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Induction of the mitochondrial permeability transition as a mechanism of liver injury during cholestasis: a potential role for mitochondrial proteases

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Abstract

As part of this thematic series on mitochondria in cell death, we would like to review our data on: (1) the role of the mitochondrial permeability transition (MPT) in hepatocyte necrosis during cholestasis; and (2) the concept that endogenous mitochondrial protease activity may lead to the MPT. Many chronic human liver diseases are characterized by cholestasis, an impairment in bile flow. During cholestasis an accumulation of toxic hydrophobic bile salts in the hepatocyte causes necrosis. We tested the hypothesis that toxic hydrophobic bile salt, glycochenodeoxycholate (GCDC), causes hepatocyte necrosis by inducing the MPT. GCDC induces a rapid, cyclosporin A-sensitive MPT. The hydrophilic bile salt, ursodeoxycholate (UDCA), prevents the GCDC-induced MPT and hepatocyte necrosis providing an explanation for its beneficial effect in human liver disease. We have also demonstrated that the calcium-dependent MPT is associated with an increase in calpain-like protease activity and inhibited by calpain inhibitors. In an experimental model of cholestasis, mitochondrial calpain-like protease activity increases 1.6-fold. We propose for the first time that activation of mitochondrial proteases may initiate the MPT and cell necrosis during cholestasis.

Keywords: Bile salt; Bile duct-ligated rat; Cytoprotection; Cbz-Leu-Leu-Tyr-CHN_2; *tert*-Butylhydroperoxide; Ursodeoxycholate

1. Cholestatic liver disease and mitochondrial dysfunction

Chronic cholestasis is a feature of many human liver diseases including primary sclerosing cholangitis, primary biliary cirrhosis, biliary atresia, and chronic allograft rejection. During cholestasis, best defined as an impairment in bile flow, toxic hydrophobic bile salts, sterol derived compounds synthesized and secreted by the liver into bile during health, accumulate in the hepatocyte [1]. The accumulation of toxic hydrophobic bile salts is thought to cause hepatocyte necrosis contributing to the pathogenesis of the cholestatic disease process [2]. The importance of bile salt-induced hepatocyte injury is highlighted by the clinical observation that exogenous administration of ursodeoxycholate (UDCA), a hydrophilic bile salt, ameliorates liver injury during cholestasis [3]. However, the subcellular mechanisms by which toxic hydrophobic bile salts cause hepatocyte necrosis or how ursodeoxycholate exerts its cytoprotective effects remain obscure.

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Several studies suggest that bile acids may be cytotoxic by causing mitochondrial dysfunction. First, enlarged, swollen mitochondria are observed in histopathologic sections obtained from bile duct ligated rats (a model of extrahepatic cholestasis) with cholestatic liver diseases [4,5]. Second, mitochondria isolated from bile duct ligated rats have impaired state III respiration [6]. The impairment of state III respiration can be duplicated by exposing mitochondria or permeabilized hepatocytes to bile salts [7,8]. Finally, we have demonstrated that ATP depletion occurs early during exposure of hepatocytes to necrotic concentrations of toxic bile salts [7]. The ATP depletion is directly due to mitochondrial dysfunction as glycolytic ATP generation is intact. Thus, as assessed using both morphologic and biochemical parameters, mitochondrial function is perturbed by cholestasis or exposure to toxic bile salts, suggesting that toxic bile salts cause hepatocyte injury via a mitochondrial-dependent mechanism.

Current concepts implicate the mitochondrial membrane permeability transition (MPT) as the cause of hepatocyte necrosis mediated by mitochondrial dysfunction [9,10]. The MPT is characterized by a rapid permeability of the inner mitochondrial membrane to ions associated with mitochondrial swelling and a collapse of the mitochondrial membrane potential [11]. Cell necrosis by the MPT is thought to occur by the opening of a 'mega channel' in the inner mitochondrial membrane causing an increase in mitochondrial permeability to small molecular mass compounds, <1500 Da [11]. Cyclosporin A inhibits the opening of this pore, an effect which is enhanced by trifluoperazine [12], and these agents prevent hepatocyte necrosis in many models of cell death [10].

Despite its appreciable scientific and clinical importance, the ability of toxic bile salts to induce the MPT has not been studied. Thus, we first sought to answer the following questions. Does the toxic hydrophobic bile salt, glycochenodeoxycholate (GCDC), induce the MPT in isolated rat liver mitochondria? Does UDCA and/or its taurine and glycine conjugates prevent the MPT induced by GCDC and other agents? We chose GCDC as the toxic hydrophobic bile salt for these studies because it is a primary bile salt whose concentrations increase >20-fold during cholestasis [1].

1.1. Experimental procedure section for bile salt induction of the MPT

1.1.1. Isolation of rat liver mitochondria and measurement of the mitochondria permeability transition

Liver mitochondria were isolated from adult male Sprague-Dawley rats (250–350 g) as previously described by us in detail with minor modifications [13]. We modified the procedure by adding digitonin to the percoll-sucrose gradient to deplete mitochondria of lysosomes. At low concentrations, digitonin abolishes lysosomal latency without affecting mitochondrial integrity [14]; therefore, digitonin can be used to deplete mitochondria of lysosomes [14]. We used a digitonin containing gradient to remove lysosomes from our mitochondrial preparation. A sucrose-percoll solution was prepared by mixing 75 ml of 250 mM sucrose containing 1 mM EGTA and 50 μM digitonin plus 25 ml of percoll (density 1.129 g/ml); a self-generating gradient was obtained by centrifuging 35 ml of the sucrose-percoll solution at $43\,000\times g$ for 20 min at 4°C in 50 ml polycarbonate tubes prior to layering on the mitochondrial suspension [13]. Mitochondria were purified by centrifugation at $43\,000\times g$ for 20 min at 4°C using a Beckman Centrifuge Model J2-21 M/E and JA 20 rotor. The mitochondrial pellet was washed free of percoll as previously described and resuspended in 2–3 ml of a buffer containing 125 mM sucrose, 50 mM KCl, 5 mM HEPES, and 2 mM KH_2PO_4, pH 7.4 [13]. The purity of the mitochondria preparation was established using marker enzymes and transmission electron microscopy [13].

The MPT was measured spectrophotometrically as previously described [15]. Isolated rat liver mitochondria were suspended in a buffer consisting of 200 mM sucrose, 10 mM Tris-MOPS, 5 mM succinate, 1 mM KH_2PO_4, 2 μM rotenone, 1 μg/ml oligomycin, 10 μM EGTA, pH 7.4 at 25°C [15]. The optical density was monitored at 540 nm in a Beckman DU 7400 Diode Array Spectrophotometer (Beckman Instruments, Palo Alto, CA) at 25°C.

1.2. Does glycochenodeoxycholate (GCDC) induce the MPT in isolated rat liver mitochondria?

GCDC induced high amplitude mitochondrial

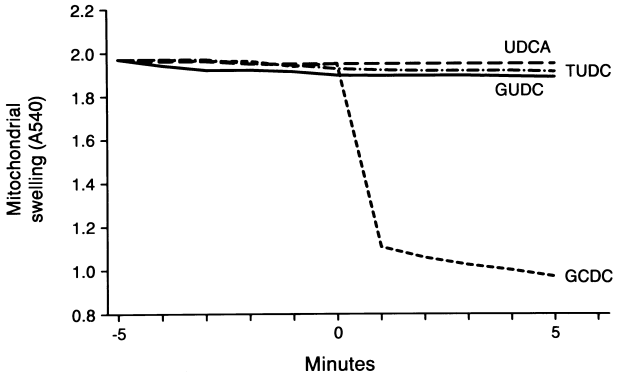

Fig. 1. GCDC, but not UDCA or its taurine (TUDC) or glycine (GUDC) conjugate, induces the MPT. Isolated mitochondria (1 mg protein/ml) were suspended in respiration buffer at 25°C. Large amplitude swelling of suspended mitochondria was measured by monitoring the optical density at 540 nm. At time zero, the bile salts were added at concentrations of 200 μM and mitochondrial swelling was monitored for an additional 5 min.

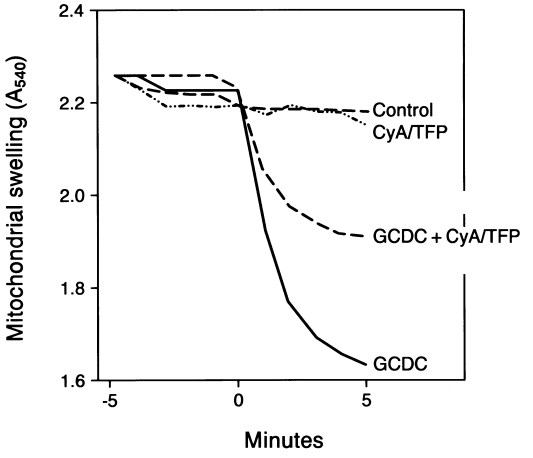

Fig. 2. Cyclosporin A (CyA) plus trifluoperazine (TFP) inhibit the GCDC-induced MPT. Isolated mitochondria (1 mg protein/ml) were suspended in respiration buffer at 25°C and preincubated for 5 min with 5 μM CyA, 10 μM TFP or 5 μM CyA plus 10 μM TFP. At time zero, 200 μM GCDC was added and mitochondrial swelling was monitored for an additional 5 min.

swelling which was both rapid (occurring within 5 min) and dose dependent (0–500 μM) [13]. Because GCDC concentrations of 200 μM are relevant to cholestatic liver injury and induced a rapid and substantial MPT, we used this concentration of GCDC for further studies (Fig. 1). In contrast to the results with GCDC, the hydrophilic bile salts ursodeoxycholate (UDCA), tauroursodeoxycholate (TUDC) and glycoursodeoxycholate (GUDC) at concentrations of 200 μM did not induce the MPT (Fig. 1). Trifluoperazine (10 μM) plus cyclosporin A (5 μM) also inhibited mitochondrial swelling caused by 200 μM GCDC (Fig. 2) demonstrating that the GCDC-induced MPT was cyclosporin A sensitive. These data directly demonstrate that the hydrophobic bile salt GCDC, but not the hydrophilic bile salt UDCA and its conjugates, induce a cyclosporin-sensitive MPT.

1.3. Does ursodeoxycholate (UDCA) inhibits the GCDC-induced MPT and hepatocyte necrosis?

Because of UDCA's therapeutic benefits in human cholestatic liver diseases, we next tested the hypothesis that UDCA exerts its beneficial effects by inhibiting the MPT induced by hydrophobic bile salts. Indeed, UDCA inhibited the GCDC-induced MPT in a dose-dependent fashion with maximum inhibition at 500 μM [13]. Pretreatment of the mitochondria for 5 min with 500 μM UDCA inhibited the GCDC-induced MPT by 69.7 ± 0.2% (Fig. 3). In addition to blocking the MPT in isolated mitochondria, UDCA strongly protected against the onset of hepatocyte necrosis during incubation with GCDC (Table 1). Hepatocyte viability was increased more than 2-fold in the presence of 500 μM UDCA plus 250 μM GCDC compared to cells incubated with GCDC alone. Cytoprotection by UDCA could not be attributed to displacement of intracellular GCDC with UDCA as intracellular accumulation of 24-^{14}C-GCDC was virtually identical in the presence or absence of a 2-fold excess of unlabeled UDCA

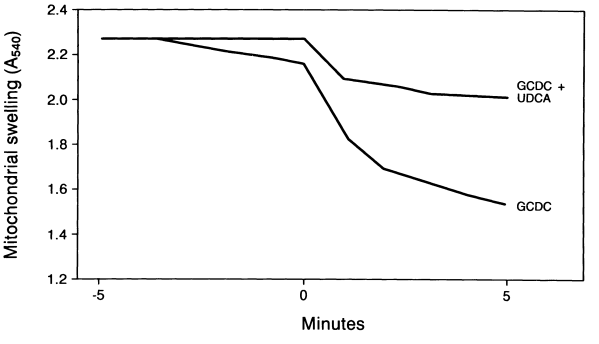

Fig. 3. UDCA inhibits the GCDC-induced MPT. Isolated mitochondria (1 mg protein/ml) were suspended in respiration buffer at room temperature and preincubated for 5 min with UDCA (500 μM). At time zero, 200 μM GCDC was added and mitochondrial swelling was monitored for an additional 5 min.

Table 1
UDCA and cyclosporin/trifluoperazine (CYA/TFP) inhibit cell necrosis by GCDC

Treatment	Cell viability (%)
Control	83 ± 3
GCDC	17 ± 2
GCDC+UDCA	49 ± 6
GCDC+CYA/TFP	68 ± 4

Rat hepatocyte suspensions (10^5/ml) were incubated in 3 ml of Krebs-Ringer-HEPES buffer at 37°C for 4 h. Cell viability was measured by propidium iodide fluorometry [7]. GCDC was used at a concentration of 250 μM, UDCA at a concentration of 500 μM, and cyclosporin plus trifluoperazine at concentrations of 5 μM and 10 μM, respectively.

(1.07 ± 0.02 vs. 1.13 ± 0.02 ^{14}C pmol/10^6 cells). If the MPT is a mechanism of GCDC-mediated hepatocyte necrosis then cyclosporin A plus trifluoperazine (well established inhibitors of the MPT) should also protect against GCDC mediated cytotoxicity. Indeed, cyclosporin A (5 μM) plus trifluoperazine (10 μM) also protected against cell death by 250 μM GCDC (Table 1). Hepatocyte viability was approx. 4-fold greater in the presence of cyclosporin A plus trifluoperazine during treatment with 250 μM GCDC. These observations suggest that GCDC-induced cytotoxicity is due to mitochondrial dysfunction with development of the MPT. Furthermore, these data suggest for the first time an intracellular pharmacologic mechanism for UDCA cytoprotection, namely, inhibition of the MPT by toxic bile salts.

2. Calpain-like protease activity and the MPT

Toxic hydrophobic bile salts are amphipathic molecules that can promote calcium flux across biomembranes, liposomes and synthetic membranes [16]. Moreover, GCDC-mediated hepatocyte necrosis is associated with increases of intracellular calcium [7]. Like many models of calcium cytotoxicity, GCDC-mediated hepatocyte necrosis is also inhibited by an acidic pH [7]. The calcium and pH dependence of GCDC-mediated hepatocyte necrosis corresponds to the calcium and pH dependence of the MPT and in this regard is consistent with the MPT as a mechanism of hepatocyte necrosis. However, the calcium and pH dependence of GCDC-mediated cell killing is also consistent with cell killing by calpains, cytoplasmic, calcium-dependent, neutral, cysteine proteases [17,18]. Indeed, several studies have implicated calpain-like protease activity in cell necrosis [9]. Therefore, we proposed a novel hypothesis to reconcile these observations. Our hypothesis is that a mitochondrial calpain-like protease activity can mediate the MPT. In support of this hypothesis is the observation that mitochondria have been reported to contain a calcium-dependent protease activity analogous to the calpains [19,20]. Thus, using the substrates and inhibitors used to identify the increase in calpain-like protease activity in cell necrosis, we performed experiments to answer the following two questions. (1) Do rat liver mitochondria contain a calcium-dependent, calpain-like protease activity? (2) Can mitochondrial protease activity induce the MPT in isolated mitochondria?

2.1. Experimental procedure section for determination of calpain-like protease activity in mitochondria

2.1.1. Measurement of calpain-like protease activity in mitochondria

Calpain-like protease activity was assayed by incubating mitochondria (1 mg/ml) in isolation media using the fluorogenic substrate Suc-Leu-Leu-Val-Tyr-AMC [21]. Fluorescence was monitored in mitochondrial suspensions following the addition of Suc-Leu-Leu-Val-Tyr-AMC in a fluorometer (Model LS-50, Perkin-Elmer, Norwalk, CT) using excitation and emission wavelengths of 380 and 460 nm respectively. Standard curves were generated using AMC. All experiments were performed at 25°C.

2.1.2. Measurement of mitochondrial membrane potential in hepatocytes

Rat hepatocytes were isolated in suspensions and cultured as we have previously described in detail [7]. Experiments measuring the mitochondrial membrane potential were performed using a multiparameter digitized video microscopy (MDVM) system employing the Metafluor quantitative fluorescence software package from Universal Imaging (West Chester, PA) [22]. The mitochondrial membrane potential was monitored in single cultured hepatocyte, using the membrane potential sensitive dye TMRE as we

have previously described in detail [22]. Briefly, cells were loaded with TMRE on the microscope stage by incubation in Krebs-Ringer-HEPES buffer (KRH buffer) containing 50 nM TMRE for 15 min at 37°C and then incubated in the presence of 50 nM TMRE throughout the experiment. Cellular fluorescence was quantitated by multiplying the average fluorescence intensity in the cell by the number of pixels above background using a threshold of zero.

2.2. Do mitochondria contain a calpain-like protease activity?

Mitochondria hydrolyzed the fluorogenic substrate in a Ca^{2+}-dependent manner. Mitochondrial hydrolysis of Suc-Leu-Leu-Val-Tyr-AMC was minimal in a calcium containing medium in the absence of a calcium ionophore, 0.7 ± 0.1 pmol AMC (mg protein)$^{-1}$ min^{-1}, but increased more than 9-fold in the presence of 50 μM 4-Br-A23187 (a non-fluorescent calcium ionophore) to 6.5 ± 0.2 pmol AMC (mg protein)$^{-1}$ min^{-1}. The K_m for the rate of hydrolysis was approx. 35 ± 4 μM. The location of the protease activity in the mitochondria was determined by fractionating the mitochondria into outer membrane, intermembrane space, inner membrane, and matrix components using the methodology validated and established by Greenawalt and co-workers [23]. The protease activity was found to be largely in the intermembrane space (Fig. 4), a position which may allow it to modify the external domains of proteins forming the permeability transition pore within the inner membrane. More importantly, however, we observed a 1.6-fold increase in mitochondrial calpain-like activity in mitochondria isolated from the bile duct ligated rat, an animal model of extrahepatic cholestasis relevant to human cholestatic liver disease. These data demonstrate that mitochondria do contain a calpain-like protease activity which increases in cholestasis. Dubiel and co-workers have also previously described a mitochondrial protease in the intermembrane space which recognizes Suc-Leu-Leu-Val-Tyr-AMC as a substrate [24]. It is likely that our protease and the protease activity described by this group are the same protease. However, the protease has not been purified nor cloned and thus its identification remains unknown.

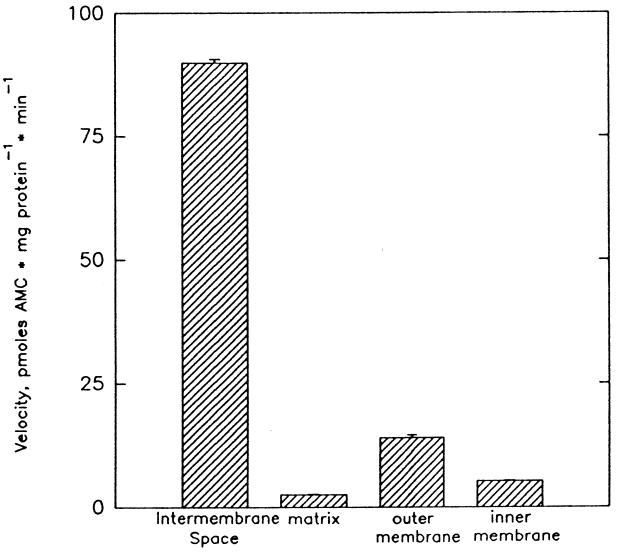

Fig. 4. The mitochondrial calpain-like protease activity is located in the intermembrane space. Mitochondria from rat liver were purified by percoll gradient centrifugation. The mitochondria were then further fractionated into outer membrane, intermembrane space, inner membrane and matrix components using an established technique employing the detergents digitonin and lubrol and ultracentrifugation procedures. The protease activity was measured in each fraction using the fluorogenic substrate Suc-Leu-Leu-Val-Tyr-AMC (50 μM).

2.3. Cysteine protease inhibitors block the calcium-induced MPT in isolated mitochondria

We next determined whether cysteine protease inhibitors would prevent mitochondrial hydrolysis of the fluorogenic substrate. Inhibition of hydrolysis of Suc-Leu-Leu-Val-Tyr-AMC was specific for cysteine protease inhibitors (leupeptin, E-64, calpain inhibitor 1, and Cbz-Leu-Leu-Tyr-CHN_2) whereas the serine (3,4-dichloroisocoumarin), aspartate (pepstatin) and metalloprotease (1,10-phenanthroline) inhibitors did not inhibit hydrolysis of the fluorogenic substrate [15]. Thus, the mitochondrial protease activity appeared to be a calpain-like protease because it recognized a calpain substrate, and was inhibited by a calpain protease inhibitor. However, further definition of this protease including its ion dependence, pH dependence, and preferred substrate will depend upon isolation and identification of the protease.

Preincubation of the mitochondria with the cys-

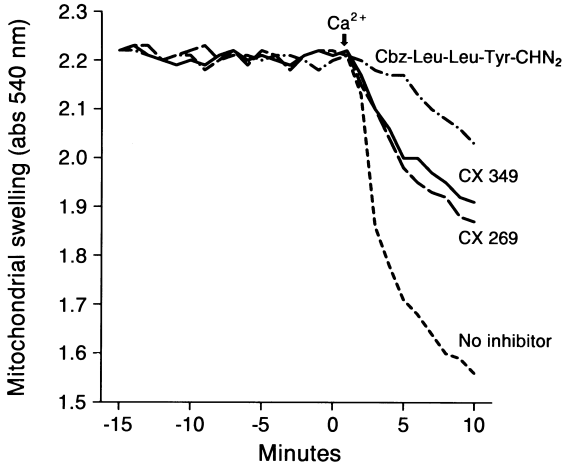

Fig. 5. The cysteine protease inhibitors, Cbz-Leu-Leu-Tyr-CHN_2, CX269, and CX349, inhibit the Ca^{2+}-induced MPT. Isolated mitochondria (1 mg protein/ml) were suspended in a sucrose-based buffer at 25°C. After 15 min of preincubation in the presence of the protease inhibitors, calcium (100 μM) was added and mitochondrial swelling was monitored for an additional 10 min.

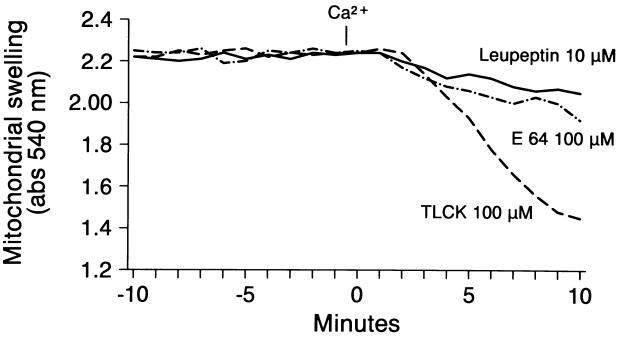

Fig. 6. The cysteine protease inhibitors, leupeptin and E-64, but not the serine protease inhibitor, TLCK, inhibit the Ca^{2+}-induced MPT. Isolated mitochondria (1 mg protein/ml) were suspended in a sucrose-based buffer at 25°C. After 15 min of preincubation in the presence of the protease inhibitors, calcium (100 μM) was added and mitochondrial swelling was monitored for an additional 10 min.

teine protease inhibitors, Cbz-Leu-Leu-Tyr-CHN_2, leupeptin and E-64, inhibited the Ca^{2+}-induced MPT (Fig. 5). Cbz-Leu-Leu-Tyr-CHN_2 was the most potent inhibitor of the MPT with maximum inhibition of $82 \pm 2\%$ at 100 μM (Fig. 5). Moreover, the highly specific calpain inhibitors Z-Leu-Phe-CONHEt (CX269) and Z-Leu-norvaline-CONH-$(CH_2)_3$-morpholine (CX349) obtained from Dr. D. Eveleth (Cortex Pharmaceuticals, Irvine, CA) also inhibited the calcium-induced MPT (Fig. 6). Inhibition of the MPT by protease inhibitors was specific for cysteine protease inhibitors as the serine protease inhibitor, TLCK, did not inhibit the Ca^{2+}-induced MPT (Fig. 6). These protease inhibitors also blocked induction of the MPT by 100μM *tert*-butylhydroperoxide (data not shown). The ability of leupeptin to inhibit the MPT induced by *tert*-butylhydroperoxide has also been observed by others [25]. Because these protease inhibitors react with cysteine residues, we cannot exclude the possibility that these inhibitors are also reacting with thiols present in the MPT pore. The role of this mitochondrial protease in mediating opening of the pore can only be rigorously tested once it has been isolated and cloned; this work is ongoing in our laboratory.

2.4. *Does the calpain-selective protease inhibitor, Cbz-Leu-Leu-Tyr-CHN_2, delay mitochondrial depolarization, a marker of the MPT, in hepatocytes?*

Finally, we sought to determine whether we could extrapolate our results in isolated mitochondria to the MPT in intact hepatocytes. We used the selective calpain protease inhibitor Cbz-Leu-Leu-Tyr-CHN_2 to determine whether it would prevent mitochondrial depolarization in intact cells. The mitochondrial membrane potential was measured as a surrogate marker for the MPT in cultured cells [26]. We chose *tert*-butylhydroperoxide (tBH) for these studies because it has been demonstrated to induce the MPT in hepatocytes [26], and we wanted to test our hypothesis in an established model before embarking on experiments with toxic bile salts. As reported by others, during exposure to 25 μM tBH, the mitochondrial membrane potential decreased to $54 \pm 3\%$ of the initial value after 1 h of incubation. However, the membrane potential was maintained in the presence of 100 μM Cbz-Leu-Leu-Tyr-CHN_2 during treatment with tBH [15]. Thus, inhibition of calpain-like protease activity appears to inhibit the onset of the MPT in hepatocytes.

3. Summary

Our studies are important for the following three

observations. First, our results demonstrate for the first time that toxic bile salts can induce the MPT, an observation relevant to hepatocyte injury during cholestasis. Second, the results suggest that pharmacologic agents such as ursodeoxycholic acid may exert their cytoprotective properties by inhibiting the MPT. Finally, our results suggest a provocative hypothesis, namely, that mitochondrial protease activity may produce the MPT under pathophysiologic conditions.

Bile salts have been previously demonstrated to cause mitochondrial dysfunction with impairment of oxidative phosphorylation [6,8]. Our data further extend these observations by demonstrating induction of the MPT by toxic bile salts. Because the MPT has been implicated as a mechanism of cell necrosis and apoptosis [10], our observations provide a mechanism for hepatocellular injury during cholestasis. Our observations of the MPT in isolated mitochondria also provide an explanation for the large, swollen mitochondria observed in histopathologic specimens from animals with cholestasis [4]. The mechanism by which bile salts induce the MPT remain unclear but may involve calcium fluxes into the mitochondria. Bile salts promote calcium flux across biomembranes and increase cytosolic free calcium [16]. Because increases of mitochondrial calcium can promote the MPT [10], it appears likely that the bile salt-induced MPT may be calcium mediated. Further experimentation will be required to test this hypothesis.

UDCA protected against induction of the MPT by the toxic hydrophobic bile salt GCDC. The protective effect of UDCA is not due to a simple biophysical interaction between a hydrophobic and a hydrophilic bile salt in the soluble phase or in the lipid membrane as an equally hydrophilic bile salt, hyodeoxycholate, did not prevent the GCDC-induced MPT (data not shown) [13]. The mechanism by which UDCA prevents the GCDC-induced MPT remains unclear. However, UDCA does not merely antagonize the effect of GCDC on inducing the MPT, but appears to be an inhibitor of the MPT. For example, UDCA prevents induction of the MPT by phenylarsine oxide and mitochondrial depolarization in a number of models of hepatocyte apoptosis [13,27]. UDCA would appear to be the first approved drug to work by such a mechanism.

An epithelial serine protease has been shown to indirectly modulate the activity of the amiloride-sensitive sodium channel [28]. Furthermore, Kroemer and co-workers have demonstrated that caspase 1 can directly induce the MPT in isolated mitochondria [29]. Thus, there is a precedent for protease modulation of ion channels and the MPT. Our data are consistent with the interpretation that an endogenous mitochondrial protease may induce the MPT. There are five conceptual mechanisms by which calpain-like proteases could lead to the MPT: (a) limited proteolysis of a channel protein causing opening of the channel; (b) proteolysis of proteins whose peptide fragments bind to channel proteins inducing conformational changes and opening of the channel; (c) proteolysis of a protein which normally inhibits opening of the channel; (d) proteolysis of proteins with the release of amphipathic peptides which directly insert into the inner membrane forming channels; and (e) proteolysis of structural proteins within the mitochondria which lead to topographical disorganization of the mitochondria and permeability changes. Current concepts suggest the low conductance state of the MPT is reversible in isolated mitochondria [30]. If the MPT is reversible during cell injury, we find it unlikely that calpain-like proteases cause pore opening by direct proteolytic cleavage of channel proteins or proteolysis of mitochondrial structural proteins. Indeed, the serine protease activating the amiloride-sensitive sodium channel does so through an indirect mechanism that does not involve proteolytic cleavage of the channel protein [28]. Proteolytic cleavage of bacterial toxins have been shown to release peptide fragments with channel forming properties [31]; thus, it is conceivable that this mechanism of channel formation (i.e., proteolytic generation of amphipathic peptide fragments which insert into the inner membrane of mitochondria forming channels) could occur during cell damage. However, this mechanism of pore formation would not be consistent with the specific effects of cyclosporin A as an inhibitor of pore opening. Peptide fragments generated from proteolytic cleavage of proteins could potentially reversibly bind to channel proteins modulating opening and closing of the channel. Indeed, mastoparan, a 14 amino acid amphipathic peptide, has been found to induce the MPT [32]. Pfeiffer and co-workers have hypothesized that

positively charged peptide fragments may allosterically modulate pore opening by binding at a site which normally binds cations [32]. Finally, the hypothesis that a protease could cleave an endogenous inhibitor of the MPT also remains a tenable hypothesis. Once the components forming the channel mediating the MPT are known, the mechanisms leading to the MPT by calpain-like protease activity can be directly elucidated. We believe it is highly likely that numerous mechanisms will be shown to cause the MPT including protease-dependent processes.

Our current work reflects a hypothesis in evolution, an we realize that the data for our model require further evidence to be compelling. At this time, our working hypothesis is that during cholestasis there is an accumulation of toxic hydrophobic bile salts within the hepatocyte. These bile salts promote increases in cytosolic free calcium and mitochondrial calcium resulting in an increase of mitochondrial calpain-like protease(s) activity. We invoke increases in calcium as the mediator for the protease activation because: (i) we have directly observed an increase in cytosolic calcium in Fura-2 loaded hepatocytes treated with GCDC [7]; and (ii) GCDC itself does not directly activate the mitochondrial protease activity (data not shown). Following protease activation, we speculate that the protease activity then acts on key inner membrane associated proteins leading to formation of the mitochondrial transition pore. The MPT would then result in failure of oxidative phosphorylation, enhanced generation of toxic oxygen species, a decrease in cellular ATP, release of mitochondrial constituents leading to activation of caspases, and perhaps other deleterious effects on the cell [9]. These adverse effects of mitochondrial dysfunction would then culminate in hepatocyte death. Our data suggest a unifying hypothesis linking the independent observations that mitochondrial protease activity and the MPT are important mechanisms causing cell necrosis. We propose for the first time that activation of mitochondrial calpain-like protease activity can function as a cytolytic trigger initiating the MPT in cell necrosis during cholestasis. The identity of the protease, its substrates and why it is increased in cholestatic liver disease are active areas of investigation in our laboratory based research program.

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