescence intensity (F/F_0) and expressed as relative fluorescence.

Measurement of acid/base transporter activity. To estimate the activity of Na^+/H^+ exchanger (NHE), the Na^+/HCO_3^- cotransporter (NBC), and Cl^-/HCO_3^- exchanger (CBE), the NH_4Cl prepulse technique was used. Briefly, exposure of pancreatic ducts to 20 mM NH_4Cl for 3 min induced an immediate rise in pH_i due to the rapid entry of lipophilic, basic NH_3 into the cells. After the removal of NH_4Cl, pH_i rapidly decreased. This acidification is caused by the dissociation of intracellular NH_4^+ to H^+ and NH_3, followed by the diffusion of NH_3 out of the cell. In standard HEPES-buffered solution, the initial rate of pH_i ($\Delta pH/\Delta t$) recovery from the acid load (over the first 60 s) reflects the activities of NHEs, whereas in HCO_3^-/CO_2-buffered solutions represents the activities of both NHEs and NBC (18, 20). To estimate CBE activity, the initial rate of pH_i recovery from alkalosis in HCO_3^-/CO_2-buffered solutions was analyzed. Previous data have indicated that under these conditions, the recovery over the first 30 s reflects the activity of CBE (18, 20).

To evaluate transmembrane base flux [$J(B^-)$], the following equation was used: $J(B^-) = \Delta pH/\Delta t \times \beta_{total}$, where $\Delta pH/\Delta t$ was calculated by linear regression analysis; the total buffering capacity (β_{total}) was estimated by the Henderson-Hasselbach equation using the following formula: $\beta_{total} = \beta_i + \beta_{HCO_3^-} = \beta_i + 2.3 \times [HCO_3^-]_i$. We denote base influx as $J(B)$ and base efflux (secretion) as $J(B^-)$.

Measurement of mitochondrial membrane potential. Mitochondrial membrane potential ($\Delta\Psi_m$) was measured using the lipophilic, mitochondria-selective fluorescence dye, TMRM. Accumulation of TMRM in the mitochondria depends on the $\Delta\Psi_m$. Pancreatic ducts were preincubated with TMRM (1 μM) for 30 min at 37°C and were transferred to a poly-L-lysine-coated coverslip (24 mm) forming the

base of a perfusion chamber. Ducts were then perfused continuously with solutions at 37°C at a rate of 2–2.5 ml/min. The perfusion solutions were complemented with 100 nM TMRM to avoid dye leakage. Changes in $\Delta\Psi_m$ were monitored using a FluoView 10i-W confocal microscope (Olympus, Budapest, Hungary). Five to ten ROIs (mitochondria) of 5–10 cells were excited with light at 543 nm, and the emitted light was captured between 560 and 650 nm. We have used the dequench method for the estimation of $\Delta\Psi_m$. At the applied concentration of TMRM, depolarization of the mitochondria causes release of the dye and its dequenching in the cytosol. Thus, increase in fluorescence intensity reflects a decrease in $\Delta\Psi_m$. Fluorescence signals were normalized to initial fluorescence intensity (F/F_0) and were expressed as relative fluorescence.

Measurement of mitochondrial permeability transition pore opening. To measure mitochondrial inner membrane permeabilization and/or the opening of the mitochondrial permeability transition pore (mPTP), we used the calcein-cobalt dequenching technique. Calcein AM is a lipid-soluble fluorescent dye that diffuses into all subcellular components, including mitochondria. Co^{2+} is a quencher of calcein, which can enter to the cytoplasm but cannot pass through the mitochondrial membrane. Upon transient opening of mPTP, Co^{2+} diffuses into the mitochondria and quenches the mitochondrial calcein fluorescence, which results in a decrease of fluorescence intensity. Pancreatic ducts were loaded with calcein-AM (1 μM) for 30 min, and then with $CoCl_2$ (1 mM) for a further 10 min. Ducts were then washed at 37°C at the perfusion rate of 5–6 ml/min and imaged using an Olympus IX71 fluorescence microscope (Olympus, Budapest, Hungary). Five or six ROIs were excited with light at 495 nm, and the emitted light was captured at 515 nm. Fluorescence signals were normalized to initial fluorescence intensity (F/F_0) and expressed as relative fluorescence.

TUNEL cell death assay. For detection of cell death, we used the terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick-end labeling (TUNEL) assay (Roche Diagnostics). Intrainterlobular pancreatic ducts were treated with bile acids, as previously described. Control and bile acid-treated isolated duct segments were fixed with 4% paraformaldehyde overnight and then cryosectioning and staining of the samples were performed, according to the manufacturer's protocol. The CDCA-treated groups were incubated for 3 h in culture media before fixation. Pictures were taken with the use of a Zeiss AxioImager fluorescent light microscope (Carl Zeiss MicroImaging, Thornwood, NY) fitted with a PixeLINK CCD camera (PixeLINK, Ottawa, ON, Canada).

Transmission electron microscopy. For electron microscopic studies, ducts were fixed in 2.5% glutaraldehyde immediately after isolation. Samples were then postfixed in 1% osmium tetroxide, dehydrated in a series of graded ethanols, and subsequently embedded in epoxy resin. Ultrathin sections were contrasted with uranyl acetate and lead citrate. Tissue sections were analyzed under a transmission electron microscope (CM10; Philips, Eindhoven, The Netherlands).

Induction of acute pancreatitis. Male Sprague-Dawley rats weighing 200–250 g were used for all experiments. Rats were kept at constant room temperature of 24°C with a 12:12-h light and dark cycle and were allowed free access to tap water and standard laboratory chow (Biofarm, Zagyvaszaántó, Hungary). Animal were fasted 12 h before the surgical procedure. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Szeged (I-74-3/2012 MÁB) and also by an independent committee assembled by national authorities (XII./3773/2012.). Rats were randomly allocated into four groups ($n = 6$) as follows: *1*) control (intraductal administration of physiological saline), *2*) UDCA (rats were treated with oral UDCA without induction of pancreatitis), *3*) CDCA (pancreatitis was induced by intraductal administration of CDCA), and *4*) UDCA+CDCA (rats were treated with oral UDCA and pancreatitis was induced with intraductal administration of CDCA). Ursodeoxycholic acid (Ursofalk) was purchased from Dr. Falk Pharma. UDCA was dissolved in 2 ml of tap water and was administered orally (gavage) 250 mg/kg body wt daily for 2 wk. The pretreatment period was chosen on the basis of studies investigating cholestatic diseases (26, 57). Control animals were only treated with the same amount of tap water. The last UDCA treatment was performed one day before the AP induction.

Rats were anesthetized with intraperitoneal injection of a cocktail containing of 50 mg/kg ketamine and 10 mg/kg xylazine. After the anesthesia, rats were shaved, and the abdominal cavity was opened with median laparotomy. The common bile duct was temporarily occluded with a vessel clip. The duodenum was punctured with a 0.4-mm diameter needle connected to polyethylene tubing, then it was placed into the pancreatic duct and 1 ml/kg 1% sodium-CDCA (dissolved in physiologic saline) was administered via retrograde ductal infusion with 1 ml/min speed with an infusion pump (TSE System, Bad Homburg, Germany). Control animals received intraductal physiological saline instead of bile acid. After bile acid or physiological saline infusion, the vessel clip and the polyethylene tube were removed, and the abdominal wall and skin were closed. Rats were killed 24 h after the surgical procedure.

Histologic examination and laboratory parameter measurements. Terminal anesthesia was performed with 50 mg/kg pentobarbital sodium. The abdominal and thoracic cavities were opened with a median laparotomy and thoracotomy and blood was collected with cardiac puncture. The pancreas was removed immediately after the blood collection, and it was trimmed from fat and lymphatic tissues on ice. Pancreata were dissected and only parts of the head and body were used for histological analysis and for laboratorial measurements. Sections from the pancreatic tail did not show any signs of necrosis or inflammation; therefore, it was not used later. The pancreatic head and body were longitudinally dissected, and one part of pancreas was put into 6% neutral formaldehyde solution, while the other part was immediately frozen in liquid nitrogen. These parts of pancreata were stored at −80°C until further use. The collected blood was centrifuged at 4°C with 2500 RCF for 15 min. Sera were eventually stored at −20°C until use.

Serum amylase activity was measured with a commercial colorimetric kit (Diagnostikum, Budapest, Hungary) with a FLUOstar OPTIMA (BMG Labtech, IronMaas Consulting, Budapest Hungary) microplate reader at 405 nm. Wet pancreatic tissues were measured, and they were dried for 24 h at 100°C. The dry weight-wet weight ratio was calculated. Pancreatic samples were prepared for hematoxylin-and-eosin staining. Pancreatic histological samples were scanned. Necrotic areas were detected and analyzed with Image J (National Institutes of Health, Bethesda, MD) software. Necrotic areas were compared with the total analyzed pancreatic area.

Statistical analysis. Data are expressed as means ± SE. Significant difference between groups was determined by ANOVA. Statistical analysis of the immunohistochemical data was performed using the Mann-Whitney *U*-test. Probability values of $P < 0.05$ were accepted as being significant.

In vivo experiments were evaluated by using ANOVA followed by Bonferroni or Dunnett's multiple-comparison post hoc test.

RESULTS

Effect of UDCA and CDCA on intracellular pH. Previous studies have shown that bile acids induce intracellular acidification in several cell types (2, 16, 32, 59). Therefore, first, we investigated the effect of CDCA and UDCA on basal intracellular pH (pH_i) of pancreatic ducts. Administration of CDCA induced a dose-dependent intracellular acidification in HEPES-buffered solution (Fig. 1*A*). The effect of CDCA on pH_i was reversible, following the removal of bile acid from the external solution, the pH_i completely returned to the basal level. UDCA also induced a dose-dependent decrease in pH_i; however, the effect of UDCA was much smaller compared with CDCA (Fig.

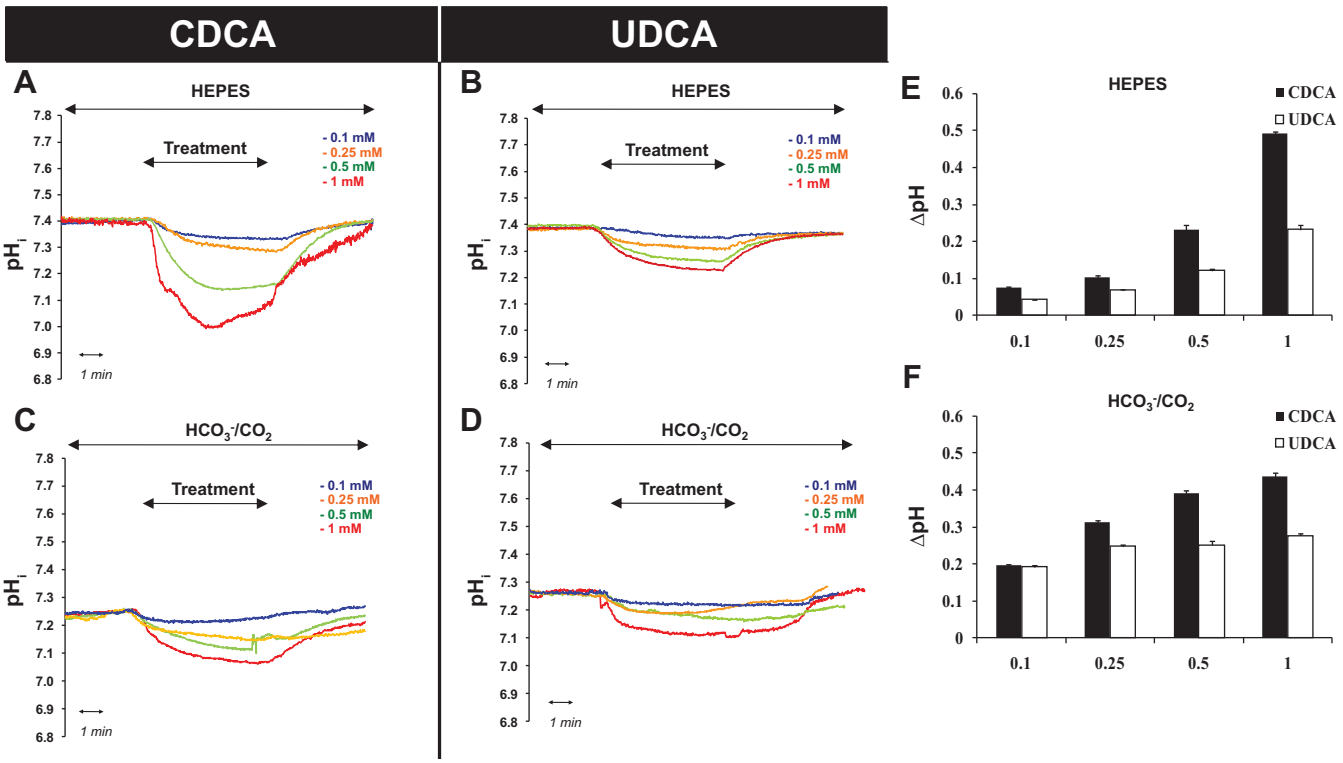


Fig. 1. Effect of bile acids on the intracellular pH (pH_i) of isolated guinea pig pancreatic ducts. Pancreatic ducts were exposed to 0.1, 0.25, 0.5, and 1 mM chenodeoxycholic acid (CDCA; *A* and *C*) or ursodeoxycholic acid (UDCA; *B* and *D*) in HEPES- (*A* and *B*) and in HCO_3^-/CO_2-buffered (*C* and *D*) solutions for 5 min. After the removal of bile acids, the intracellular pH (pH_i) spontaneously recovered. Bar charts show summary data for the maximal pH_i change (ΔpH_{max}) induced by bile acids in HEPES- (*E*) and in HCO_3^-/CO_2-buffered (*F*) solutions. The starting pH_i for the measurement of ΔpH was the pH_i immediately before exposure to bile acids. Data are presented as means ± SE; n = 32–34 regions of interest from 4 or 5 ducts.

1*B*). We repeated these experiments in HCO_3^-/CO_2-buffered solution, where the two bile acids induced similar degrees of pH_i decrease (Fig. 1, *C* and *D*). Summary of the changes of pH_i is shown in Fig. 1, *E* and *F*.

UDCA pretreatment prevents the inhibitory effect of CDCA on acid-base transporters. To investigate the acid-base transporters of pancreatic ducts, we used the ammonium prepulse technique. In the absence of HCO_3^-, the initial recovery from the acid phase is due to the activity of the NHE (Fig. 2*A*). In the presence of HCO_3^-, the recovery from alkalosis reflects the activity of the Cl^-/HCO_3^- exchanger (CBE), whereas the recovery from acidosis results from the activity of both NHE and Na^+/HCO_3^- cotransporter (NBC) (Fig. 2*B*). Two NH_4Cl pulses were applied: the first was the control, and the second was the test. To estimate the effect of bile acids on the activity of acid-base transporters CDCA (1 mM) and UDCA (0.1, 0.25, 0.5, and 1 mM) were administered 3 min before the pulse, during the pulse, and 5 min after the pulse. Acute administration of UDCA did not affect the rate of recovery from the acid or alkali load either in HEPES-buffered nor in HCO_3^-/CO_2-buffered solutions (data not shown). In contrast, 1 mM CDCA strongly inhibited the activity of the acid-base transporters. (Fig. 2, *A* and *B*) Next, we tested whether UDCA administration can influence the inhibitory effect of CDCA. When UDCA and CDCA were added simultaneously, UDCA was unable to prevent the inhibitory effect of CDCA on the ion transporters in all of the investigated concentration (data not shown). Several studies have shown that prolonged preincubation of the cells with UDCA is needed to exert its protective effect (21, 22, 48, 50). Therefore, in the next step, we pretreated the ducts with UDCA for various time periods (5 h and 24 h), and the effect of CDCA on the ion transporters was examined. A 5-h preincubation of the ducts with UDCA (0.1, 0.25, 0.5, and 1 mM) did not affect the response to CDCA. However, 24-h pretreatment with UDCA significantly decreased the toxic effect of CDCA both in HEPES (Fig. 2*A*) and in HCO_3^-/CO_2-buffered (Fig. 2*B*) solutions. The lowest concentration of UDCA, which had a protective effect was 0.5 mM (Fig. 2, *A* and *B*), whereas at higher concentrations (1 mM), the protective effect of UDCA was significantly decreased. Therefore, we decided to use 0.5 mM UDCA in the subsequent experiments. Summary data of the base fluxes [$\pm J(B^-/min)$] are shown on Fig. 2, *C–E*. As shown, pretreatment of the ducts with 0.5 mM UDCA prevented the inhibitory effect of CDCA on NHE (Fig. 2*C*), CBE (Fig. 2*D*), and NBC (Fig. 2*E*).

UDCA pretreatment had no effect on the CDCA-induced calcium signaling. Several studies have indicated that bile-induced toxic calcium signaling is an initial step in the development of AP (25, 31, 38, 58). We have previously shown that high concentration of CDCA induces (Ca^{2+}) elevation in ductal cells (59). So, in the next step, we have examined whether UDCA pretreatment has any effect on the CDCA-induced Ca^{2+} signaling. Administration of 0.5 mM UDCA alone had no significant effect on $[Ca^{2+}]_i$ (data not shown). In contrast, 1 mM CDCA induced high and partially reversible Ca^{2+} signaling in pancreatic ducts. (Fig. 3*A*) Preincubation of

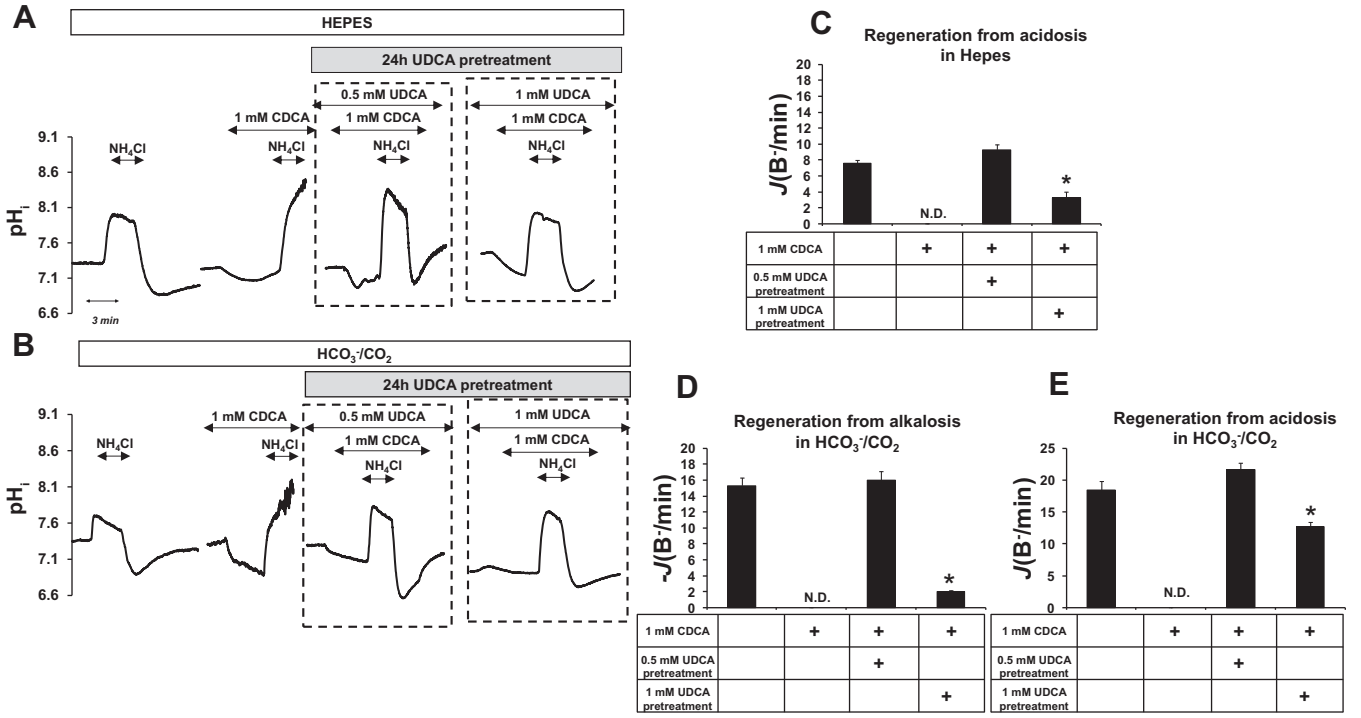


Fig. 2. Effect of bile acids on the acid-base transporters of isolated guinea pig pancreatic ducts. The activities of the ion transporters were investigated by the ammonium prepulse technique (see MATERIALS AND METHODS). Administration of 1 mM CDCA was proven to be toxic on acid-base transporters both in standard HEPES- (*A*) and HCO_3^-/CO_2-buffered (*B*) solutions. 0.5 and 1 mM UDCA pretreatment ameliorated the CDCA-induced toxicity on the transporters both in HEPES- (*A*) and in HCO_3^-/CO_2-buffered (*B*) solutions. Regeneration from acidosis in standard HEPES-buffered solution reflects the activity of Na^+/H^+ exchanger (NHE; *C*), whereas the recovery from acidosis (*D*) and alkalosis (*E*) in HCO_3^-/CO_2-containing solutions reflects the activities of Na^+/HCO_3^- cotransporter and Cl^-/HCO_3^- exchanger, respectively. $-J(B^-)$ and $J(B^-)$ were calculated from the $\Delta pH/\Delta t$ obtained by linear regression analysis of pH_i measurements made over the first 30 or 60 s, respectively. Data are presented as means ± SE. $^*P \leq 0.05$ vs. the control; $n = 36–41$ regions of interest from 6 or 7 ducts. N.D., not detectable.

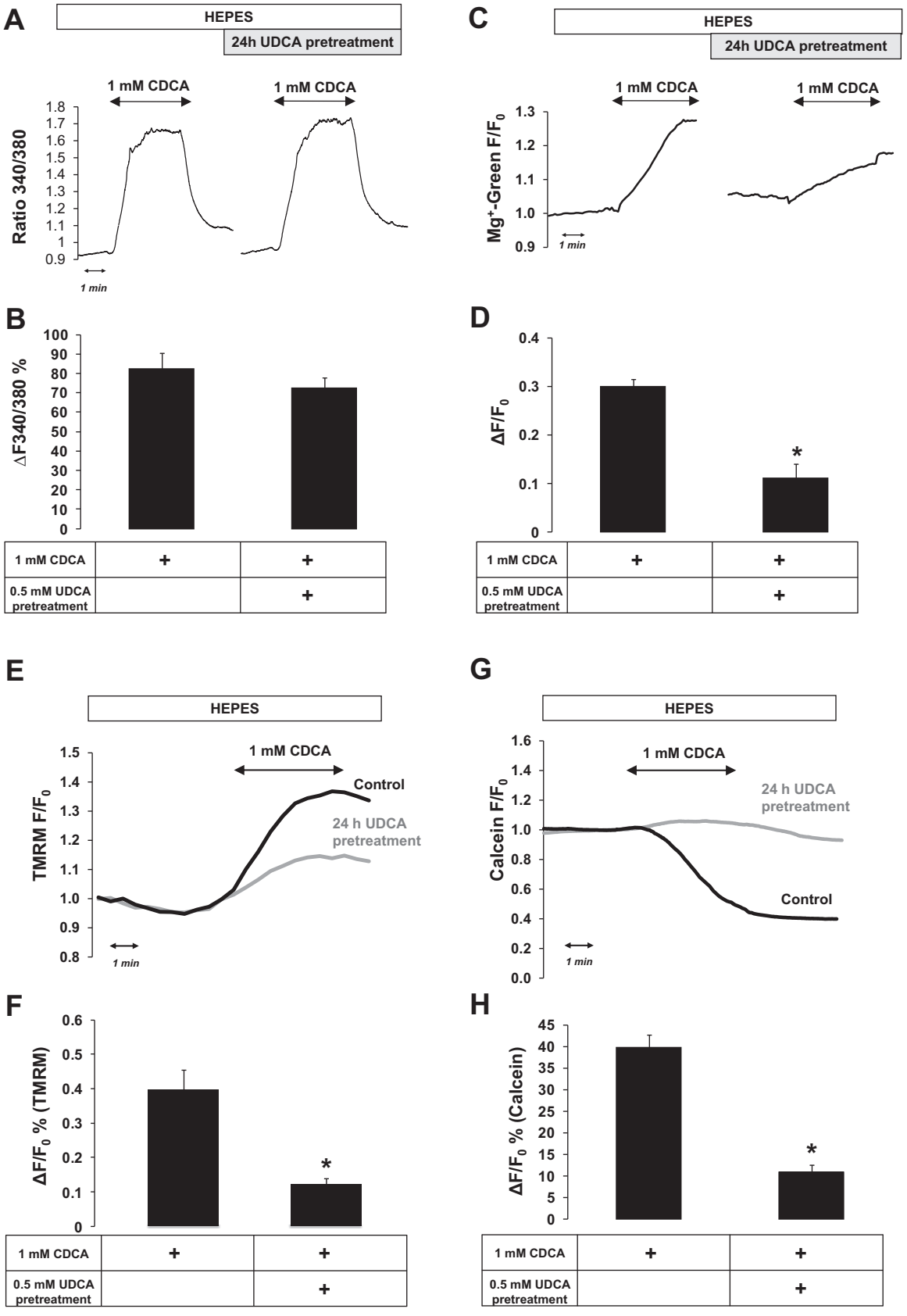
the ducts with 0.5 mM UDCA for 24 h did not affect the CDCA-induced increase in $[Ca^{2+}]_i$, (Fig. 3, *A* and *B*), indicating that the protective effect of UDCA is unlikely to be caused by the prevention of the elevated $[Ca^{2+}]_i$.

UDCA pretreatment decreased the rate of CDCA-induced mitochondrial injury. Increasing number of studies indicate the importance of mitochondrial injury in cell death (27, 28). We have previously shown that beside the elevated Ca^{2+} signaling, CDCA strongly damages mitochondria and causes ATP_i depletion in pancreatic ducts, which may contribute to the inhibitory effect of CDCA on ion transporters due to bioenergetic failure (29). Therefore, in the next series of experiments we performed both functional and morphological studies to characterize the effect of UDCA pretreatment on CDCA-induced mitochondrial injury.

The level of ATP_i is a good indicator of mitochondrial function; therefore, in the first step, we measured changes in ATP_i in cells exposed to CDCA with or without UDCA pretreatment. As shown on Fig. 3*C*, administration of 1 mM CDCA caused a huge and irreversible ATP_i loss in PDECs (Fig. 3*C*). In contrast, 24 h preincubation of the cells with 0.5 mM UDCA reduced the rate of CDCA-induced ATP_i decrease by 57.6 ± 3.6% (Fig. 3, *C* and *D*). UDCA administration alone did not affect ATP_i.

To further analyze the protective effect of UDCA on ductal mitochondria, we also investigated $\Delta\Psi_m$ and mPTP upon administration of bile acids. Ducts were incubated with 1 μM TMRM to investigate the changes in $\Delta\Psi_m$ (see MATERIALS AND METHODS). After the stabilization of the mitochondrial fluorescence, 1 mM CDCA was applied and the fluorescence signal was monitored. As shown in Fig. 3*E*, the administration of CDCA resulted in a huge increase in TMRM fluorescence intensity, indicating that this bile acid induced marked mitochondrial depolarization. Preincubation of the ducts

Fig. 3. Effect of bile acids on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), ATP level (ATP_i), mitochondrial membrane potential ($\Delta\Psi_m$), and mitochondrial permeability transition pore (mPTP) opening of pancreatic ducts. Representative experimental traces showing the effect of 1 mM CDCA on $[Ca^{2+}]_i$ (*A*), ATP_i (*C*), $\Delta\Psi_m$ (*E*), and mPTP opening (*G*) in nonpretreated and 24-h UDCA pretreated pancreatic ducts. *A*: acute administration of CDCA caused Ca^{2+} signalization in pancreatic ductal cells, which was not affected by UDCA pretreatment. *C*: decrease in ATP_i is shown by an increase in fluorescence intensity. Twenty-four hours of UDCA pretreatment markedly decreased the CDCA-induced ATP depletion. *E*: decrease in $\Delta\Psi_m$ is shown by an increase in tetramethylrhodamine methyl ester (TMRM) fluorescence intensity. Administration of 1 mM CDCA caused rapid depolarization of the mitochondrial membrane, which was decreased by UDCA pretreatment. *G*: mPTP opening is shown by a decrease in fluorescence intensity. CDCA-induced a marked mPTP opening that resulted in significant influx of Co^{2+} into the mitochondria causing decrease in calcein fluorescence. Twenty-four hours of UDCA pretreatment reduced CDCA-induced mPTP opening resulting in milder decrease in calcein fluorescence. Summary data for the maximal fluorescence intensity changes are shown in *B*, *D*, *F*, and *H*. Data are presented as means ± SE. $^*P \leq 0.05$ vs. 1 mM CDCA; $n = 35–40$ regions of interest from 6 or 7 ducts.


A
HEPES
24h UDCA pretreatment
1 mM CDCA
1 mM CDCA
Ratio 340/380
1 min
B
ΔF340/380 %
1 mM CDCA + +
0.5 mM UDCA pretreatment + +
C
HEPES
24h UDCA pretreatment
1 mM CDCA
1 mM CDCA
Mg+-Green F/F0
1 min
D
ΔF/F0
*
1 mM CDCA + +
0.5 mM UDCA pretreatment + +
E
HEPES
1 mM CDCA
Control
24 h UDCA pretreatment
TMRM F/F0
1 min
F
ΔF/F0 % (TMRM)
*
1 mM CDCA + +
0.5 mM UDCA pretreatment + +
G
HEPES
1 mM CDCA
24 h UDCA pretreatment
Control
Calcein F/F0
1 min
H
ΔF/F0 % (Calcein)
*
1 mM CDCA + +
0.5 mM UDCA pretreatment + +

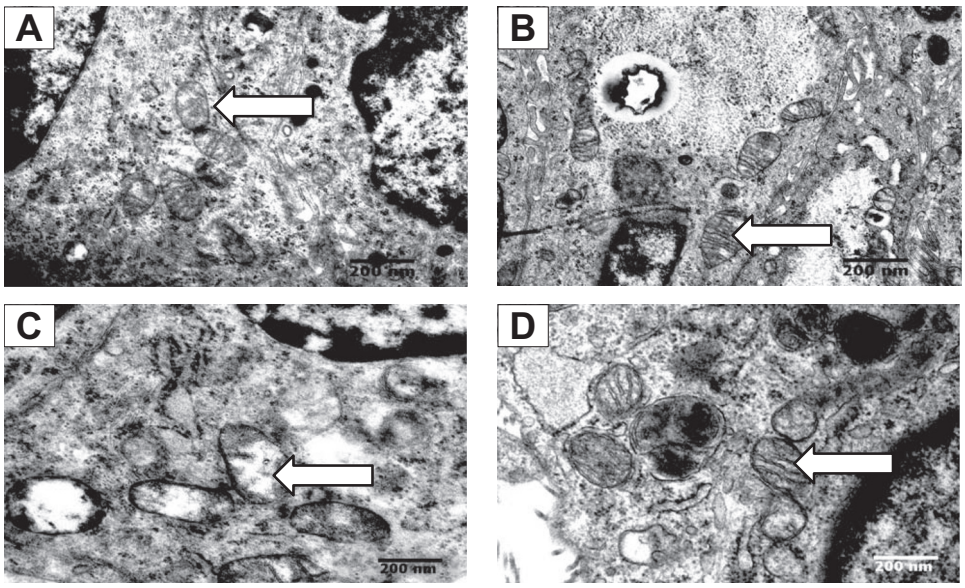


Fig. 4. Effect of bile acids on the morphology of pancreatic ductal mitochondria. Representative electron micrograph images of pancreatic ductal mitochondria show normal mitochondria with intact inner membranes in the control (*A*) and 24 h UDCA (0.5 mM) pretreated ducts (*B*). Treatment with 1 mM CDCA caused mitochondrial swelling after 5 min (*C*), which was completely prevented by 24 h UDCA (0.5 mM) pretreatment (*D*). Arrows show mitochondria.

with 0.5 mM UDCA reduced the CDCA-induced depolarization by 69.4 ± 4.6% (Fig. 3, *E* and *F*).

Because ATP depletion and mitochondrial depolarization are caused by mPTP induction, next we investigated the effect of CDCA on the opening of mPTP using the calcein-cobalt technique. Treatment of the calcein-loaded pancreatic ducts with 1 mM CDCA decreased the fluorescence excitation of calcein, ~1 min after the addition of the bile acid. (Fig. 3*G*) Similarly to the $\Delta\Psi_m$ experiments, 24-h pretreatment of the ducts with 0.5 mM UDCA had a protective role in mitochondria and decreased the CDCA-induced mPTP opening by 72.1 ± 4%. (Fig. 3, *G* and *H*) Administration of UDCA alone did not affect mPTP.

We also examined the morphology of the ductal mitochondria using electron microscopy (Fig. 4, *A–D*). The mean number of mitochondria in the section of the control, the CDCA, the UDCA, and the CDCA+UDCA groups was nearly the same. No morphological alterations were observed in the control and UDCA-treated groups. (Fig. 4, *A* and *B*) In contrast, incubation of the ducts with 1 mM CDCA for 5 min resulted in mitochondrial swelling and the loss of the mitochondrial inner membrane (Fig. 4*C*). This swelling could be prevented by UDCA pretreatment. Moreover, the integrity of the mitochondria was also maintained in the CDCA+UDCA-treated ducts compared with the ducts only treated with CDCA (Fig. 4*D*).

UDCA pretreatment prevented the CDCA-induced cell death. In the next step, we tested whether the toxic effect of CDCA on the mitochondria is associated with cell death. Ducts were treated with 1 mM CDCA for 5 min, then they were incubated in culture media for further 3 h to leave time for development of cell death. Cell death was assessed by TUNEL staining (Fig. 5, *A* and *B*). This method is based on the labeling of the 3′-OH ends of the fragmented DNA, which are generated during cell death. Incubation of the pancreatic ducts with CDCA resulted in a significant increase in cell death compared with control, nontreated ducts. (Fig. 5, *A* and *B*) Although, both apoptosis and necrosis can be characterized by DNA fragmentation (17), the presence of intact cell organelles, cellular shrinkage, and the lack of cellular content release indicate that CDCA rather induces apoptotic cell death than necrosis. Twenty-four hour preincubation with UDCA (0.5 mM) alone caused only a small degree of DNA fragmentation in the ductal cells,

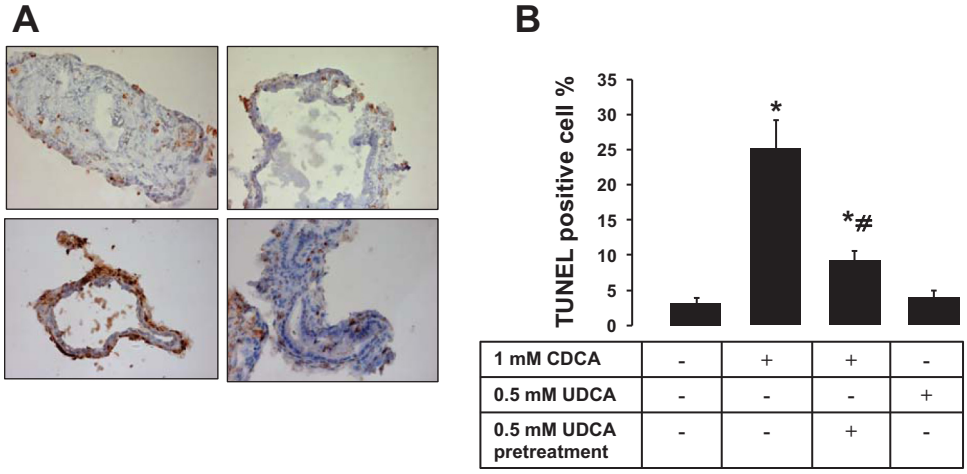


Fig. 5. Effect of UDCA pretreatment on CDCA-induced cell death on pancreatic ductal epithelial cells (PDECs). *A*: representative pictures of TUNEL-positive and eosin-stained pancreatic duct segments show that incubation of the pancreatic ducts with 1 mM CDCA (*left bottom*), increased the rate of dead cells compared with control (*top*, *left*) nontreated ducts. Five-minute administration of UDCA (0.5 mM) alone, had no significant effect on the cells (*top*, *right*), whereas 24-h pretreatment of the ducts with 0.5 mM UDCA (*bottom*, *right*) decreased the rate of dead cells in the CDCA-treated group. *B*: bar chart shows the percentage of total cell counts that are TUNEL positive. Data are presented as means ± SE. $*P \leq 0.05$ vs. Control, $\#P \leq 0.05$ vs. 1 mM CDCA, $n = 3$ or 4.

but significantly reduced the CDCA-induced apoptotic cell death by 63.3 ± 5.7%. (Fig. 5, *A* and *B*)

Oral administration of UDCA attenuates CDCA-induced pancreatitis in vivo. To investigate the protective effect of UDCA under in vivo conditions, we utilized a CDCA-induced pancreatitis model (53, 54). We used rats as the model animals because we were unable to administer CDCA intraductally in guinea pigs due to the anatomical topography of the main duct.

Serum amylase activities were significantly elevated after retrograde infusion of CDCA (CDCA group: 983 ± 100 U/l) compared with intraductally administered physiological saline (control group: 396 ± 50 U/l). Pretreatment of UDCA for 2 wk did not influence the serum amylase activities (UDCA group: 424.7 ± 20) in control animals; however, it was significantly decreased (582 ± 50 U/l) in the UDCA+CDCA group vs. the CDCA group (Fig. 6*A*).

Pancreatic water content was significantly elevated after retrograde infusion of CDCA (80 ± 1%) compared with intraductally administered physiological saline (60 ± 1%). A 2-wk pretreatment with UDCA showed no influence on the pancreatic water content (61 ± 2%) in the UDCA group. However, in the UDCA+CDCA group, it was significantly decreased (65 ± 2%) vs. the CDCA group (Fig. 6*B*).

Pancreatic damage was characterized by determining the extent of tissue necrosis (Fig. 6, *C* and *D*). Intraductal infusion of physiological saline with or without UDCA pretreatment did not cause any acinar cell necrosis. However, retrograde infusion of CDCA caused markedly elevated (14 ± 2%) acinar cell damage, which was significantly ameliorated (6 ± 2%) in the UDCA+CDCA group (Fig. 6*C*).

DISCUSSION

Bile reflux into the pancreas may lead to pancreatic injury and as a result can induce AP (34). Therefore, a better understanding of the mechanisms underlying bile-induced pancreatic injury and its prevention may provide novel therapeutic tools for the treatment of AP. In the present study, we have shown for the first time that the hydrophilic bile acid UDCA is able to attenuate the toxic effect of CDCA on pancreatic ducts by preventing CDCA-induced mitochondrial injury.

We used isolated intrainterlobular pancreatic ducts, which is a reliable in vitro model for investigating pancreatic ductal damage. The CDCA concentration used in this study was previously shown to induce intracellular Ca^{2+} signaling, mitochondrial injury, and inhibition of the acid-base transporters (29, 59), whereas the concentrations of UDCA were chosen on the basis of the literature data (42, 43). Examinations were performed using 0.5 mM UDCA, since the protective effect of UDCA was not increased by using higher concentrations (1 mM), whereas lower concentrations of this bile acid (0.1 and 0.25 mM) had no detectable protective effect. This concentration of UDCA is one magnitude higher than the physiological concentration of bile acid in the blood (9) and several orders of magnitude lower than the concentration of bile acids in the gall bladder or duodenum (10–100 mM). The optimal preincubation time of pancreatic ducts with UDCA was found to be 24 h, indicating that the development of protective action is a complex mechanism, which probably includes changes at the transcriptional level that modulate various signaling and apoptotic pathways (42, 43, 51, 52).

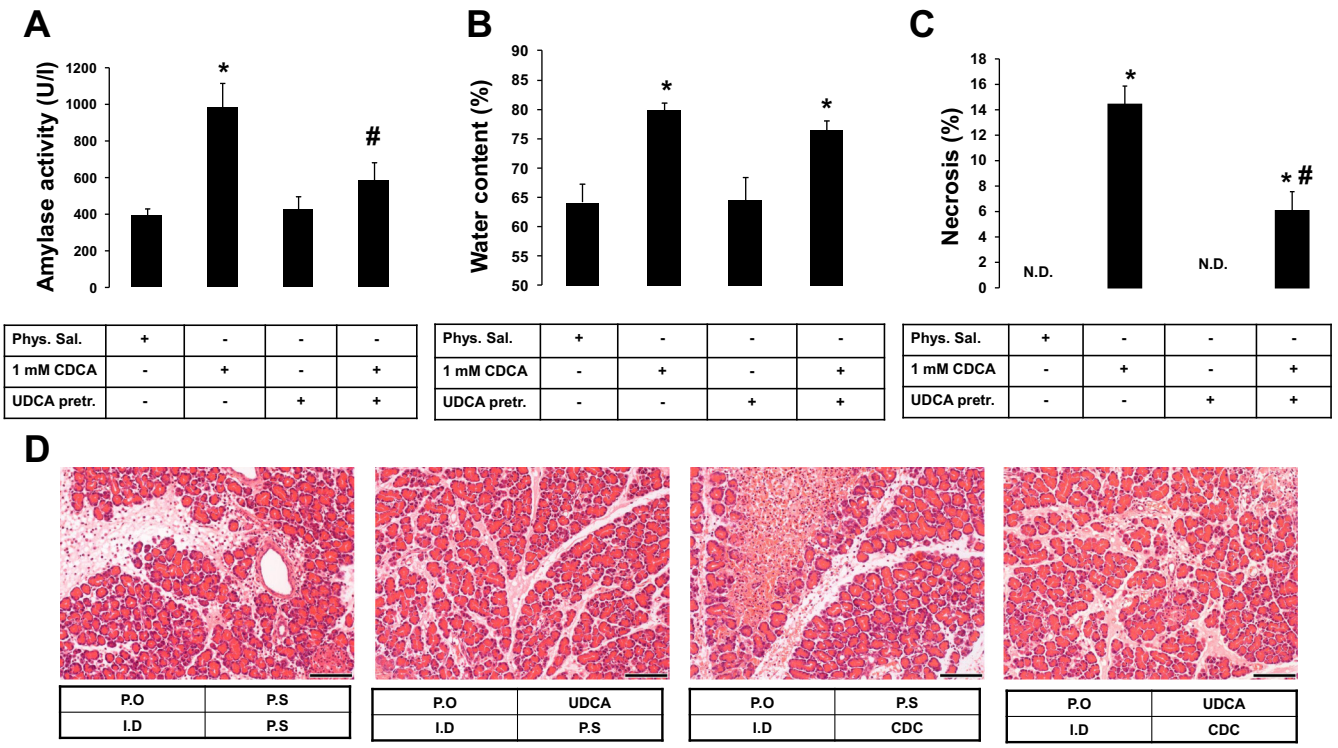


Fig. 6. Effect of UDCA pretreatment on CDCA-induced pancreatitis in rats. Serum amylase activity (U/l) (*A*), pancreatic edema (*B*), and cell necrosis (*C*) were significantly increased by intraductal administration of CDCA. However, 2 wk of UDCA pretreatment ameliorated each of these severity parameters. *D*: representative histological images of control, UDCA-pretreated, CDCA-induced acute pancreatitis, and UDCA-pretreated, CDCA-induced acute pancreatitis animals. Control rats were given physiological saline instead of CDCA. PS, physiological saline; P.O., per os; I.D., intraductal. Data are presented as means ± SE. *$P \leq 0.05$ vs. Control. #$P \leq 0.05$ vs. 1 mM CDCA; $n = 6$ animals in each groups. Scale bar = 100 μm.

In the present study, we demonstrated that 24-h pretreatment of pancreatic ducts with 0.5 mM UDCA significantly reduced the inhibitory effect of CDCA on the acid/base transporters. Studies on pancreatic acinar cells have indicated that the toxic effect of hydrophobic bile acids is mediated by a sustained Ca^{2+} signaling (15, 61). We have previously shown that a high concentration of CDCA induces a huge and long-lasting elevation of $[Ca^{2+}]_i$ in pancreatic ducts.(59) Therefore, we tested whether the protective effect of UDCA on the acid/base transporters is due to the prevention of CDCA-induced calcium signaling. When pancreatic ducts were exposed to UDCA for 24 h and CDCA was then added, the extent of calcium elevation did not change, indicating that the protective effect of UDCA is unlikely to be caused by the reduction of Ca^{2+} signaling. This finding is in accordance with our previous observation on pancreatic ducts, where preincubation of the cells with a specific calcium chelator, BAPTA AM was unable to prevent the inhibitory effect of CDCA on acid-base transporters (59).

An increasing number of studies support the concept that mitochondrial damage plays a central role in the bile-induced cellular injury and that UDCA pretreatment is able to attenuate the toxic effect of hydrophobic bile acids on mitochondria.(8, 41–43) Therefore, in the next step, we investigated the protective effect of UDCA on the function and morphology of pancreatic ductal mitochondria. Administration of CDCA alone induced mPTP opening in the ductal cells. This is an early event in cell death that leads to mitochondrial swelling due to an increase in the mitochondrial inner membrane permeability. mPTP is also characterized by the loss of membrane potential, which leads to mitochondrial dysfunction and consequently inhibition of ATP synthesis. We did not investigate the exact mechanism by which CDCA induces mPTP opening. Studies on rat hepatocytes demonstrated that in the presence of the specific mPTP inhibitor, cyclosporine A, the effect of CDCA was completely ameliorated, indicating that CDCA selectively acts on mPTP (45). It has been also demonstrated that CDCA induces mPTP due to increased membrane fluidity and cytochrome-*c* release in calcium-loaded hepatic mitochondria (44). In contrast to the effects of CDCA, UDCA alone caused no significant changes in mitochondrial function. However, in combination with CDCA, UDCA was able to prevent the CDCA-induced mPTP, mitochondrial membrane perturbation, and the consequently formed decrease in membrane potential. Moreover, UDCA prevented the CDCA-induced ATP_i loss, which provides further evidence that UDCA pretreatment is beneficial to avoid mitochondrial injury. This conclusion was confirmed by electron microscopic studies, which showed normal appearance of mitochondria in the UDCA+CDCA group compared with the CDCA group, where mitochondrial swelling and disruption of the inner mitochondrial membrane were observed. The mechanism underlying the mitochondrial protective effect of UDCA is not clear. One of the main inducers of mPTP is Ca^{2+} overload and oxidative stress. Although we have shown that UDCA pretreatment had no effect on the extent of CDCA-induced Ca^{2+} elevation, we did not investigate the effect of UDCA on the total Ca^{2+} load. It is possible that the protective effect of UDCA is due to the reduction of Ca^{2+} overload or by the inhibition of reactive oxygen species (ROS) production; however, further studies are needed to confirm these hypotheses.

Mitochondrial dysfunction is often associated with cell death either by the reduction of ATP_i levels or by irreversible alterations in the mitochondrial membrane permeability, which induces the release of apoptotic signaling molecules from the mitochondria. Since mitochondria play a central role in cell survival, we wanted to determine whether the CDCA-induced mitochondrial injury would result in cell death and to examine the possible protective role of UDCA in this process. Administration of CDCA induced marked DNA fragmentation in intact pancreatic ducts. We speculate that impairment of mitochondrial function plays a central role in this mechanism, but other signaling pathways may be involved. The apoptotic effect of CDCA and other hydrophobic bile acids have been examined in more detail in hepatocytes. ROS generation, mPTP induction, cytochrome-*c* release and activation of downstream caspases have been shown to be associated with apoptosis (65, 66). It has been also demonstrated that the glycine-conjugated form of CDCA, directly stimulate Fas-dependent cell death due to the activation of the Fas receptor, which is independent from the mitochondrial pathway (13). Twenty-four hour pretreatment with UDCA effectively reduced the CDCA-induced apoptosis as indicated by significantly decreased DNA fragmentation, which further confirms the cytoprotective effect of UDCA. The exact mechanism by which UDCA exerts its protective effect was not investigated by our group; however, our results and previous studies on hepatocytes strongly indicate that mPTP inhibition by UDCA is one of the key mechanisms in the reduction of CDCA-induced cell death in PDECs.

To extend our study, we also tested the protective effect of UDCA in vivo. There is no accepted pancreatitis model in guinea pigs; therefore, we induced AP in rats by intraductal injection of low concentration of CDCA (53, 54). Under these experimental conditions, CDCA induced acinar cell damage and also increased serum amylase activity. Previous studies on

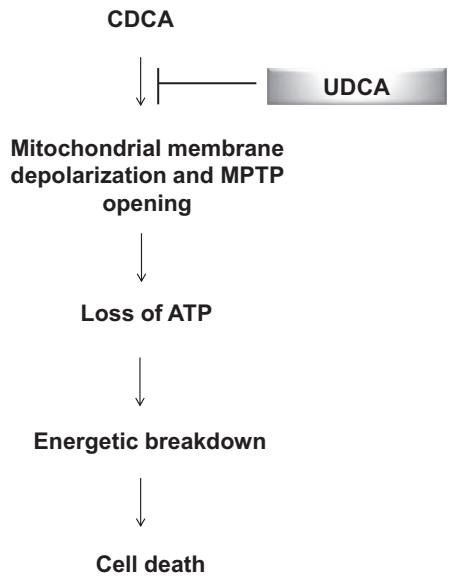


Fig. 7. Proposed model for the protective effect of UDCA. CDCA induces mitochondrial damage by decreasing mitochondrial membrane potential and opening of mitochondrial permeability transition pore (MPTP). UDCA is able to prevent the toxic effect of CDCA by the stabilization of the mitochondrial membrane. →, induction; |——, inhibition.

isolated rat and mouse acini demonstrated that the hydrophobic bile acid, TLC-S induces mitochondrial injury, pathological Ca^{2+} signaling, and the generation of ROS (7, 61–63). We speculate that similar intracellular mechanisms may also have arisen in response to CDCA. The CDCA-induced acinar cell injury, hyperamylasemia, and pancreatic edema were markedly reduced in the UDCA-treated group. The protective effect of UDCA can be attributed to its ability to reduce mitochondrial injury both in acinar and ductal cells. Several attempts have been made to investigate the effect of UDCA on isolated pancreatic acinar cells. Using rat and mouse acinar cells, we found that isolated acinar cells are more sensitive to long-term bile acid treatment than ductal cells, which made the investigation of the protective effect of UDCA very difficult. Cell viability experiments showed that even at low concentrations, bile acids decreased the viability of isolated acinar cells, and in contrast to ductal cells, UDCA pretreatment had no protective effect on acinar cells under our experimental conditions (data not shown).

Lastly, our results may have important clinical implication in patients with gallstone obstruction, where oral administration of UDCA, or its metabolically stable form, may reduce the risk of pancreatitis development. The beneficial effect of UDCA treatment has been demonstrated in idiopathic recurrent pancreatitis, in which long-term UDCA treatment reduced the rate of recurrence by prevention of the formation of gallstones (46, 55, 60). The effect of orally administered UDCA is highly dependent on its metabolism. In rats, most of the UDCA is metabolized to TUDCA (40), which also has a cytoprotective effect as demonstrated in the liver (4, 5, 37, 50). In humans, one of the major metabolites of UDCA is isoursodeoxycholic acid (or isoUDCA) (6), which is the 3β-hydroxy epimer of UDCA and has been shown to be more effective than UDCA (30). In the current study, we have not investigated the serum concentration of UDCA after oral administration. Notably, since UDCA was administered in an excess dose, we believe that sufficiently high concentration of UDCA (or TUDCA) was present in the serum to exert its protective effect.

Understanding the early injury mechanisms induced by hydrophobic bile acids is extremely important to find a therapeutic target to reduce pancreatic injury. In this study, we confirm and extend our previous observations that mitochondria is a key target in the CDCA-induced cellular injury in PDECs. The hydrophilic bile acid UDCA inhibits CDCA-induced apoptosis probably by the stabilization of mitochondrial membrane via blockade of membrane depolarization and mPTP and also by prevention of mitochondrial swelling (Fig. 7). Several studies have focused on the inhibition of cellular injury during AP to stop or delay the progression of the disease. UDCA may represent a novel option against the bile-induced ductal injury; however, issues for the therapeutic application of this bile acid in AP need further investigation.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

M.K., B.K., Z.B., and J.M. performed experiments; M.K. analyzed data; P.H., Z.R.J., Z.R., L.T., and V.V. interpreted results of experiments; V.V. conception and design of research; V.V. drafted manuscript; V.V. approved final version of manuscript.

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