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Bile Acids Affect Liver Mitochondrial Bioenergetics: Possible Relevance for Cholestasis Therapy

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It has been pointed out that intracellular accumulation of bile acids cause hepatocyte injury in cholestatic disease process. This study was aimed to test if cytotoxicity of these compounds is mediated through mitochondria dysfunction. Bile acids effects on isolated rat liver mitochondrial were analyzed by monitoring changes in membrane potential and mitochondrial respiration, as well as alterations in H^+ membrane permeability and mitochondrial permeability transition pore induction. Increasing concentrations of the bile acids litocholic (LCA), deoxycholic (DCA), ursodeoxycholic (UDCA), chenodeoxycholic (CDCA), glycochenodeoxycholic (GCDC), or taurochenodeoxycholic (TCDC) decrease transmembrane potential ($\Delta\Psi$) developed upon succinate energization. These compounds also decreased state 3 respiration and enhanced state 4. We have also demonstrated that the observed concentration-dependent stimulation of state 4 by LCA, DCA, CDCA, TCDC, and GCDC, is associated with an enhanced permeability of mitochondria to H^+. Addition of LCA, DCA, CDCA, TCDC, GCDC, and UDCA to mitochondria energized with succinate resulted in a dose-dependent membrane depolarization and stimulation of mitochondrial permeability transition. Tauroursodeoxycholate (TUDC) elicited no significant effect on succinate-supported mitochondrial bioenergetics. In contrast, in the presence of glycoursodeoxycholic (GUDC), $\Delta\Psi$ increases as a function of bile salt concentration. The results of this investigation demonstrate that at toxicologically relevant concentrations, most but not all bile acids alter mitochondrial bioenergetics, so impairment of mitochondrial function can be clinically relevant for patients with cholestasis.

Key Words: **mitochondria; bile acids; permeability transition pore; membrane potential; respiration.**

Liver disease ranks as the third leading cause of death in the world. The formation of bile is a vital function. Although several pathogenic processes (e.g., immunological, inflammatory, genetic, and obstructive) in the liver may cause its impairment (Javitt, 1982), the mechanisms leading to hepatocellular damage in cholestatic liver disease are not completely characterized, nor the exact therapy that should be prescribed for these patients. Furthermore, the use of bile acid therapy (Beuers *et al.,* 1998), has increased, but the mode of action remains poorly understood.

One of the postulated factors contributing to hepatocyte injury is the intracellular accumulation of toxic, hydrophobic bile acids observed during cholestasis (Greim *et al.,* 1972). The tendency to cause cytotoxicity has been correlated with the hydrophobicity of these compounds (Armstrong and Carey, 1982; Attili *et al.,* 1986). Bile acids, in particular the lipophilic dihydroxy and monohydroxy species, are surface-active substances and can damage biological membranes (Schölmerich *et al.,* 1984). Alteration of bile canalicular membrane molecular organization has been pointed out as a mechanism for the impaired secretion of bile observed in intrahepatic cholestasis (Plaa *et al.,* 1982). However, although bile acids accumulate in the liver during the cholestatic disease process, it has been considered unlikely that they reach sufficient levels for the detergent properties to lead to massive solubilization and disruption of cell membranes (Attili *et al.,* 1986).

Alternatively, several reports related with both morphological and biochemical observations support the concept that bile acids may be cytotoxic by causing mitochondrial dysfunction. Long-term cholestasis caused by bile duct ligation is known to lead to impaired hepatic mitochondrial function in the rat (Krähenbühl *et al.,* 1992) and enlarged, swollen mitochondria are observed in histopathologic sections obtained from this model of extrahepatic cholestasis (Schaffner *et al.,* 1971). It has also been demonstrated that ATP depletion, a process involved in cellular necrosis, occurs early when hepatocytes are exposed to necrotic concentrations of toxic bile salts (Spivey *et al.,* 1993). As mitochondria are provided with a variety of bioenergetic functions mandatory for the regulation of intracellular aerobic energy production and electrolyte homeostasis, impairment of mitochondrial function by bile acids may have drastic consequences on cellular function through the perturbation of the bioenergetic charge and balance of the cell.

The mitochondrial inner membrane can undergo a permeability increase specifically inhibited by the immunosupressive agent Cyclosporine A (Cy A) (Broekemeier *et al.,* 1989). This transition is manifested by the transformation of a calcium-dependent, thiol-regulated, voltage-gated complex of membrane-spanning proteins into a nonspecific pore capable of

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conducting solutes of < 1500 Da (Zoratti and Szabo, 1995). Experimentally, the permeability transition is characterized by an abrupt swelling and depolarization of the mitochondria, reflecting the loss of ability to maintain ion and solute gradients across the inner membrane. The result is inhibition of oxidative phosphorylation, bioenergetic deficits, and presumably cell death. Induction of the permeability transition pore (PTP) is widely implicated in the mechanism by which many agents interfere with mitochondrial bioenergetics *in vitro* (Bernardi *et al.*, 1994; Gunter *et al.*, 1994). Recent data have reported the induction of the PTP as a mechanism of cytotoxicity of bile acids (Botla *et al.*, 1995; Gores *et al.*, 1998) but no systematic and exhaustive study on the ability of these agents to induce the PTP has been done.

Bernardi (1992) demonstrated that depolarization of the membrane increases the probability of pore opening. Therefore, prior to studying the effects of bile acids on the PTP, it was first necessary to study a direct effect on mitochondrial bioenergetics independent of this phenomenon. The analysis of bile acid effects on the chemical and physical processes of oxidative phosphorylation will therefore be meaningful to understand underlying mechanisms responsible for cell damage and the resulting disturbances of liver function.

The current investigation was designed to examine whether primary and secondary bile acids, with varying degrees of hydrophobicity, and their glycine/taurine conjugates cause impairment of mitochondrial function. Interactions on mitochondrial respiration and membrane potential, as well as alterations on H^+ membrane permeability and mitochondrial permeability transition pore induction are reported.

MATERIALS AND METHODS

Chemicals. UDCA, LCA, CDCA, DCA, all used as ethanolic solutions, were purchased from Sigma Chemical Co. (St. Louis, MO) as well the sodium salts TCDC, GCDC, and GUDC, which were dissolved in water. TUDC, as sodium salt, was obtained from Cal Biochem (La Jolla, CA). Cyclosporine A was a generous gift from Novartis Pharma AG (East Hanover, NJ). All other chemicals were of the highest grade of purity commercially available.

Isolation of mitochondria. Mitochondria were isolated from liver of male Wistar rats by conventional methods (Gazotti *et al.*, 1979) with slight modifications. Homogenization medium contained 250 mM sucrose, 10 mM HEPES (pH 7.4), 0.5 mM EGTA, and 0.1% fat-free bovine serum albumin. EGTA and bovine serum albumin were omitted from the final washing medium, adjusted at pH 7.4. The mitochondrial pellet was washed twice, suspended in the washing medium, and immediately used. Protein content was determined by the biuret method (Gornall *et al.*, 1949) calibrated with bovine serum albumin.

Mitochondrial respiration. Oxygen consumption of isolated mitochondria was determined polarographically with a Clark oxygen electrode (Estabrook, 1967) connected to a suitable recorder in a 1-ml thermostated water-jacketed chamber with magnetic stirring, at 25^0C. The standard respiratory medium consisted of 130 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 5 mM KH_2PO_4, 50 μM EDTA, 5 mM HEPES (pH 7.4), and 3 μM rotenone. Bile acids were added to the reaction medium with mitochondria (1 mg) and allowed to incubate for 3 min before the addition of succinate (5 mM). To induce state 3 respiration, 50 nmol ADP was used. The respiratory control ratio (RCR) was calculated using oxygen consumption rates during state 3 and subsequent state 4 respiration.

Membrane potential ($\Delta\Psi$) measurements. The mitochondrial transmembrane potential was estimated using an ion-selective electrode to measure the distribution of tetraphenylphosphonium (TPP^+) according to previously established methods (Kamo *et al.*, 1979; Palmeira *et al.*, 1994). The reference electrode was Ag/ $AgCl_2$. Mitochondria (1 mg) were suspended with constant stirring, at 25^0C, in 1 ml of the standard respiratory medium supplemented with 3 μM TPP^+ and were energized by adding succinate to a final concentration of 5 mM. The distribution of TPP^+ was allowed to reach a new equilibrium (ca. 2 min) before making any further addition. The electrode was calibrated with TPP^+ assuming Nerstian distribution of the ion across the synthetic membrane, and $\Delta\Psi$ is expressed in mV. A matrix volume of 1.1 μl/mg protein was assumed. Respiratory rates and $\Delta\Psi$ were simultaneously measured.

Measurement of the permeability of mitochondrial membrane to H^+. Mitochondrial osmotic volume changes were measured spectrophotometrically by the apparent absorbance changes at 540 nm. The reactions were carried out at 25^0C in 2 ml of 135 mM NH_4NO_3, 10 mM HEPES, and 0.1mM EDTA (pH 7.4). Mitochondria (0.5 mg/ml) were suspended with constant stirring.

Measurement of the mitochondrial permeability transition. Mitochondrial swelling was estimated by changes in light scattering, as monitored spectrophotometrically at 540 nm (Palmeira *et al.*, 1997). The incubation medium contained 200 mM sucrose, 10 mM Tris-Mops (pH 7.4), 1mM KH_2PO_4, 5 mM succinate, and 10 μM EGTA supplemented with 2 μM rotenone and 0.5 μg/ml oligomycin. The reaction was stirred continuously and the temperature maintained at 25^0C. The experiments were started by the addition of 1 mg of mitochondria (final volume 2 ml). Ca^{2+} (50 μM $CaCl_2$) was added prior the addition of bile acids.

Statistical analysis. Results are presented as percentage of control ± SEM. Statistical evaluation was performed using the two-tailed Student's *t* test. A p value < 0.05 was considered statistically significant.

RESULTS

Effects of Bile Acids on Mitochondrial Membrane Potential

The effect of bile acids (in the micromolar range) on the energization and phosphorylation capacities of mitochondria was investigated by following the transmembrane potential ($\Delta\Psi$) developed by mitochondria upon succinate oxidation (Fig. 1). After succinate addition, mitochondria developed a $\Delta\Psi$ of about –217 mV (without correction for passive membrane binding). ADP addition caused an expected drop in the membrane potential, because ATP synthase uses $\Delta\Psi$ to phosphorylate added ADP; after a short lag phase, where ADP phosphorylation takes place, the transmembrane potential repolarizes close to the initial value.

Incubation of isolated rat liver mitochondria with increasing concentrations of UDCA, DCA, LCA, CDCA or its conjugates TCDC and GCDC, decreased the transmembrane potential developed upon succinate energization. The most effective toxic compound under study was LCA, a monohydroxy bile acid that is more lipophilic than the dihydroxy bile acids CDCA, DCA, and UDCA.

Concentrations of 15 and 100 μM of litocholic and deoxycholic acids, respectively, completely abolished $\Delta\Psi$. A similar effect was observed when mitochondria were incubated with 50 μM CDCA, as shown in Figure 1B. In contrast, the taurine/glycine conjugates of chenodeoxycholate exhibited no mitochondrial toxicity for concentrations ranging from 0 to 50 μM.

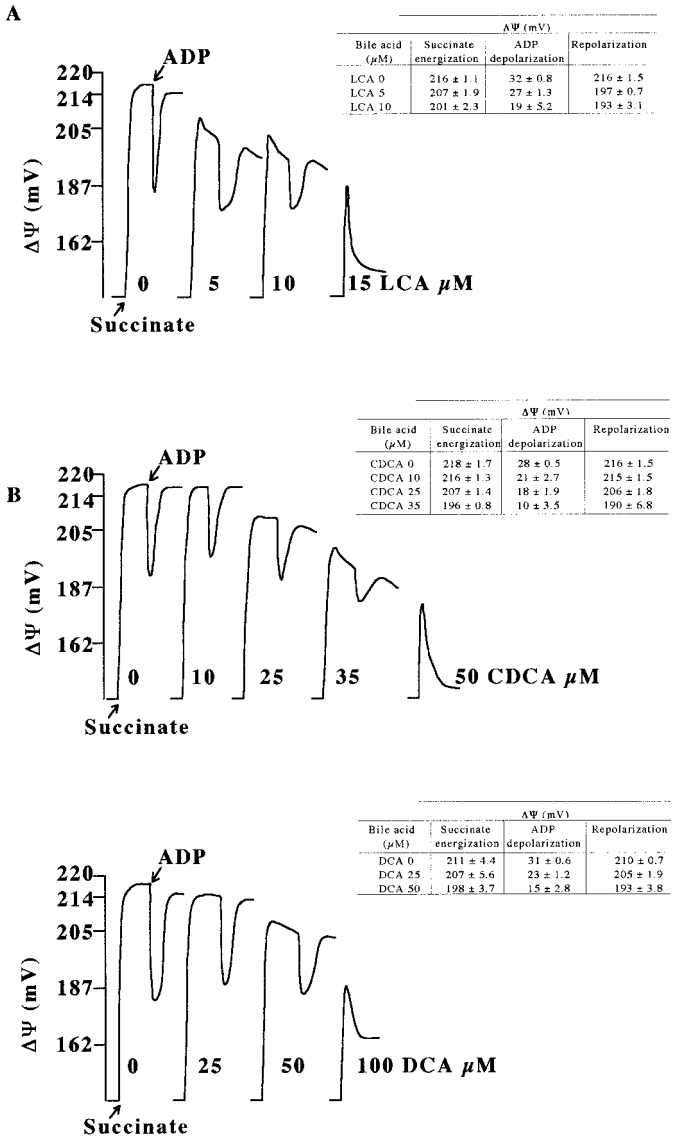

FIG. 1. Effect of litocholic (LCA), chenodeoxycholic (CDCA), and deoxycholic (DCA) acids on mitochondrial transmembrane potential ($\Delta\Psi$). Mitochondria (1 mg) in 1 ml of the standard respiratory medium supplemented with 3 μM TPP^+ were energized with 5 mM succinate. Addition of ADP (50 nmol) induces state 3 condition. The various concentrations of bile acids added are indicated on the traces. The traces represent typical direct recordings expressive of 3–4 independent experiments with different mitochondrial preparations. Table inserts show the average response (mean ± SE) of membrane potential developed with succinate, the drop in membrane potential after ADP addition, and the repolarization value after all the added ADP being phosphorylated.

This is in accord with the fact that taurine/glycine conjugates are generally less toxic than the primary bile acids. However, in the presence of higher concentrations of TCDC and GCDC, such as 250 μM, $\Delta\Psi$ that developed after succinate energization was decreased, probably due to nonspecific side effects. The addition of 300 μM UDCA decreased mitochondrial membrane potential; the effect was quite clear at 400 μM ursodeoxycholic acid (Fig. 2B).

The depolarization induced by ADP decreased in the presence of these bile acids, reflecting the incapacity of mitochondria to phosphorylate all added ADP. Furthermore, the lag phase preceding repolarization steadily increases and the rate of repolarization is progressively decreased. Additionally, at the end of repolarization, the transmembrane potential was lower as compared to the characteristic state 4 value prior to ADP addition.

$\Delta\Psi$ was not significantly affected by TUDC at either concentration. GUDC showed a stimulatory effect on transmembrane potential (Fig. 2C). In the presence of glycoursodeoxycholate, $\Delta\Psi$ increased as a function of bile salt concentration. The depolarization after ADP addition and the lag phase preceding repolarization was not affected. The repolarization following ADP phosphorylation nearly returns the $\Delta\Psi$ recorded prior ADP addition.

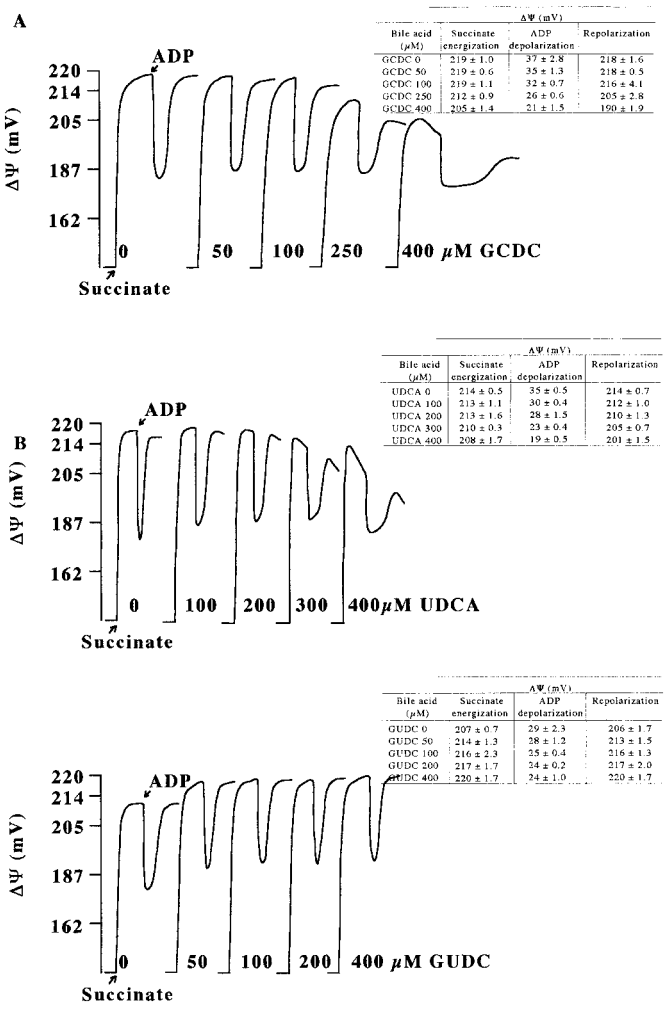

FIG. 2. Effect of glycochenodeoxycholate (GCDC), ursodeoxycholate (UDCA), and glycoursodeoxycholate (GUDC) on mitochondrial transmembrane potential ($\Delta\Psi$). The experimental conditions were the same as those reported in Figure 1.

The hepatoprotective effects of UDCA and its conjugates were evaluated in terms of development of $\Delta\Psi$ by energized rat liver mitochondria in the presence of the other bile acids. Although the known beneficial action of UDCA in cholestatic liver diseases (Beuers *et al*., 1998) is recognized, we didn't observe any protection in terms of $\Delta\Psi$ effects promoted by the other bile acids (data not shown). In this case, we preincubated mitochondria with 100 or 300 μM UDCA for 3 or 5 min before exposure to LCA, DCA, CDCA, TCDC, or GCDC for 3 min. Preincubation with 200 μM TUDC or GUDC also had no effect (data not shown).

Similarly, preincubation with 1 μM CyA did not prevent impairment of mitochondrial function, reflected from the monitoring of mitochondrial transmembrane potential alterations by bile acids.

Effects of Bile Acids on Mitochondrial Respiration

To investigate possible alterations on oxygen consumption of rat liver mitochondria exposed to bile acids, respiratory rates characteristics of state 4, state 3 (ADP-stimulated respiration), and RCR were evaluated in the presence of a FAD-linked substrate (succinate). The respiratory control rates for controls were indicative of good mitochondrial preparations (data from three to four independent preparations).

As can be seen in Fig. 3, isolated mitochondria showed significantly decreased state 3 respiration rate and RCR and increased state 4 for all bile acids except TUDC and GUDC, compared to controls.

Mitochondria incubated with 35 μM CDCA (Fig. 3C) exhibited a high increase in state 4 respiration and a 30% decrease in ADP-stimulated state 3 respiration compared to mitochondria from control. This resulted in a significantly lower (20%) RCR. In contrast, the taurine and glycine conjugates of this bile acid and ursodeoxycholate only showed similarly drastic effects for higher concentrations (400 μM). GUDC (data not shown) and TUDC, however, elicited no dramatic alteration in state 3 or state 4 respiration.

For the higher concentrations considered of LCA, DCA, and CDCA, respectively, 15, 100, and 50 μM, the transfer of energy between electron flow, and ATP synthesis was totally prevented, as the addition of ADP didn't give rise to state 3 respiration.

When the concentration of the bile acid in the mitochondrial incubation was lowered to 10 μM, only LCA (Fig. 3A) caused state 3 decrease (by about 55%) and largely increased rate of substrate oxidation. In contrast, the other bile acids studied exhibit no mitochondrial effects under these conditions.

Effects of Bile Acids on Membrane Permeability to Protons

The observed bile acids concentration-dependent increase in the rate of state 4 respiration, suggested a possible uncoupling effect. The uncoupling action can be related to the increased permeability of mitochondrial membrane to H^+ induced by these compounds. To further explore and understand these findings we monitored the degree of mitochondrial swelling in isoosmotic NH_4NO_3 medium, in the presence of bile acids (Fig. 4).

Despite the permeability of the inner mitochondrial membrane to NO_3^- (Mitchell and Moyle, 1969), mitochondria do not swell in isoosmotic solutions of NH_4NO_3 because the membrane is impermeable to NH_4^+ and H^+ (Mitchell and Moyle, 1967). The observed carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP)-induced mitochondrial swelling in the NH_4NO_3 medium (Fig. 4) reflects an increased permeability of mitochondrial membrane to H^+ ions due to the protonophore behavior of FCCP.

As expected, LCA, DCA, CDCA, TCDC, and GCDC-induced decreased absorbance in NH_4NO_3 medium behaves similarly to FCCP-induced swelling, because they exhibit increased membrane permeability to protons. This contributes to the observed stimulation of the state 4, which is characteristic of proton uncoupler agents. Particularly, 400 μM GCDC induced high and rapid mitochondrial swelling, as can be seen in Fig. 4C.

In contrast, addition of UDCA or its taurine/glycine conjugates to hepatic mitochondria did not induce absorbance changes *in vitro*. There was no discernible difference in the trace compared to that for the control sample (Fig. 4D). Additionally, pretreating mitochondria with 400 μM TUDC, GUDC, or UDCA did not prevent LCA, DCA, CDCA, TCDC, or GCDC-induced decreased absorbance at 540 nm (data not shown).

Effects of Bile Acids on Mitochondrial Permeability Transition Pore Induction

Mitochondria possess a finite capacity for accumulating calcium before undergoing the calcium-dependent mitochondrial permeability transition pore (PTP). Based on these reports we evaluated the calcium concentration that should be applied to assess the effect of bile acids on PTP induction.

Figure 5 illustrates the results achieved when we follow mitochondrial swelling as the indicator for induction of the PTP by bile acids. Adding LCA, DCA, and CDCA to calcium-loaded, succinate-energized rat liver mitochondria caused a dose-dependent stimulation of mitochondrial swelling. The induction of the PTP by these compounds was evident by the fact that pretreatment with 1 μM CyA completely prevented mitochondrial swelling. Increasing concentrations of TCDC (Fig. 5C) and GCDC also elicited PTP. Adding 300 μM UDCA to mitochondria energized with succinate (Fig. 5D) resulted in an immediate PTP, as reflected by the rapid and profound decrease in light scattering. In this case, 1 μM CyA also prevented mitochondrial swelling. TUDC and GUDC at concentrations of 400 μM did not induce the PTP.

Regardless of the therapeutic benefits of UDCA in human cholestatic liver disease (Beuers *et al*., 1998), we next tested

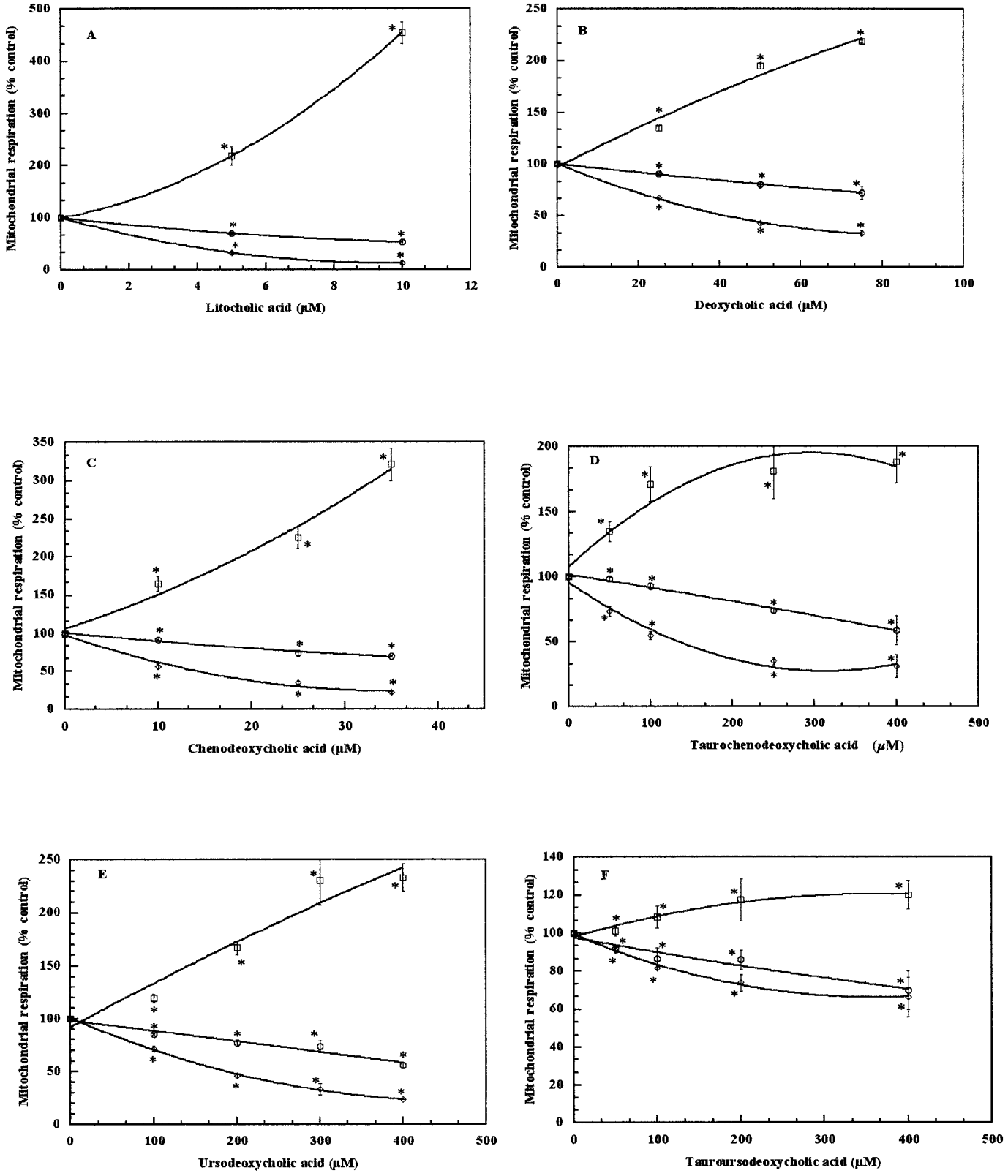

FIG. 3. Effect of bile acids on respiratory rates of rat liver mitochondria. Mitochondria (1 mg) were incubated in 1 ml of the respiratory standard medium. State 4 respiration was initiated by the addition of 5 mM succinate after 3 min of incubation. The state 3 respiration was initiated by the addition of 50 nmol ADP 2 min after energization. Absolute values for control state 4 and state 3 respiratory rates are 9.4 ± 0.8 and 55.5±1.8 nmol O_2/min/mg protein, respectively. Values are the mean ± SEM of three to four independent experiments with different mitochondrial preparations: circle, state 3; square, state 4; diamond, RCR. Asterisk indicates values statistically different from control ($p < 0.05$).

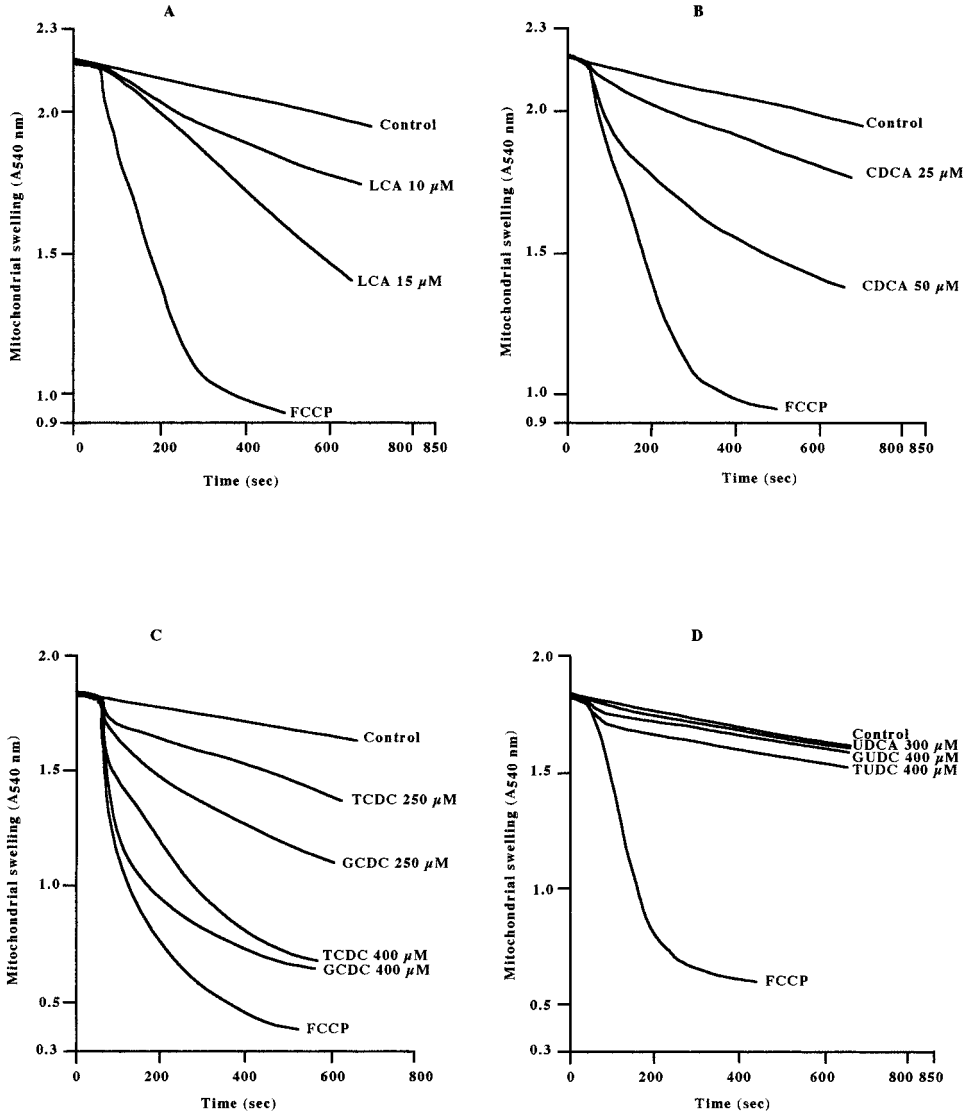

FIG. 4. Effect of bile acids on the mitochondrial swelling in isoosmotic NH_4NO_3 medium. Mitochondria (0.5 mg) were suspended in 2 ml of 135 mM NH_4NO_3, 5 mM HEPES, 0.1 mM EDTA (pH 7.4) at 25^0C. Bile acids and FCCP (1 μM) were added after 2 min of incubation. The traces represent typical recordings expressive of several independent experiments with different mitochondrial preparations (3–4).

the hypothesis that UDCA or its conjugates exert their beneficial effects by interfering with the PTP induced by hydrophobic bile acids. There were no appreciable differences in the traces representative of these experiments, showing clearly no protective effects by UDCA and its conjugates on the capability of hydrophobic bile acids to induce PTP. Conversely, our data (Figs. 5 A and 5B) clearly demonstrate that the combination of 100 μM UDCA plus hydrophobic bile acids is additive in inducing a larger degree of mitochondrial swelling.

DISCUSSION

Because of the importance of mitochondria in cellular energy metabolism (they provide about 90% of the total ATP necessary to liver cells), alterations in normal oxidative phosphorylation may play an important role in cell pathologies. Indeed, a number of observations suggest that mitochondria are a primary target of chemical-induced injury and that their dysfunction ultimately leads to cell death (Petit *et al.*, 1995).

Our data clearly demonstrate that bile acids elicited perturbations in mitochondrial bioenergetics. The study of transmembrane electrical potential ($\Delta\Psi$) is essential for an integrated appraisal of the mitochondrial function, as it is the main component of the electrochemical gradient (μH^+), accounting for greater than 90% of the total proton motive force (Nicholls, 1982). LCA, DCA, UDCA, CDCA, TCDC, and GCDC decreased the potential generated by succinate, which is associated with the stimulation of the respiratory rates sustained by this substrate. Resting coupled respiration is controlled by the proton leak through the mitochondrial inner membrane and, to a lesser extent, by the respiratory chain (Brand *et al.*, 1988). The observed uncoupling may be a consequence of several interactions of these compounds with the mitochondrial mo-

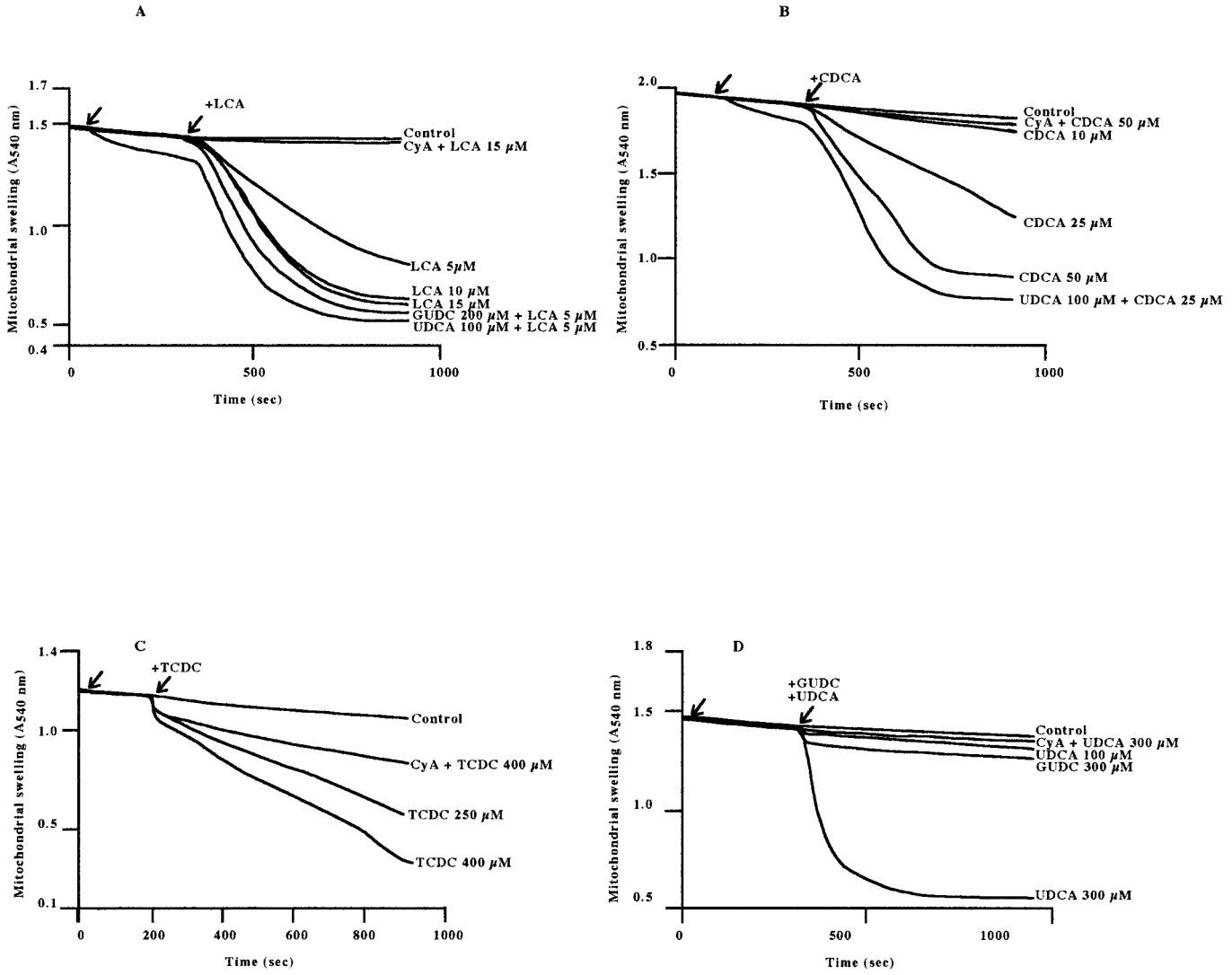

FIG. 5. Effects of bile acids on PTP induction. The incubation medium (final volume 2 ml at 25°C) contained 200 mM sucrose, 10 mM Tris-Mops (pH 7.4), 1 mM KH_2PO_4, and 10 μM EGTA supplemented with 2 μM rotenone, 0.5 μg/ml oligomycin, and 8 mM succinate. The experiments were started by the addition of 1 mg of mitochondria (not shown). Ca^{2+} (50 μM) was added prior the addition of bile acids. Where indicated (first arrow), 1 μM CyA, 100 μM UDCA or 200 μM GUDC were included in the reaction medium to evaluate possible protection of pore opening induced by bile acids. The traces are typical of several independent experiments with different mitochondrial preparations (3–4).

lecular system. TCDC, GCDC, DCA, LCA, and CDCA, induced permeability of the membrane to protons, either by a protonophoric action or by disruption of membrane order. It is worth nothing that LCA strongly enhanced state 4, compared to other bile acids. This can be related with the induction of slippage of proton pumps reducing the protonmotive force. In contrast, UDCA does not cause an increased H^+ leak, although the observed drop of $\Delta\Psi$ accompanied a stimulation of the respiratory rate. Such effects can be explained by an interaction at the level of the PTP, facilitating its opening. The results obtained in this study show that in isolated liver mitochondria, LCA, DCA, CDCA, TCDC, GCDC, and UDCA elicited a concentration-dependent induction of PTP, as already described for GCDC (Botla *et al.*, 1995; Gores *et al.*, 1998). Pretreatment with Cy A, a specific and potent inhibitor of the PTP (Broekemeier *et al.*, 1989), completely protected against the Ca^{2+}-dependent swelling. As cyclosporine A did not prevent the stimulation of state 4 respiration nor the membrane depolarization induced by these compounds, we conclude that the induction of PTP opening is not a direct effect on the pore itself, rather it is related to membrane depolarization that increases the probability of pore opening. Although the protective effect of UDCA on the GCDC-induced PTP has been described (Botla *et al.*, 1995; Gores *et al.*, 1998), our data clearly demonstrate ursodeoxycholate is an inducer of the permeability transition. Indeed, the transmembrane potential decrease and state 4 respiration increase support this observation. Conversely, taurine/glycine conjugates of UDCA have no

effect on PTP induction. The stimulatory effect of GUDC on mitochondrial potential is tentatively explained as a coupling action of the bile salt exerted on ATP synthase itself, with a decrease of ΔpH dissipated through the subunities of the enzyme complex. On the other hand, in the presence of LCA and GCDC, the recovery following ADP addition was lengthened and did not resume completely. The enhanced lag phase suggest that these two bile acids have a disturbing effect on the ATP synthase itself.

Furthermore, bile acids depress state 3 respiration, indicating that the inhibitory action of these compounds on state 3 should be related to the phosphorylation system. Our observations are in agreement with previous reports (Krähenbühl *et al*., 1994; Spivey *et al*., 1993) which indicate that GCDC, DCA, LCA inhibited state 3 mitochondrial respiration in a dose-dependent manner. In contrast, increase in state 4 respiration and the effect of GCDC on mitochondrial membrane potential has not been described. One possible line of reasoning can be specific differences in experimental protocols, as Spivey *et al*. (1993) determined the effect of GCDC on $\Delta\Psi$ and on state 4 *in situ* using cultured hepatocytes permeabilized with digitonin.

The mechanism by which bile acids promote the onset of PTP remains unclear. It is known that induction of this phenomenon involves a membrane potential-dependent transformation of a specific channel within the inner mitochondrial membrane, depolarization increasing the probability of pore opening (Bernardi, 1992; Bernardi *et al*., 1994; Gunter *et al*., 1994). Furthermore, oxidative stress or, in general, oxidizing conditions appear to act as inducers of the PTP (Zoratti and Szabo, 1995). It has been suggested (Krähenbühl *et al*., 1994; Shivaram *et al*., 1998; Sokol *et al*., 1993) that oxygen free radicals may be involved in the pathogenesis of bile acid acids toxicity by causing increased leak from the altered electron transport chain. In this case, a possible involvement of a peroxidative process may also be implicated on the mitochondrial permeabilization membrane to H^+, contributing to the observed uncoupling effect.

It is well established that hydrophobicity, as demonstrated by its close correlation with the partition coefficient of compounds, is often associated with biological action. (Hansch and Dunn, 1972). Thus, the cytotoxicity potential is related to the hydrophobicity and detergent properties of each bile acid (Armstrong and Carey, 1982; Attili *et al*., 1986). UDCA is more hydrophilic than the two major dihydroxy bile acids CDCA and DCA. LCA is the more lipophilic one. Additionally, conjugation of bile acids with glycine and taurine in human liver reduces hepatotoxicity. Our results are consistent with this report, as hydrophobic species disrupted mitochondrial function at concentrations far below those observed for hydrophilic bile acids.

Spivey and collaborators (Spivey *et al*., 1993) report intracellular CDCA, TCDC, and GCDC concentrations during *in vivo* cholestasis to be less than 0.1 mM (≈800 μM), values in accordance with the *in vitro* bile acid concentrations used in this study. This study therefore suggests that physiologically relevant bile acid concentrations induce cell damage, as has been suggested as a mechanism for hepatocyte injury in cholestatic disease, and is putatively preceded by bioenergetic lesion. We conclude that at toxicologically relevant concentrations, most but not all bile acids alter mitochondrial function. The cytotoxicity is characterized by mitochondrial membrane depolarization and increased state 4 respiration, followed by induction of the mitochondrial permeability transition.

In conclusion, impairment of mitochondrial function by bile acids can be clinically relevant for patients with cholestasis. Although exogenous administration of UDCA ameliorates liver injury during cholestasis, our data clearly demonstrate that ursodeoxycholate or its conjugates TUDC and GUDC do not exert their cytoprotective action by preventing alteration of mitochondrial function caused by other bile acids. Further studies are being conducted in our laboratory in order to better elucidate the specific biochemical mechanisms involved in cholestasis as well as in bile acid therapy.

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