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Protective effect of carvedilol on chenodeoxycholate induction of the permeability transition pore

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Abstract

Intracellular accumulation of toxic, hydrophobic bile acids has been proposed as one of the putative final common pathways leading to cholestatic liver injury. Furthermore, bile acids have been proposed as a causative factor for hepatic cardiomyopathy. Hepatic tissue concentrations of chenodeoxycholic acid (CDCA) during cholestasis are greater than those of other toxic bile acids. In the presence of calcium and phosphate, CDCA induced the permeability transition pore (PTP) in freshly isolated rat liver mitochondria. In this study, we evaluated the effects of carvedilol, a multirole cardioprotective compound, on CDCA-induced PTP. Mitochondrial membrane potential, osmotic swelling, and calcium fluxes were monitored. CDCA-induced PTP, characterized by membrane depolarization, release of matrix calcium, and osmotic swelling, was prevented by carvedilol. Under the same conditions, its hydroxylated analog BM-910228 did not reveal any protective effect. This finding reinforces carvedilol's therapeutic interest, because it may potentially prevent mitochondrial dysfunction associated with cardiomyopathy in the pathophysiology of cholestatic liver disease. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Chenodeoxycholic acid; Carvedilol; BM-910228; Liver mitochondria; Permeability transition; Cardiomyopathy

1. Introduction

Cholestasis, an arrest or stagnation in bile flow, is one type of liver disease of unknown etiology. There are a variety of possible causes, but one of the putative final common pathways leading to cholestatic liver injury is the intracellular accumulation of bile acids [1,2]. Bile acids, the water-soluble, amphipathic end products of cholesterol metabolism, are involved in liver, biliary, and intestinal disease [3]. These compounds have the capacity to induce both choleresis and cholestasis, depending on the concentrations and hydrophobic properties of individual bile acids [4,5]. Chenodeoxycholic acid is a primary bile acid mostly implicated in liver injury. Hepatic tissue concentrations of CDCA increase 20-fold during cholestasis and are greater than those of other toxic bile acids such as lithocholate (LCA) and deoxycholate (DCA) [6].

In a number of studies, impairment of mitochondrial function has been proposed as a cause of hepatocyte dysfunction leading to cholestatic liver injury [7–11]. The major function of mitochondria in human cells is to provide ATP by oxidative phosphorylation. However, mitochondria have many other roles, including the modulation of intracellular calcium concentration and participation in the process of apoptotic cell death [12]. Consequently, mitochondrial dysfunction contributes to a great number of human diseases [13].

Recent data pointed out induction of the PTP as a mechanism of cytotoxicity of bile acids [11,14,15]. Mitochondrial permeability transition is a calcium-dependent process linked to the opening of a non-specific channel into the inner membrane. The mitochondrial PTP is normally accompanied by mitochondrial swelling, a precipitous drop in membrane potential ($\Delta\Psi$), and the spontaneous release of accumulated calcium [16] and other gradients. This phenomenon is stimulated by the presence of inorganic phosphate, oxidative stress, or dithiol reagents [16–19], and is typically inhibited by cyclosporine A [20]. PTP activation is voltage-dependent, i.e. opening is facilitated by depolarization [17,21], and is now recognized to be closely involved in cell death either by necrosis or by apoptosis [22].

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Abbreviations: CyA, cyclosporine A; CDCA, chenodeoxycholic acid; PTP, permeability transition pore; TPP^+, tetraphenylphosphonium; and $\Delta\Psi$, transmembrane electric potential.

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A possible relationship between plasma accumulation of bile acids and the cardiomyopathy of cholestatic liver disease has been proposed [23]. A recent report indicates that bile acid-induced modification of cardiac β-adrenoreceptor activity may represent a mechanism whereby these compounds exert their effects on cardiac performance [24]. Carvedilol ({1-[carbazolyl-(4)-oxy]-3-[2-methoxyphenoxyethyl)amino]-propanol-(2)}) is a lipophilic, vasodilating β-adrenoreceptor blocking agent with known cardioprotective properties. Among the various metabolites of carvedilol, BM-910228 ({1-[3 hydroxycarbazolyl-(4)-oxy]-3-[2-methoxyphenoxyethyl)amino]-propanol-(2)}) is one of the most important.

Although there are still considerable uncertainties about the mechanisms underlying the multiple cardioprotective effects of carvedilol, its action could be related to protection of mitochondrial function. Thus, it is reasonable to hypothesize that carvedilol may potentially prevent bile acid-induced mitochondrial dysfunction.

The aim of this study was to investigate the *in vitro* effects of carvedilol and its metabolite BM-910228 on the CDCA-induced PTP in rat liver mitochondria. Within this context, mitochondrial swelling, membrane potential, and mitochondrial calcium fluxes were monitored.

2. Materials and methods

2.1. Chemicals

CDCA, used as ethanolic solution, was purchased from Sigma Chemical Co. Carvedilol and BM-910228 were obtained from Boehringer. All other chemicals were of the highest grade of purity commercially available.

2.2. Isolation of mitochondria

Mitochondria were isolated from liver of male Wistar rats by conventional methods [25] with slight modifications. Homogenization medium contained 250 mM sucrose, 10 mM HEPES (pH 7.4), 0.5 mM EGTA, and 0.1% fat-free BSA. EGTA and BSA 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 [26] calibrated with BSA.

2.3. Membrane potential measurements

The mitochondrial transmembrane potential was estimated using an ion-selective electrode to measure the distribution of TPP^+ according to previously established methods [27,28]. The reference electrode was $Ag/AgCl_2$. The incubation medium contained 200 mM sucrose, 10 mM Tris–Mops (3-[*N*-morpholino]propanesulfonic acid) (pH 7.4), 1 mM KH_2PO_4, and 10 μM EGTA supplemented with 2 μM rotenone and 0.5 μg/mL of oligomycin. Mitochondria (0.5 mg) were suspended with constant stirring, at 25°, in 1 mL of the standard incubation medium supplemented with 3 μM TPP^+ and 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. A matrix volume of 1.1 μL/mg of protein was assumed.

2.4. Measurement of mitochondrial permeability transition

Mitochondrial swelling was estimated by changes in light scattering as monitored spectrophotometrically at 540 nm [29]. The reaction medium was stirred continuously and the temperature maintained at 25°. The experiments were started by the addition of 1 mg of mitochondria to a final volume of 2 mL of the standard incubation medium, supplemented with 2 μM rotenone, 0.5 μg/mL of oligomycin, and 5 mM succinate. Calcium (50 μM $CaCl_2$) was added prior to all the other compounds.

2.5. Measurement of mitochondrial calcium fluxes

The accumulation and release of calcium by isolated rat liver mitochondria was determined using a calcium-sensitive fluorescent dye, calcium green-5N [30]. The reactions were carried out at 25°, in 2 mL of the standard incubation medium, supplemented with 2 μM rotenone, 0.5 μg/mL of oligomycin, and 0.1 μM calcium green-5N. Mitochondria (0.4 mg) were suspended with constant stirring. Fluorescence (excitation 505 nm; emission 531 nm) was monitored continuously for an additional 1.5 min prior to adding calcium ($CaCl_2$) to a final concentration of 15 μM. Fluorescence was monitored continuously for an additional 3 min, at which time 8 mM succinate was added to the suspension. At each experiment, fluorescence was monitored for an additional 10 min before the reaction was stopped with excess EGTA. Calcium fluxes are expressed as relative fluorescence units (RFU). At the concentration examined, CDCA did not interfere with the experimental assay.

3. Results

3.1. Effect of CDCA on mitochondrial membrane potential

Fig. 1 reports $\Delta\Psi$ dissipation caused by CDCA (50 μM) in succinate-energized, isolated rat liver mitochondria. In the presence of calcium (50 μM), mitochondria depolarized immediately upon addition of CDCA, and resting membrane potential was never restored. Such changes are characteristic of an increased permeability of the mitochondrial inner membrane. To ascertain whether the decrease could be

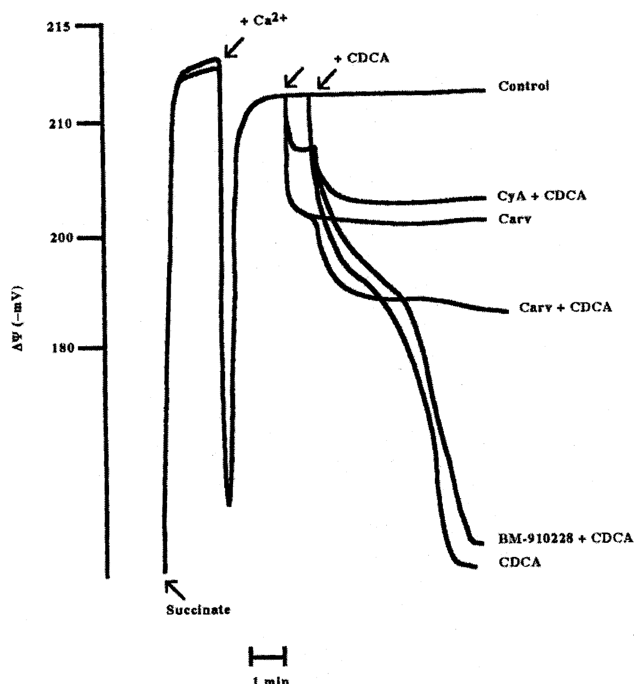

Fig. 1. Effects on membrane potential ($\Delta\Psi$) of mitochondria energized with succinate. Mitochondria (0.5 mg) were incubated in 1 mL of the standard incubation medium supplemented with 2 μM rotenone, 0.5 μg/mL of oligomycin, and 3 μM TPP^+, and energized with 5 mM succinate. Calcium was added at a final concentration of 50 μM. When indicated, 1 μM CyA, 10 μM carvedilol (Carv), or 10 μM BM-910228 was included in the reaction medium prior to addition of 50 μM CDCA. The traces are typical of several independent experiments with four different mitochondrial preparations.

attributed to the PTP, we treated the mitochondria with CyA. Under these conditions, mitochondria were able of sustaining membrane potential after the addition of CDCA. A similar result was observed for carvedilol (10 μM). Indeed, after preincubation with carvedilol, and upon addition of CDCA to calcium-loaded mitochondria, an immediate depolarization of membrane potential occurred, but it was incomplete and $\Delta\Psi$ remained stable within the time monitored. In contrast, in the presence of BM-910228 and upon addition of CDCA, mitochondria were not capable of maintaining membrane potential, and a complete dissipation was observed. Control experiments showed that carvedilol alone induced a slight decrease in $\Delta\Psi$ (Fig. 1). Additionally, its hydroxylated analog, BM-910228, had a similar behavior only for higher concentrations than that used for carvedilol (data not shown).

3.2. Effect of CDCA on calcium-dependent mitochondrial swelling

In order to support the suggestion of the protective effects of carvedilol on the induction of permeability transition by CDCA, we performed swelling experiments. Succinate-energized, hepatic mitochondria underwent a large-amplitude decrease in light scattering (Fig. 2) upon addition of CDCA. This CDCA-induced mitochondrial swelling required mitochondria preloaded with calcium (data not shown). No swelling was observed in the control experiment following calcium addition (Fig. 2), showing that mitochondria loaded with 50 μM calcium maintained their permeability barrier. The fact that the CDCA-induced decrease in absorbance was inhibited by cyclosporine A indicates that this was the result of the calcium-dependent PTP induction. Additionally, CDCA-induced mitochondrial swelling was strongly prevented by pretreating mitochondria *in vitro* with 10 μM carvedilol, which is what would be predicted from the results of the membrane potential experiments. In contrast, BM-910228 had no effect on the mitochondrial response to CDCA addition (Fig. 2). Control experiments were designed to assess if addition of carvedilol (Fig. 2) or BM-910228 (not shown) to hepatic mitochondria induces changes in absorbance. There was no discernible difference in the trace compared to that of the control sample.

3.3. Effect of CDCA on mitochondrial calcium release

The experiment reported in Fig. 3 shows the ability of CDCA to induce mitochondrial calcium efflux. Measurement of calcium fluxes indicated that unlike control mitochondria (which accumulated all of the added calcium and retained it for the entire period monitored), addition of CDCA induced release of matrix calcium. The disturbing action of CDCA on mitochondrial calcium homeostasis was partially prevented by carvedilol, whereas BM-910228 had no significant effect (Fig. 3). Control experiments showed no interference with calcium fluxes when BM-910228 (data not shown) or carvedilol (Fig. 3) was added to calcium-loaded, succinate-energized mitochondria.

4. Discussion

The plasma accumulation of bile acids has been proposed by several investigators as a causative factor for hepatic cardiomyopathy [23]. However, the mechanism whereby bile acids exert these cardiac effects is unclear. Mitochondrial dysfunction is central to a range of important human disorders. In order to treat these disorders and develop mitochondrial medicine, the enhancement of the ability to manipulate mitochondrial function is essential to protect against mitochondrial damage in rational ways.

The observation that CDCA, a primary bile acid, increases the sensitivity of isolated mitochondria to calcium-dependent induction of the PTP provides a physicochemical explanation for the bioenergetic form of cell death caused by toxic bile acids. Classically, PTP opening has been associated with generalized mitochondrial dysfunction, which is consistent with a role of the PTP in cell death. This phenomenon is implicated in the mechanism of cell killing by a number of agents, including heavy metals, pesticides,

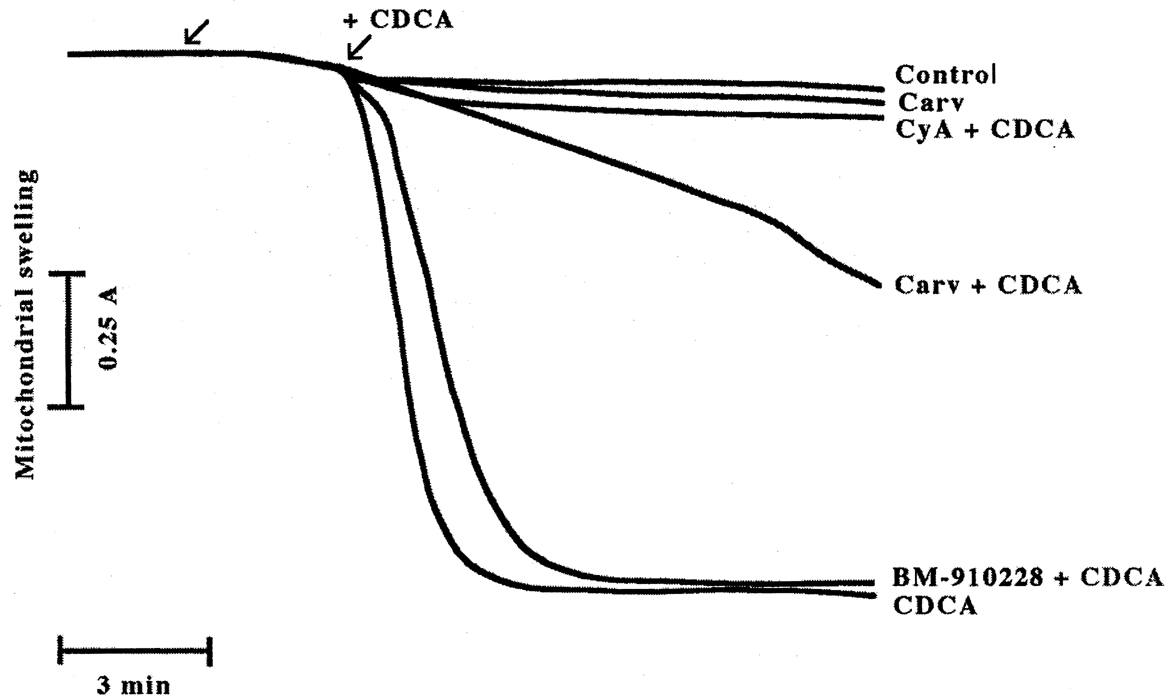

Fig. 2. Effects on mitochondrial swelling. The incubation medium (final volume 2 mL at 25°) contained 200 mM sucrose, 10 mM Tris–Mops (pH 7.4), 1 mM KH_2PO_4, and 10 μM EGTA supplemented with 2 μM rotenone, 0.5 μg/mL of oligomycin, and 5 mM succinate. The experiments were started by the addition of 1 mg of mitochondria (not shown). Calcium (50 μM) was added prior to all other compounds. Where indicated (first arrow), 1 μM CyA, 10 μM carvedilol (Carv), or 10 μM BM-910228 was included in the reaction medium prior to 50 μM CDCA. The traces are typical of several independent experiments with four different mitochondrial preparations.

and selected pharmaceuticals [21,31]. Mitochondrial permeability transition is due to the formation of a non-specific pore in the inner membrane which renders it permeable to solutes smaller than about 1.5 kDa, including H^+, thus uncoupling electron transport from oxidative phosphorylation [16]. The structure of the pore is still not certain, but is thought to be a supramolecular complex comprising proteins of the matrix (cyclophilin D), of the inner (adenine nucleotide translocator) and outer (voltage-dependent anion channel, perhaps Bax) mitochondrial membranes, and of the periplasmic space (creatine kinase) [16,32]. Such a complex constitution of the pore is reflected by the multitude of regulatory factors that influence the open/closed probability of the pore [16].

Carvedilol, a multiple-action neurohormonal antagonist with known cardioprotective effects, has been previously described [33] as a specific inhibitor of endogenous NADH dehydrogenase in heart mitochondria. In this study, the measurements of light scattering, membrane potential, and calcium movements indicated that carvedilol prevents CDCA-induced PTP. The effect of many inducers or inhibitors of the PTP may actually consist in a modulation of the "sensitivity" of the pore to voltage [17,21,31]. Due to its protonophoric effect [34], carvedilol and, to a lesser extent its metabolite BM-910228, caused a direct and slight depolarization of membrane potential, which by itself was insufficient to cause the PTP. A possible mechanism whereby carvedilol would be effective at preventing induction of the mitochondrial PTP could be attributed to a successful competition (with CDCA) for a specific voltage-sensing element that could be responsible for the induced shift in gating potential of the PTP. The demonstrated different action of carvedilol and BM-910228 against the induction of permeability transition by CDCA did not confirm the proposed interaction with the voltage-sensing element of the PTP.

The mechanism by which bile acids promote the onset of the PTP remains unclear. It is known that oxidative stress or, in general, oxidizing conditions appear to act as inducers of the PTP [16]. It has been suggested [10,35,36] that oxygen free radicals may be involved in the pathogenesis of bile acid toxicity by causing increased leaking from the altered electron transport chain. It is proposed that a critical factor regulating induction of the PTP is the oxidation state of cysteine residues in the immediate vicinity of the voltage-sensing element of the pore [37]. Thus, the reported beneficial effects of carvedilol could be due to its potent antioxidant activity [38] in interfering with a hypothetical oxidative mechanism of induction of permeability transition by CDCA. By contrast, BM-910228 (shown to have higher antioxidant activity than carvedilol [39]) did not afford protection against CDCA-induced membrane depolarization, mitochondrial swelling, or matrix calcium release, the antioxidant effect of carvedilol not being valid to justify this result.

The hypothesis of a direct interaction with the pore assembly could also explain the action of carvedilol in

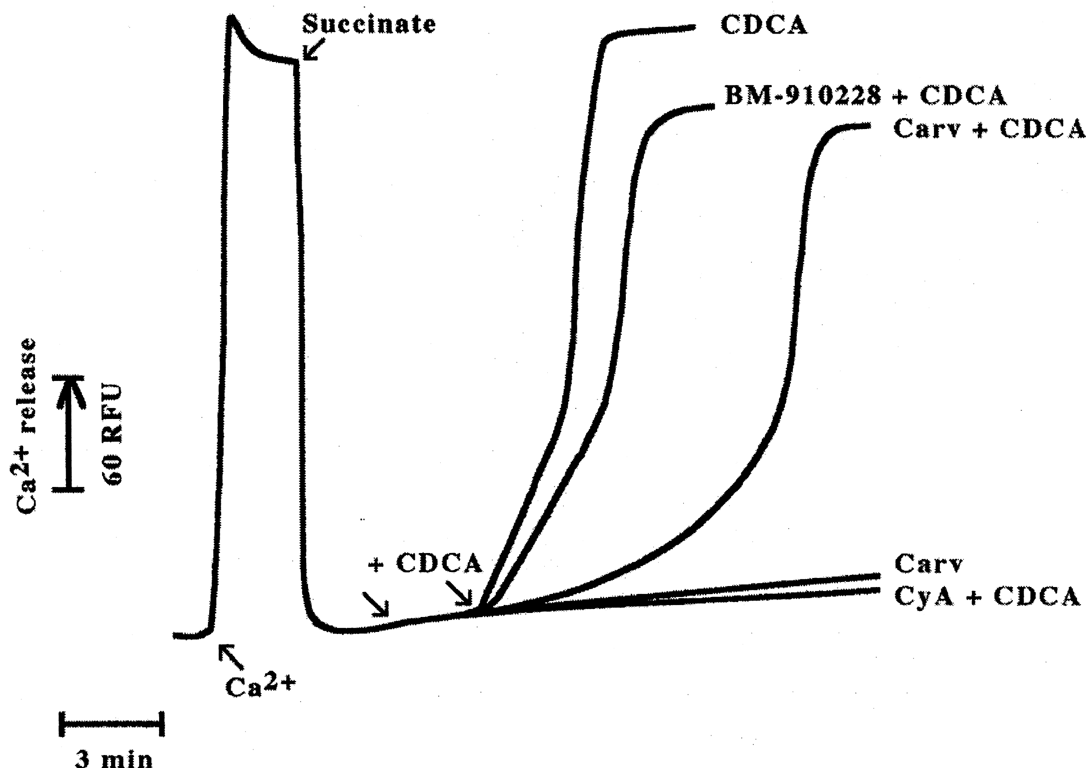

Fig. 3. Effects on mitochondrial calcium release. Mitochondria (0.4 mg) were incubated in 2 mL of the standard incubation medium prior to calcium (15 μM). Calcium fluxes, expressed as relative fluorescence units (RFU), were recorded for an additional 13 min. When indicated, CyA (0.4 μM), 5 μM carvedilol (Carv), or 5 μM BM-910228 was added 1 min prior to CDCA (20 μM). Excess EGTA was added at the end of the experiment. The traces are typical of several independent experiments with three different mitochondrial preparations. An upward pen deflection is indicative of calcium release.

modulating the ability of CDCA to induce PTP opening. Due to its great lipophilicity, carvedilol has a considerable tendency for membrane insertion [40] and may disturb the correct assemblage of the pore proteic components.

In summary, our data provide strong evidence for prevention by carvedilol against mitochondrial damage induced by the toxic bile acid, chenodeoxycholate. Thus, it is possible that such protection may modulate the injurious action of bile acids, providing an alternate means for protecting cell damage. This new observation with potential therapeutic interest may provide an important opportunity to intervene in the clinical management of cholestatic disease and its associated cardiomyopathy.

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