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Mitochondrial dysfunction in cholestatic liver diseases

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1. ABSTRACT

Cholestatic liver diseases are characterized by blockade of bile flow from the liver to the intestine, and accumulation of hydrophobic bile acids in the liver and plasma. As a consequence an inflammatory response evolves associated with increased apoptosis, oxidative stress, and eventually fibrosis. Cholestasis is associated with profound metabolic changes, alterations in the mitochondrial function, decreased fatty acid oxidation, and increased glycolisis. Mitochondria play a central role in the development of this liver disease because they mediate death receptor signaling - triggered by inflammatory cytokines or bile acids - and contribute to oxidative damage, metabolic disorder, and onset of fibrosis. During the pathogenesis of biliary cirrhosis mitochondria's need for renewal is hampered by a blunted mitochondrial biogenesis. Lack of stimulation of mitochondrial renewal helps to explain mitochondrial impairment in long-term cholestasis. The marked depletion of mitochondrial DNA and occurrence of mitochondrial DNA deletions are probably relevant contributors to the progression of this severe disease. All these findings certainly support the consideration of long-term cholestasis as a secondary mitochondrial hepatopathy.

2. CHOLESTATIC LIVER DISEASES

Cholestasis literally means "standing still of bile", and is defined as any condition that causes retention and accumulation of potentially toxic bile acids in the liver and the systemic circulation (1). Cholestasis can be triggered by different causes, such as inflammation, viral infection, autoimmune diseases, gallstones, or tumors of the pancreas, liver or biliary tree. The origin of the hepatic damage can generally be hepatocellular – ascribed to a functional defect in bile formation mainly due to genetic mutations – or ductular/ductal (referred as obstructive jaundice) – caused by an impairment of bile secretion at the level of the bile ducts – (2-4).

Cholestasis is responsible for severe biochemical and structural alterations of several organs, being the liver the most affected. Chronic cholestasis results in profound changes in liver architecture – fibrosis and increase in bile ductules – (5, 6), morphology – hepatomegaly and appearance of regenerative nodules – (6), and physiology – decreased enterohepatic circulation of bile acids and synthesis of albumin, portal hypertension – (6, 7). From a biochemical point of view, in cholestasis there is a shift in the mechanisms of energy production, since an alteration of

mitochondrial function in the liver is associated with an increase in the glycolitic activity to cope with energy needs.

Experimentally, cholestasis is usually studied in rodents, but also in rabbits and dogs, by the use of surgical intervention of common bile duct ligation (BDL or CBDL) (8, 9). This technique simulates an extrahepatic cholestasis, as could occur because of gallstones or tumors, and causes accumulation of bile acids and other compounds in the liver and in plasma. In order to discriminate systemic effects (e.g. inflammatory mediators, hormonal regulation) from organ specific effects of cholestasis (e.g. mechanisms of apoptosis), ligation of just one bile duct (partial bile duct ligation, PBDL) can be performed (10, 11).

The BDL model exhibits similarities and differences with the human cholestatic liver disease. Like humans, rodents develop bile duct and septal proliferations, inflammatory cell infiltration and liver fibrosis. However, the BDL model shows a more aggressive phenotype of the pathology, characterized by extensive areas of necrosis and formation of regenerative nodules, and death occurs within weeks or months (depending on the species) (12). Bile duct ligation is still the most used and better characterized model of cholestasis.

Growing availability of genetic mouse models, like the Mdr2 null mice, allows addressing specific questions on biliary phospholipid excretion. This model closely resembles the phenotype observed in a subtype of progressive familial intrahepatic cholestasis (13). Because of the large use of the BDL animal model, most (if not all) the studies cited in this review refer to it.

Several studies have already assessed mitochondria as relevant organelles in the pathophysiology of liver cholestasis (14-19). Mitochondria are key components of the cholestatic liver diseases, being involved in several steps of the progression of the pathology. Overall mitochondrial function is reduced during experimental cholestasis, and this is associated with an important metabolic disturbance, characterized by reduced fatty acid oxidation and ketone body formation, both during short- and long-term cholestasis (20, 21). Increased anaerobic glycolisis as a compensatory energy-producing mechanism in the cholestatic liver (17) is associated with depletion of glycogen stores (22).

Profound metabolic alterations are also aggravated by inflammatory cell infiltration, release of proapototic factors and cell death. Mitochondria play a key role in the cholestatic liver because they link inflammation with apoptosis and energy production. Mitochondrial damage is not paralleled by proper mitochondrial biogenesis to promote restoration of mitochondrial function. Non-functional mitochondria are important sources of reactive oxygen species (ROS), which in turn can promote the onset of apoptosis and are also responsible for the activation of pro-fibrogenic mechanisms (23, 24). This is aggravated by a highly hydrophobic cellular milieu due to accumulation of potentially toxic bile acids, which further enhance mitochondrial dysfunction. The specific mechanisms involved in the onset and development of mitochondrial dysfunction in liver cholestasis are still controversial and will be the focus of this review.

3. IMPAIRMENT OF MITOCHONDRIAL FUNCTION IN CHOLESTATIC LIVER DISEASE

Direct and indirect evidences support a profound alteration of the energy homeostasis during cholestasis (17, 20, 21, 25). Major alterations of energy metabolism in experimental cholestasis resemble metabolic alterations observed in patients with liver cirrhosis (26). Nevertheless, in the human pathology a systematic assessment of the energy metabolism and mitochondrial bioenergetics in patients with cholestasis alone is difficult to be obtained. Thus, experimental data from animal models are generally required to get a deep understanding of cholestatic liver disease.

After the onset of cholestasis, bile acids accumulate in the liver promoting cellular signaling but also exerting cytotoxic actions in hepatocytes (27). Hydrophobicity of these compounds seems to determine the degree of toxicity, being probably CDCA, DCA and litocholic (LCA) acids the most potent molecules in this regard (28). Part of the adverse effects of bile acids on mitochondrial bioenergetics could be related to the perturbation of mitochondrial membrane composition, as in fact CDCA and LCA can incorporate into mitochondrial membranes and reduce its content in phospholipids (19). Thus, this property of hydrophobic/lipophilic bile acids could alter membrane fluidity, mitochondrial membrane potential (Ψ_m), ATP levels and ROS production, and increase the propensity to apoptosis.

Accumulation of bile acids negatively affects mitochondrial function and bioenergetics by directly impairing activities of the enzyme complexes of the ETC (19). Complex I and III activities in isolated mitochondria were negatively affected in presence of less than 100 mM of DCA, CDCA or LCA, and complex IV was also inhibited by bile acids but higher concentrations were required (19). These concentrations are within the pathological range found in the liver during cholestasis in rodents (29), and support a pathophysiological relevant role of these findings.

In the incubation of isolated rat mitochondria with succinate to assess complex II activity, addition of individual bile acids induced a decrease in ATP synthesis, ratio between ATP synthesis and oxygen consumption (P:O ratio), and respiratory control index (RCI) (30, 31). In another study, bile acids induced a loss of Ψ_m under similar experimental conditions, and the effect was larger using CDCA, DCA and LCA than using less hydrophobic bile acids (30). These findings support our experimental observations in long-term bile duct ligated rats. In these studies a decrease in Ψ_m was found in isolated hepatocytes by flow cytometry analysis (18) and confirmed in isolated mitochondria by polarographic determination (17).

ADP-stimulated mitochondrial respiration (state 3) is largely affected by bile acids and by cholestasis. In

fact, addition of individual bile acids suppressed mitochondrial respiration in state 3 using succinate as substrate (30, 31). In an independent study, state 3 mitochondrial respiration was also decreased by incubating mitochondria with hydrophobic bile acids using L-glutamate, succinate, duroquinol or ascorbate/TMPD as substrates (19).

State 4 (non-ADP stimulated) mitochondrial respiration either does not change or increases in long-term cholestasis, as previously observed in mitochondria isolated from short-term cholestatic rats and dogs (17, 19, 30, 32, 33). The increase in state 4 mitochondrial respiration found in some studies could be explained by an increased proton leak through the inner mitochondrial membrane, which pushes more electrons throughout the ETC without a parallel raise in ATP synthesis. The observed increase in state 4 respiration is already evident at 1 day after bile duct ligation, or even after few minutes after the addition of bile acids *in vitro* (30, 33). A short-term mechanism of ETC uncoupling induced by bile acids has been demonstrated, yet uncoupling protein 2 (UCP-2) already increases in the liver at transcript level at 4 and 8 days of extrahepatic cholestasis in rats (34). Given a direct effect of bile acids on state 4 mitochondrial respiration, it is reasonable to suggest that these compounds could directly modulate UCP protein activity, but to date experimental evidence to support this hypothesis is lacking.

Several lines of evidence sustain an overall decrease in mitochondrial function during experimental cholestasis. An early study by Ozawa and colleagues showed that various respiratory parameters of liver mitochondria change in response to acute biliary obstruction in rabbits (35). Despite a transient increase at 3 h from bile duct ligation, ATP production was already reduced at 6 h, reached a minimum level at 24 h, and maintained a plateau level for 7 days (35). More recent studies reported biochemical alterations of the mitochondrial function in response to acute and chronic cholestasis. Mitochondrial function decreased during short and long-term cholestasis (1 to 6 weeks) in rats (31). In this study it was reported a progressive decrease in RCI, oxygen consumption in ADP-stimulated respiration (state 3), and ATP synthesis using succinate or α-ketoglutarate as substrates (31).

More recent research confirms some previous findings on mitochondrial respiration in cholestasis. ADP-stimulated oxygen consumption and RCI were markedly reduced using glutamate and malate as substrates, but not using succinate (17). Thus, after long-term cholestasis there seems to exist a major impairment of complex I activity of the ETC rather than a general impairment of the respiratory function. Moreover, some compensatory adaptations of mitochondrial respiration could be responsible for maintaining coupling of mitochondrial respiration to ATP synthesis, at least under basal conditions. Despite a major overall impairment of the mitochondrial function during long-term cholestasis, the liver seems to cope to a certain extant with its metabolic requirements by adaptive hepatomegaly. Accordingly, at 5 weeks of cholestasis, oxygen consumption in perfused rat liver does not differ in BDL from sham-operated rats (36).

In the cholestatic liver some substances may exert an inhibitory effect on mitochondrial respiration, decreasing ETC activity and ATP synthesis. A progressive decrease in ATP synthesis in isolated liver mitochondria was observed throughout 3 weeks of cholestasis progression, but the decrease was less marked when ATP synthesis was determined in mitochondria isolated from hepatocytes (37). The authors proposed the existence of inhibitory compounds in the liver, which might impact on mitochondrial respiration even in competent organelles. In a different study it was found a 34% decrease in ATPase activity in the liver after 5 weeks of cholestasis, but this parameter did not change significantly in isolated liver mitochondria (36). This discrepancy can be partly explained by the different source of mitochondria, which were from hepatocytes of cholestatic animals in the first study and from the whole liver (which includes all liver cells) in the second study.

Controversial results have also been presented about the activity of respiratory complexes. In one study, mitochondrial preparations from BDL rats presented significantly higher activity of complex III (+35%), complex IV (+40%) and citrate synthase (+25%) than those from sham animals (38). In contrast, in another study cytochrome c oxidase (complex IV) and ATPase (complex V) activity did not show any change in cholestatic versus control rats (39). Finally, other data reported a decrease in the activity of complexes II, III, IV and V, but no change in citrate synthase activity after 90% ligation of the biliary tree (40).

Mitochondrial function was also decreased in tissues other than the cholestatic liver lobes (40). Kanai and colleagues showed that 70% or 90% bile duct ligation produces a decrease in the mitochondrial oxygen consumption in state 3 at 4 weeks from the surgical procedure in the obstructed lobes (40). Importantly, after 90% partial cholestasis significant changes were also observed in the non-obstructed liver lobe, which included a decrease in the RCI, in the P:O ratio, and in the activities of the five respiratory complexes (40). Another study showed decreased mitochondrial respiration in state 3 and RCI in heart mitochondria at 1 week of BDL in rats (41). These results suggest that compounds (e.g. bile salts) released in the circulatory system during cholestasis may produce mitochondrial dysfunction in extrahepatic tissues.

4. MITOCHONDRIAL BIOGENESIS IN CHOLESTATIC LIVER DISEASE

Mitochondrial function and mass have to be tightly regulated at the cellular level because of their vital role in energy production, metabolism, and signaling (42). The active process of biosynthesis of new mitochondria - called mitochondrial biogenesis - has to cope with the specific energy demand of the cell, so it requires a complex interaction between nuclear and mitochondrial genomes. The vast majority of the mitochondrial proteome (1100-

1500 proteins, 43 is encoded by nuclear DNA (nDNA), but 13 essential proteins of the electron transport chain (ETC) are encoded by the mtDNA (44). The whole process of mitochondrial biogenesis involves synthesis, import, and incorporation of proteins and lipids to the existing mitochondria, as well as replication of the mitochondrial DNA (mtDNA). Mitochondrial biogenesis is finely coordinated through the activity of several transcription factors at the nuclear level, being the most relevant nuclear respiratory factor 1 (NRF-1) and 2 (NRF-2/GABPα), estrogen related receptor a (ERR-α), the nuclear coactivators peroxisome proliferator activated receptor g coactivator 1a (PGC-1α) and β (PGC-1β), and PGC-1α-related coactivator (PRC) (44). These proteins promote the transcription of several mitochondrial genes in the nucleus, among which mitochondrial transcription factor A (TFAM), B1 (TFB1M), and B2 (TFB2M) are critical because they bind mtDNA and control mtDNA genes transcription and replication (45, 46).

During the progression of experimental cholestasis, it was observed an increase in mitochondrial protein content in the liver until day 14 after bile duct ligation, consistent with the observed increase in liver mass (47). In turn, in comparison to pair-fed rats, mitochondrial mass per gram of liver did not change until day 14, but it decreased by 35% at day 28 (47). After long-term partial cholestasis - that allows comparisons between obstructed and non-obstructed liver lobes – it was found a decrease in some subunits of the ETC in both the obstructed and the non-obstructed lobes (40). The authors proposed that an inhibition of the expression of nuclear and mitochondrial genes and/or an accelerated proteolysis might occur (40). Two studies confirmed the hypothesis of an impairment of mitochondrial and nuclear genes expression observed during short-term and long-term cholestasis (16, 17). However, no studies have so far supported the hypothesis that increased proteolysis may be the cause of mitochondrial dysfunction during the progression of cholestasis.

A decrease in mitochondrial biogenesis has been reported to appear very early in cholestasis. During the first 3 days of bile duct ligation, TFAM transcript and protein levels were lower in cholestatic animals than in control ones (16). Nonetheless, in this study control rats were not pair-fed to BDL ones. Our group characterized most of the process that brings about a decrease in mitochondrial biogenesis during long-term cholestasis, and found that the problem arises both at nuclear and mitochondrial levels (17). NRF-2/GABPα levels were markedly decreased in the cholestatic rat liver and NRF-1 seemed to have reduced activity and was unable to maintain an active transcription of nuclear encoded-mitochondrial genes. This resulted in a decrease in TFAM, which is a target gene of NRF-1 and NRF-2/GABPa, at mRNA and protein levels. Mitochondria themselves further contribute to the reduced biosynthetic potential, since synthesis of mitochondrial proteins in isolated liver mitochondria is largely decreased in the cholestatic rat starting from 2 weeks of bile duct ligation (47).

One major event associated to TFAM loss in the cholestatic liver was a marked (i.e. ~65%) decrease in mtDNA/nDNA ratio (17). Three different situations can explain this finding: 1) a decrease in the copy number of mtDNA per mitochondrion, 2) a loss of mitochondria, which is in turn paralleled by a decrease in mtDNA amount per cell, 3) an increase in cell ploidy, which affects the mtDNA to nDNA ratio, independent of absolute abundance of mtDNA in the cell. To check for the third situation, we assessed cell ploidy in liver from cholestatic and sham operated-pair fed rats. Our results showed a non-significant increase in cell ploidy during cholestasis (unpublished results) that cannot account for the marked reduction (~65%) in mtDNA observed after 4 weeks of bile duct ligation (17). According to previous studies, it was found that the decrease in mtDNA observed during cholestasis should be ascribed to a loss of mitochondria rather than to a decrease in mtDNA per mitochondria. Indeed, mtDNA content per mitochondria did not change throughout progression of cholestasis (40). Taken together, these results suggest that mtDNA loss is secondary to an overall deregulation of mitochondrial biogenesis.

A special consideration should be made regarding mitochondrial protein import, a mechanism that promotes renewal of functioning organelles. Mitochondrial import is a process mediated by cytosolic chaperones, which allow contact between proteins containing mitochondrial target sequences and mitochondrial membrane proteins with receptor function (48). Upon recognition by the receptor, the imported protein is channeled through pore membrane proteins of the outer membrane, and subsequently of the inner mitochondrial membrane (48). After shuttling, the protein is cleaved by mitochondrial peptidases to give rise to a mature form, and then delivered to the correct mitochondrial location. To be imported into the mitochondrion, most proteins require ATP, GTP and an active proton-motive force (49, 50). The import pore includes protein translocases of the outer membrane (TOMs), which are required to take the initial interaction with cytosolic proteins. Among them, TOM20 shows higher affinity for targeting signals contained in the cleavable presequences, and TOM70 is usually required for proteins carrying internal targeting signals (48, 51, 52). During the progression of experimental cholestasis in rats, TOM20 levels increase in liver mitochondria but TOM70 levels are strongly reduced (17). Regardless possible compensatory mechanisms, loss of TOM70 in cholestatic liver could have an important impact on mitochondrial biogenesis and bioenergetics. Deletion of TOM70 in yeast demonstrated that this receptor has a role as docking site for cytosolic chaperones and also maintains solubility of proteins targeted to the mitochondrion (53). Indeed, a yeast mutant in TOM70 shows a reduced import of several mitochondrial proteins, including ATPase subunits and the chaperone Hsp60 (53). In agreement with this report, Hsp60 decreases in liver mitochondria from cholestatic rats at 2 and 4 weeks after bile duct ligation, in association with the decrease in TOM70 (17).

We also found that other mitochondrial proteins are differently regulated in the cholestatic liver. TFAM

levels are largely decreased after 4 weeks of bile duct ligation, even to a greater extent than the decrease in the mRNA level (17). So far, it is unclear if there is a specific role for protein degradation in this regard. Eventually, TFAM depletion could be secondary to a decreased mitochondrial import, as evidenced by a decreased level of other mitochondrial proteins. Defects in mitochondrial protein import could indeed contribute to the development of the disease. Nonetheless, whatever the relevant mechanism in cholestasis, loss of mitochondrial proteins is not a general phenomenon. In fact, cytochrome c is a specific example of a mitochondrial protein that may even increase during cholestasis (17). To date, specific studies aimed at understanding the contribution of altered mitochondrial import machinery to the defective mitochondrial biogenesis and bioenergetics in the development of cholestatic liver disease are lacking.

5. MITOCHONDRIAL-MEDIATED APOPTOSIS IN CHOLESTATIC LIVER DISEASE

5.1. General aspects

Apoptosis is a ubiquitous form of programmed cell death occurring in human liver diseases (54-57). Hallmarks of apoptosis are cytoplasmic shrinkage, chromatin condensation, nuclear fragmentation, presence of plasma membrane blebbing, and fragmentation of the cell into apoptotic bodies (58). Upon completion of the process, ordered resorption of individual unwanted or dangerous cells minimizes leakage of intracellular components into the extracellular space and attenuates inflammatory responses. Apoptosis is generally initiated by external stimuli converging on death receptors (DRs) like tumor necrosis factor (TNF) receptor superfamily, member 6 (FAS/APO1/CD95) or TNF (ligand) superfamily, member 10 (TRAIL/APO2L), that oligomerize and lead to the formation of a death initiating signaling complex (DISC) consisting of Fas (TNFRSF6)-associated via death domain (FADD) and an initiator caspase, pro-caspase 8/10 (59). In type I cells, processed caspase-8 is able to directly activate other effector caspases (i.e. CASP-3, 6 and 7), which in turn trigger the execution of apoptosis of the cell. Conversely, in type II cells caspase-8 mediates the activation of the BH3 interacting domain death agonist (BID) leading to the activation of BCL2-associated X protein (BAX) and/or BCL2-antagonist/killer 1 (BAK) to the mitochondrion, the release of pro-apoptotic factors from mitochondria, formation of the apoptososme, and then activation of caspase-9 and effector caspases (60, 61). In the latter case, a mitochondrial amplification loop becomes essential to the execution of apoptosis.

Hepatocytes have for long time been considered type II cells because experimental evidences supported the notion that CASP-3 is activated through the mitochondrial pathway in these cells (62). Nonetheless, more recent research defines that hepatocytes behave as type I (mitochondrial-independent apoptosis) or type II cells (mitochondrial-dependent apoptosis) depending on the intensity of the apoptotic stimulus (63, 64). In one study it has been shown that loss of X-chromosome linked inhibitor of apoptosis (XIAP) or an increase in second mitochondria-derived activator of caspases (SMAC/DIABLO) in mice rendered hepatocytes susceptible to apoptosis independent of BID upon Fas stimulation, like type I cells (63). In another study it has been proposed that BID is an amplifier of a weak DR signal, and upon increased Fas stimulation, quiescent and non-quiescent hepatocytes are more sensitive to Fas ligand-induced (FasL-induced) apoptosis. Furthermore, hepatocytes' cross-talk with the extracellular matrix can switch the mode of apoptosis from type II to type I. Hepatocytes cultured on collagen, which more closely resemble physiological conditions, undergo mitochondrial-independent apoptosis upon FasL stimulation (65). Thus, despite a recognized paramount role for mitochondria in the induction of apoptosis in hepatocytes, they can undergo apoptosis also in a mitochondrial-independent fashion depending on the intensity of the stimulus and on the interaction with the extracellular environment.

5.2. Apoptosis in liver cholestasis: time course and mechanisms

Liver injury during cholestasis is characterized by hepatocyte and cholangiocyte apoptosis. Caspase-3 immunostaining and activity have been previously used to assess the evolution of apoptosis in the cholestatic rat liver. These results showed that apoptosis is significantly elevated during the first week after bile duct ligation, declining thereafter (66, 67). Though probably less abundant, apoptosis is still present in the cholestatic liver during mid- and long-term evolution of the disease (17, 18). Indeed, flow cytometric evaluation of apoptosis in isolated hepatocytes from cholestatic rat liver revealed that apoptosis is still significant at 4 weeks from bile duct ligation (18).

During progression of cholestasis, multiple mechanisms of apoptosis are likely to act synchronously to promote cell death in the liver parenchyma. At three days from the onset of cholestasis, apoptosis in the liver seems to be predominantly Fas-dependent as assessed by a reduced level of apoptosis in Fas-deficient lpr (lymphoproliferation) mice (68). Nonetheless, at day 7 from the bile duct ligation, apoptosis was attenuated to a lesser extent in Fas-deficient mice, indicating that other mechanisms were promoting apoptosis after bile duct ligation (68). Consistent with molecular alterations of mitochondrial physiology, mitochondrial-dependent mechanisms are largely contributing to cell death after bile duct ligation. An alteration of the ratio between BAX and B-cell CLL/lymphoma 2 (BCL-2), which peaks at 3 days after bile duct ligation, has been described during cholestasis in rats (69). Despite the majority of apoptosis fades away at the end of the first week from bile duct ligation, residual liver apoptosis is still detectable after 2 weeks, with increased BAX/BCL-2 ratio and cleaved caspase-3 in rat liver (17).

Further evidence supports a central role of the mitochondrion as a contributor to the apoptotic response in the cholestatic liver. Loss of Ψ_m during long-term cholestasis has been clearly demonstrated by two independent techniques, namely flow cytometry and

polarography (17, 18). Under proapoptotic stimuli, opening of mitochondrial permeability transition (MPT) pores develops and causes collapse of the Ψ_m with release of propoaptotic factors that bring about apoptosome formation and caspase activation. In isolated mitochondria from rat liver, chenodeoxycholic acid (CDCA) has been found to be a direct inducer of MPT, accompanied by membrane depolarization, release of matrix calcium, and osmotic swelling (70). Other hydrophobic bile acids are similarly able to cause mitochondrial depolarization and MPT in isolated mitochondria (30, 71-73), thus creating a direct link between bile acids and mitochondrial-mediated apoptosis. In this regard, mitochondria are likely to play a key role in apoptosis progression and during long-term cholestasis. A direct causal effect of hydrophobic bile acids on Ψ_m has also been demonstrated in different studies using isolated hepatocytes (74, 75) or HepG2 hepatocytes (76). After bile duct ligation, Bid promotes apoptosis in wild type and Fas-deficient mice, and Bid antisense administration to wild type mice promotes a reduction of apoptosis and serum AST levels (75). Similarly, Bid antisense reduces apoptosis in GCDC treated hepatocytes (75). Despite clear evidence of the involvement of the mitochondrial pathway during cholestasis-induced apoptosis, the role of Bid can be controversial (see section 4.4), and more studies are needed to clarify the role of this pro-apoptotic protein in chronic cholestasis.

5.3. Bile acids promote apoptosis in liver

Primary bile acids are synthesized exclusively in the liver using cholesterol as precursor. In the intestine, bacterial flora further processes these compounds to form secondary bile acids, which also have relevant roles in mitochondrial-dependent cell death. Given that bile acids accumulate in the liver and in plasma during cholestasis, they are likely to play an important role in the progression of the pathology (77-79). High concentration of potentially toxic bile acids in the liver can elicit diverse necrotic and apoptotic mechanisms, likely independent of the detergent action of bile acids (27). Exposure of primary hepatocytes and hepatocyte cell lines to bile acids promotes cell death by either apoptosis or necrosis, depending on the concentration of these compounds (74, 75, 77). The mechanisms whereby bile acids damage hepatocytes are not fully understood. However, they are able to promote apoptosis by stimulation of both extrinsic (i.e. DR-mediated) pathways (68, 80) and intrinsic (i.e. mitochondrially-mediated) mechanisms of apoptosis (75) (Figure 1).

Bile acids are direct inducers of apoptosis in hepatocytes through ligand-dependent and ligand-independent activation of Fas (77). GCDC treatment increases oligomerization of Fas, recruitment of FADD, mitochondrial permeabilization and activation of effector proteases, in the absence of detectable FasL mRNA (77). In McNtcp.24 cells, which resemble hepatocyte behavior, GCDC promotes intracellular trafficking of Fas by a Golgi- and microtubule-dependent pathway, to finally increase Fas density on the plasma membrane (81).

The hydrophobic bile acid glycochenodeoxycholate (GCGC) has been proposed to be a key player in the induction of hepatocyte apoptosis, because exposure of hepatocytes to GCDC results in the induction of cell surface receptor TRAIL-R2/DR5 at transcriptional level, and also inactivation of FADD-like apoptosis regulator (cFLIP/cFLAR) by phosphorylation (75, 82, 83). Thus, GCDC dually sensitizes hepatocytes to cell-induced apoptosis. Bile acids are important mediators of the apoptotic response, as TRAIL alone does not induce cell death in normal hepatocytes (75). During experimental cholestasis induced by bile duct ligation, hepatocytes overexpress TRAIL-R2 and are sensitized to exogenous administration of TRAIL (84). Accordingly, both hepatocyte apoptosis and liver injury are significantly reduced in TRAIL null mice during cholestasis (80).

Thus, death receptors such as Fas and TRAIL-R2 are involved in liver injury induced by cholestasis (s Figure 1). Livers from mice deficient in Fas exhibited less cell death by necrosis and apoptosis as well as reduced fibrosis after bile duct ligation (68, 85). Collectively, these data suggest that GCDC-induced hepatocyte apoptosis involves ligand-independent oligomerization of Fas, as it occurs without significant elevations of FasL levels (81).

5.4. Anti-apoptotic response to cholestatic injury

Cell injury triggers not only apoptosis but also a pro-survival response, which is dependent on different mechanisms of regulation, either at transcriptional or post-transcriptional level. After bile duct ligation, activation of nuclear factor-kappa B (NF-κB) in hepatocytes promotes expression of anti-apoptotic proteins and survival (66), whereas overexpression of IκB (a NF-κB repressor) in the liver increases apoptosis and liver damage (67). NF-κB-mediated survival was dependent on the regulation of anti-apoptotic genes A1/Bfl and cIAP2 at the transcriptional level. Apparently, bile acids are direct mediators of this pro-survival response (66, 86). An increase in the level of the anti-apoptotic BCL-2 has also been observed in liver mitochondria during the progression of the pathology (17, 69), possibly to withstand the increase in Bax incorporation in the mitochondrial membrane.

At an early stage of cholestasis some kind of resistance to cell death may develop. During this early stage, increased cardiolipin content in the mitochondrion alters the composition of the mitochondrial membrane and decreases the probability of MPT pore formation, and the threshold of apoptosis increases (87). Another adaptive response against cell death that accounts for apoptosis resistance is the phosphorylation of Bid, which impairs truncation (tBid) and activation of the protein and hence the promotion of apoptosis 88. Paradoxically, mitochondrial dysfunction observed during cholestasis might act as a defense mechanism against bile acids toxicity. In HCT-116 colon carcinoma cells, inhibitors of complex I, III and V largely decrease deoxycholic acid (DCA)-induced cell apoptosis (89). Thus, several mechanisms of mitochondrial and extra-mitochondrial origin cooperate to confer resistance against apoptosis in the liver, but risking to maintain damaged (potentially dysfunctional or even malignant) cells alive.

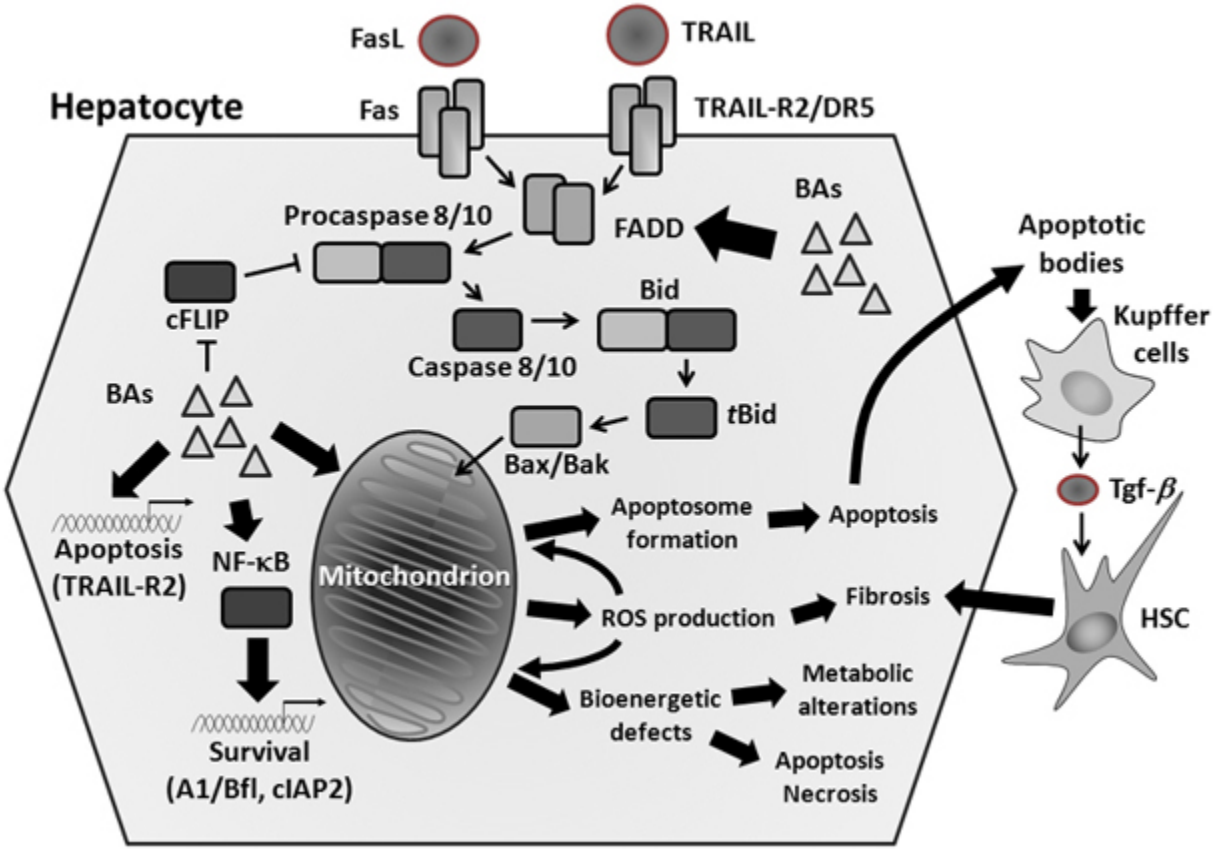

Figure 1. Bile acids together with death ligands receptors (Fas and TRAIL-R2/DR5) casuse apoptosis in hepatocytes during bile duct ligation. Inflammatory cells promote release of cytokines and death receptors ligands (FasL and TRAIL), which activate proapoptotic caspases, truncation of Bid (tBid) and activation of Bax/Bak. Bile acid accumulation in the cholestatic liver promotes apoptosis by different mechanisms: a) FADD accumulation on the plasma membrane; b) phosphorylation of cFLIP (an anti-apoptotic protein), yielding to an inactive form; c) increase of TRAIL-R2 mRNA, which potentiates the apoptotic stimulus. Nonetheless, bile acids also favor survival of the hepatocyte by activation of NF-κB. Mitochondria, stimulated by bile acids and/or proapototic Bax/Bak, trigger apoptosis. The result is a loss of mitochondrial membrane potential, decrease in ATP synthesis, and increase in ROS production. These mechanisms lead to metabolic alterations, apoptosis, and fibrosis. Abbreviations: BAs, bile acids; HSC, hepatic stellate cells; ROS, reactive oxygen species.

Despite apoptosis is largely considered a process to restore liver function homeostasis, there is a strong mechanistic link between hepatocyte apoptosis, inflammation, and fibrosis in chronic liver diseases. Engulfment of apoptotic bodies by phagocytic cells leads to their activation and in turn to secretion of chemokines as well as recruitment of leukocytes and inflammatory cells into the liver (90-92). Apoptosis contributes to hepatic fibrogenesis through activation of hepatic stellate cells (HSC) by direct and indirect mechanisms related to the destiny of apoptotic bodies. Phagocytosis of apoptotic bodies by Kupffer cells results in the release of transforming growth factor β (TGF-β), which activates HSC (93). If HSC are responsible for phagocytosis of the apoptotic bodies then the up-regulation of TGF-β in the same HSC leads directly to their activation through an autocrine mechanism (94). Indeed, engulfment of apoptotic bodies by HSC is a stimulus for HSC activation and cause secretion of increasing amounts of collagen, leading to liver fibrosis (91).

6. MITOCHONDRIAL OXIDATIVE STRESS IN CHOLESTATIC LIVER DISEASE

Oxidative stress is a common feature of obstructive cholestasis (95), primary biliary cirrhosis (96, 97), and sepsis-induced cholestasis (98), and it has been linked to the progression of cholestatic liver injury. During cholestasis, oxidative stress is a systemic phenomenon since lipid peroxidation increases in plasma, kidney, brain and heart from rats at 24 h after bile duct ligation (99). Oxidative stress is the imbalance between pro-oxidant agents and antioxidant agents in favor of the formers (100). Increases in oxidized glutathione (GSSG) – an index of glutathione oxidation -, 8-hydroxy-2'-deoxyguanosine – an index of DNA oxidation -, and lipid oxidation occur in rat liver at 15 days of bile duct ligation or thereafter (101-103).

Hepatic GSH is depleted in long term cholestasis (18, 102) due to lower GSH synthesis and down-regulation of GSH synthetic enzymes (18, 104). During cholestasis,

tumor necrosis factor alpha (TNF-α) and toxic bile acids have been involved in liver injury and hepatocyte apoptosis (68, 80). It is known that mitochondrial glutathione depletion sensitizes hepatocytes to apoptosis induced by TNF-α (105). In the long term cholestasis there is depletion of reduced glutathione (GSH) in liver mitochondria (18) and consequently hepatocytes should be prone to cell death under this condition. Oxidative stress is especially marked in liver mitochondria in the long term experimental cholestasis, i.e. at 14 days after bile duct ligation or thereafter, as evidenced by a remarkable increase in lipid peroxidation and GSSG together with depletion of GSH (18, 101, 106). Furthermore, high-fat diets increased lipid oxidation in liver mitochondria and hepatic injury after bile duct ligation (107).

Nrf2, nuclear factor erythroid derived 2 like 2 (Nrf2) is one of the transcription factors regulating inducible and constitutive gene expression mediated by the antioxidant response elements (ARE), which are found in the promoter regions of two major detoxication enzymes, namely glutathione S-transferase A2 (GSTA2) and NADPH:quinone oxidoreductase (NQO1) (108). It is noteworthy that toxic bile acids induce a switch from Nrf2 to c-avian musculoaponeurotic fibrosarcoma/V-maf musculoaponeurotic fibrosarcoma oncogene homolog G (c-Maf/MafG) ARE nuclear binding, leading to decreased expression of GSH synthetic enzymes and contributing to liver injury during chronic cholestasis (104). Ursodeoxycholic acid (UDCA) and S-adenosyl methionine treatment prevented this decrease preserving GSH levels and preventing liver injury (18, 104). The beneficial effects of UDCA are mediated by activation of the PI3K/Akt/Nrf2 pathway (97, 109, 110). In agreement with an important contribution of oxidative damage to the progression of the cholestatic diseases, pretreatment with vitamin E significantly diminished mitochondrial lipid peroxidation and hepatocyte damage in rats receiving intravenous taurochenodeoxycholic acid (TCDCA) (111). The antioxidants idebenone – a coenzyme Q analogue - or α-tocopherol inhibited the generation of reactive oxygen species (ROS) in isolated rat liver mitochondria and in isolated hepatocytes as well as the mitochondrial membrane permeability transition and subsequent apoptosis induced by GCDCA in rat hepatocytes (74, 112). Summarizing, these data support the relevancy of ROS in liver cholestasis.

Mitochondria are likely major producers of ROS during cholestasis. In agreement, adenoviral therapy to overexpress MnSOD was more effective than Cu/ZnSOD to reduce liver damage, fibrosis and 4-hydroxynonenal (a marker of lipid peroxidation) (113). Nevertheless, these findings cannot exclude a significant role of other sources of ROS in cholestatic injury apart from liver mitochondria. Accordingly, inhibition of the ROS generating enzyme xanthine oxidase by oxypurinol reduced hepatocellular injury after bile duct ligation in rats without affecting mitochondrial lipid peroxidation (114). These authors suggested that a component of cholestatic injury might be caused by oxidative stress from a source outside of the hepatocyte. In this regard, the increase in plasma homocysteine that occurs after bile duct ligation may contribute to oxidative stress and tissue damage (115).

In contrast to these results, Baron and Muriel reported that treatment with the antioxidants vitamin E or trolox prevented the increase in lipid peroxidation and GSSG levels in the liver without affecting tissue injury assessed by serum enzyme activities and histology after 7 days of bile duct ligation in rats (116). This apparent controversy might be explained by the adaptive response that occurs in the liver in the short term of cholestasis, evidenced by the increase in hepatic GSH levels at 1 and 5 days after bile duct ligation (102), which may uncouple oxidative stress and tissue damage at this stage. In addition, the experiments mentioned previously on the beneficial effects of vitamin E were performed in hepatocytes *in vitro* or in perfused liver, whereas the experiments by Purucker and colleagues were performed *in vivo*.

Mitochondrial ROS trigger signal transduction pathways under physiological conditions, but in excess they promote oxidative stress and oxidative damage. Bile acids such as DCA and taurodeoxycholic acid (TDCA) increased mitochondrial ROS production in hepatocytes (117). Mitochondrial ROS may also cause inactivation of protein tyrosin phosphatases (PTPase) that triggers tyrosin phosphorylation of the epidermal growth factor receptor ERBB1 and subsequently activation of mitogen-activated protein kinase kinase 1/2 and mitogen-activated protein kinase 1/2 (MEK1/2-ERK1/2) 117. Activation of the ERK1/2 and AKT pathways by bile acids protects against hepatocyte apoptosis through reduction of the FAS-R-caspase 8/9/3 pathway (118, 119). Activation of these pathways was abolished in Rho 0 cells or by cyclosporine A and bongkrekic acid, which inhibit mitochondrial permeability transition and electron transport (117).

However, this protection is ineffective during chronic cholestasis due to generation of large amount of mitochondrial ROS. In turn, ROS could contribute to maintain apoptosis active, as observed during long-term cholestasis (17, 18). Bile acid toxicity in hepatocytes seems to be dependent on the generation of mitochondrial ROS and accordingly, UDCA reduced DCA-induced hepatocyte killing at least in part by inhibiting DCA-induced mitochondrial permeability transition and ROS generation (72, 73). Chronic oxidative stress may also contribute to apoptosis by inducing a severe depletion of mitochondrial cardiolipin content found in the long term cholestasis (18). Cardiolipin normally protects against apoptosis by providing an increased pool of negative ions that bind Ca^{2+}, thereby preventing it from binding to protein sites that induce opening of mitochondrial permeability transition pores (120).

Dent and coworkers proposed that during reabsorption of bile acids with nutrients after feeding, bile acid-induced mitochondrial ROS modulate AKT/glycogen synthase activity as well as ERK1/2/LDL receptor helping in the regulation of glucose and lipid metabolism (117). However, during chronic cholestasis prolonged exposure to high concentrations of bile acids causes oxidative stress,

uncoupling growth factor receptors from protective downstream signaling pathways (117). Thus, marked glutathione depletion and oxidation as well as lipid peroxidation occur in liver mitochondria upon chronic cholestasis (18, 106) without activation of the AKT pathway (17). It is worth noting that the mitochondrial concentration of GSH exhibited a positive correlation with the activity of some respiratory chain complexes, particularly with succinate:ferricytochrome c oxidoreductase, whereas the mitochondrial concentration of thiobarbituric acid reacting substances (TBARS) - as an index of lipid peroxidation - showed a negative correlation. Serum ALT and alkaline phosphatase activities as well as bilirubin levels correlated significantly with mitochondrial levels of conjugated dienes and TBARS (107). Furthermore, the beneficial effects of UDCA in the treatment of chronic cholestasis are associated with a partial prevention of both glutathione depletion and oxidation in liver mitochondria as well as with a decrease in mitochondrial ROS generation (18).

In addition, oxidative stress contributes to the onset and progression of liver fibrosis induced by chronic cholestasis (24). ROS and lipid peroxidation products, such as 4-hydroxynonenal, promote fibrogenesis through redox signalling that leads to up-regulation of the transforming growth factor beta, activation of HSC, and modulation of the expression of metalloproteinases and their specific inhibitors (24, 121).

Oxidative stress and particularly the increased mitochondrial ROS production that occurs in biliary cirrhosis 18 should induce mitochondrial biogenesis through activation of NRF-1 and PGC-1α (122). Indeed, oxidative stress normally induces NRF-1 phosphorylation and up-regulation of TFAM expression via phosphatidylinositol 3-kinase (PI3K) and AKT/protein kinase B (123). However, AKT was not phosphorylated and PGC-1α and TFAM were down-regulated in biliary cirrhosis avoiding the stimulation of mitochondrial biogenesis through this pathway (17).

In addition to be a consequence of cholestasis, oxidative stress can also promote cholestasis on its own (124). Oxidative stress impairs the hepatocyte capability to secrete and retain bile salts in the bile canaliculus and induces actin-cytoskeletal disarrangement causally linked to internalization of canalicular transporters and disarrangement of tight-junctional structures (124). Indeed, oxidative stress triggers reversible retrieval of the multidrug resistance-associated protein 2 (Mrp2), a biliary transporter involved in bile salt-independent bile flow, from the canalicular membrane of hepatocytes and its translocation into cytosolic putative vesicles via the redox-sensitive balance of protein kinase A/C (PKA/PKC) activation (125).

7. DUAL ROLE OF INFLAMMATION IN CHRONIC CHOLESTASIS

In cholestasis the accumulation of bile acids – especially hydrophobic ones - causes hepatocyte injury that results in the activation of Kupffer cells and infiltration of other inflammatory cells into the liver, particularly neutrophils (126). This second phase of generalized injury triggered by the innate immune system aggravates liver injury promoting fibrosis and eventually chronic liver failure (80, 85). Consequently, long term cholestasis is a chronic inflammatory condition characterized by liver fibrosis together with maintained expression of pro-inflammatory cytokines and inducible nitric oxide synthase (iNOS) (17, 126, 127).

Inflammation seems to play a dual role in the development of liver damage during cholestasis. On the one hand, neutrophils and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) significantly promote liver injury after bile duct ligation, whereas on the other hand Kupffer cells protect against cholestatic liver injury (128). TRAIL is a death ligand released by cells of the innate immune system - especially natural killer cells, natural killer T cells, and monocytes - which plays a key role in liver injury during cholestasis (80). Accordingly, apoptosis was markedly reduced in TRAIL knockout mice subjected to bile duct ligation, and it was associated with reduced liver fibrosis and serum ALT values (80).

Kupffer cells play distinct roles in the cholestatic liver disease. Phagocytosis of apoptotic bodies by Kupffer cells results in the release of TGF-β, which activates HSC (93). Nevertheless, Kupffer cell-depleted mice and interleukin-6-deficient mice exhibited more liver injury, particularly necrosis, than wild type animals after bile duct ligation (128). It was suggested that Kupffer cells produce cytokines, chemokines and toxic products that would contribute to hepatocyte death, inflammatory infiltrate and activation of HSC (129, 130). However, Kupffer cells reduce cholestatic liver injury by cytokine-dependent mechanisms that mainly involve secretion of interleukin 6 (128). Indeed, exogenous IL-6 completely reversed the consequences of Kupffer cell depletion (128). It seems that Kupffer cells protect against the increase in bacterial endotoxin derived from the gut that occurs during cholestasis due to impairment in the integrity of the intestinal mucosa (131).

Pro-inflammatory cytokines, particularly TNF-α and interleukin-2, are potent inducers of intrahepatic cholestasis (132). Indeed, TNF-α inhibits bile flow and hepatocellular bile salt uptake in the rat (133, 134). This inflammation-induced cholestasis results mainly from inhibition of expression and function of hepatocellular transport systems for bile salts (132). Consequently, chronic inflammation associated with chronic cholestasis may generate a deleterious positive feed-back mechanism that potentiates each other.

However, the role of TNF-α in chronic cholestasis is dual and paradoxically this pro-inflammatory cytokine seems to be more beneficial than detrimental. TNF-α may promote hepatocyte apoptosis via TNFRSF1A-associated via death domain (TRADD), receptor (TNFRSF)-interacting serine-threonine kinase 1 (RIP), and mitogen-activated protein kinase 8 and 9 (JNK1 and JNK2)

(135) and thus aggravate cholestasis (132), but paradoxically TNF-receptor-deficient mice subjected to chronic cholestasis showed enhanced hepatocellular necrosis (127). TNF-α protects by inducing the expression of multidrug resistance-associated protein 3 expression on the basolateral membrane of hepatocyes, favoring the efflux of bile salts and glucuronide conjugates (127). In addition, TNF-α may also trigger activation of an anti-apoptotic pathway mediated by NF-κB transcription of survival genes, such as growth arrest and DNA-damage-inducible protein beta (GADD45beta), manganese superoxide dismutase (MnSOD), and CASP8 and cFLIP/cFLAR (135).

8. CHOLESTATIC LIVER DISEASE AS A SECONDARY MITOCHONDRIOPATHY

Disorders that affect mitochondrial oxidative phosphorylation usually have an important impact on cellular metabolism, leading to liver diseases and often to multisystem alterations, and they have been called mitochondrial hepathopaties (136). They have been divided into primary disorders (137, 138), in which the mitochondrial defect is the primary cause of the liver disease, and secondary disorders that are caused either by genetic defects originally of non-mitochondrial proteins or by acquired injury to mitochondria (139). Most primary mitochondrial hepatopathies are caused by mutations in nuclear genes, such as mitochondrial polymerase g (POLG), rather than by deletions or rearrangements of mitochondrial DNA (136). They include electron transport defects, fatty acid oxidation and transport defects, disorders of the mitochondrial translational process, urea cycle enzyme deficiencies and phosphoenolpyruvate carboxykinase deficiency (136). Among them, the mitochondrial DNA depletion syndrome should be highlighted because it is characterized by a dramatic reduction in the mtDNA copy number in different tissues (< 10% of the normal amount of mitochondrial DNA vs nuclear DNA), insufficient synthesis of mitochondrial complexes I, II, IV, and V, together with lactic acidosis, cholestasis, steatosis, and fibrosis (136).

It is well known that severe mitochondrial dysfunction occurs in chronic cholestasis in rodents and in humans. Indeed, this liver disorder leads to a marked loss of Ψ_m as well as to decreased ATP production, fatty acid oxidation, and ketone body formation (18-21, 140-142). In addition, cholestasis and progressive liver fibrosis have been associated with mtDNA depletion in infants (143). Recently, we have reported that long-term cholestasis is also characterized by a remarkable reduction in the mitochondrial DNA to nuclear DNA ratio, i.e. 35% of the normal ratio (17), probably due to a reduced TFAM protein import. TFAM, which regulates mtDNA transcription and replication, also functions as histone-like protein that protects mtDNA (Figure. 2). Hence, a marked reduction in TFAM levels would lead to mtDNA instability. Accordingly, we have shown that long-term cholestasis leads to mtDNA deletions (17). Chronic mitochondrial oxidative stress might be responsible, at least in part, for the increased frequency of mtDNA deletions (144). It is noteworthy that somatic mtDNA mutations are associated with hepatocarcinogenesis (123).

Therefore and taken into account these mtDNA mutations together with the severe mtDNA depletion and mitochondrial impairment, we have proposed that long-term cholestasis should be considered a secondary mitochondrial hepatopathy (17). Other secondary mitochondrial hepatopathies are Reye's Syndrome, Wilson's Disease, valproic acid hepatotoxicity, and liver diseases induced by nucleoside reverse transcriptase inhibitors (136). It is worth noting that identical mutations in mitochondrial DNA may give rise to great variations in phenotype and severity due to heteroplasmia (136). Indeed, cells may harbor both normal and mutant mitochondrial DNA in various amounts due to random partitioning during cell division.

9. MITOCHONDRIA AS PUTATIVE TARGETS FOR THERAPEUTIC INTERVENTION IN CHOLESTASIS

The knowledge and understanding of cholestatic liver diseases is growing, especially at the experimental level. Nonetheless, pharmacological treatments have not evolved so much since the introduction of UDCA as a therapeutic agent. UDCA is one of the major compounds found in the bear bile and it is currently used for curing hepatic and biliary disorders. Bear bile has been used in traditional Chinese medicine for thousands of years, because it was found to have a wide range of pharmacological actions with little toxicological side effects. Professor Olof Hammarsten from the University of Uppsala (Sweden) was the first to isolate an unknown bile acid from the bile of the Polar bear and named it "ursocholeinsäure", later renamed ursodeoxycholic acid by Prof. Shoda (145). Since mid 1950's, Japanese scientists succeeded in chemically synthesizing UDCA from cholic acid (146), which is currently made synthetically in large quantities and widely used in Western medicine. Nowadays, UDCA is the only drug approved by the Food and Drugs Administration (FDA) for the treatment of primary biliary cirrhosis (PBC) patients without liver transplantation. The efficacy of UDCA for treatment of recurrent PBC after liver transplantation remains less well known.

Putative mechanisms of action of UDCA (or its taurine-conjugated form TUDCA) include: anti-apoptotic (147, 148), regulation of immune function (149, 150), anti-inflammatory (151), promotion of choleresis (152-154), coordination and maintenance of mitochondrial integrity (18) and alteration of cell signaling (155, 156). Despite several relevant studies *in vivo* with animals and *in vitro* with cells and isolated organelles (e.g mitochondria), still poor is the knowledge of the mechanisms of action of this molecule in the human being. UDCA has been proposed to protect hepatocytes by decreasing mitochondrial peroxide production in BDL rats (18), a mechanism that probably contributes to the anti-apoptotic action of UDCA. UDCA could have a have a beneficial role by increasing antioxidant defenses in liver cells (157). This mechanism of

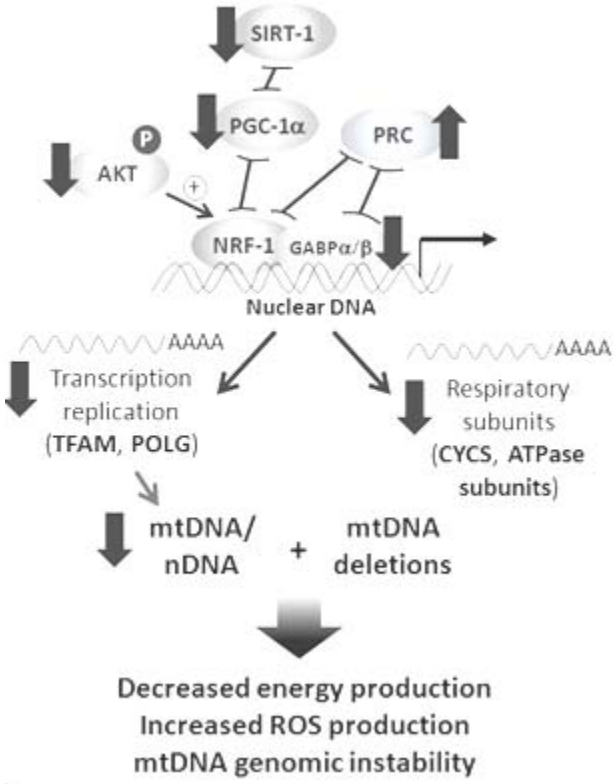

Figure 2. Mitochondrial biogenesis is defective during cholestasis. Renewal of mitochondria is brought about by redox-sensitive NRF-1 and GABPα, which promote transcription of nuclear encoded-mitochondrial genes. The dehacetylase SIRT1, which participates in the regulation of PGC-1α activity, PGC-1α and GABPα are decreased in the cholestatic liver. PRC and AKT do not cooperatively support NRF-1 activity during cholestasis. In this situation transcription of TFAM, POLG, CYCS, and some ATPase subunits is blunted, and mitochondrial biogenesis is not effective. Long-term cholestasis is characterized by a marked loss of mtDNA and increased frequency of mtDNA deletions. Abbreviations: CYCS, cytochrome c somatic; mtDNA, mitochondrial DNA; POLG, mitochondrial polymerase gamma; TFAM, transcription factor A, mitochondrial.

protection is supported by experimental evidence with PBC patients (97). Kawata K. and collaborators studied a group of 13 PBC patients before and after UDCA treatment. Compared with pre-treatment, UDCA was able to increase total and phosphorylated levels of Nrf2. This was associated with an increase in the levels of thioredoxin (Trx) and thioredoxin reductase 1 (Trxr1), the only enzyme in charge of reducing Trx using NAPDH as a cofactor (97). These findings support previous experimental knowledge of an antioxidant effect of UDCA (18, 157). Thus, by this mechanism UDCA is suggested to protect mitochondria from oxygen radical-induced dysfunction. Other mechanisms through which UDCA ameliorates liver function in humans are yet to be established.

Given the clear implication of mitochondrial dysfunction in the progression of cholestatic liver diseases, novel therapeutic approaches could be considered. Selective delivery of mitochondrial antioxidants such as MitoQ - or related compounds (158-160) -, could be considered alone or in combination with UDCA. MitoQ, which consists of a quinone/quinol moiety linked to a triphenylphosphonium (TPP) moiety through an alkyl chain (159), easily passes through the phospholipid bilayer and accumulates in the mitochondrial matrix driven by the Ψ_m (161). MitoQ is an effective antioxidant against lipid peroxidation (162, 163) and peroxinitrite formation (164), but minor is its reactivity towards hydrogen peroxide (164). This compound has been shown to have other biological activities, such as preventing the loss of Ψ_m and decreasing caspase activity in response to apoptotic stimuli (162). Conversely to MitoQ, uptake of other mitochondrial antioxidants like SS peptides (tetrapeptides with alternating aromatic residues and basic amino acids) was shown to be almost independent of Ψ_m. These compounds also have important scavenging effects and can be used to reduce apoptosis (160).

Long-term oral administration of MitoQ to mice, rats and humans has been proven to be safe (165-167). After oral administration, it accumulates in heart, liver, kidney, skeletal muscle, brain and fat (165). Experimentally, this mitochondrial antioxidant has been successfully used to protect against cardiac ischemia reperfusion (I/R) injury in rats (168) and mice (169), increased blood pressure in spontaneously hypertensive rats (170), and organ damage in a lipopolysaccaride-peptoglycan model of sepsis (171). In humans, MitoQ has

been used to treat chronic hepatitis C virus (HCV), a disease with evidence for increased oxidative stress and mitochondrial damage (172). In this study MitoQ was proven effective to reduce hepatic damage (assessed as serum ALT), without alteration of the viral load. Because of the safety of this mitochondrial antioxidant, MitoQ could be a possible therapeutic approach to test for the treatment of cholestatic liver diseases, which have been shown to be associated with high levels of mitochondrial oxidative damage.

In conclusion, mitochondrial dysfunction may cause oxidative stress, apoptosis and fibrosis. Mitochondrial dysfunction is also central to a severe metabolic alteration in the cholestatic liver, a condition worsened by a defective induction of mitochondrial biogenesis at nuclear and mitochondrial level. Therapeutic approaches to treat the cholestatic liver and associated diseases (like PBC) rely on the partial and not well understood efficacy of UDCA, the only approved treatment at present. A more targeted antioxidant therapy to rescue mitochondrial function, such as MitoQ or other mitochondrial antioxidants, might have beneficial effects that complement UDCA treatment.

10. CONCLUDING REMARKS

In this review we have focused on the contribution of mitochondria to the pathogenesis of liver disease in long-term cholestasis, elucidating the major mechanisms known so far to explain the mitochondrial impairment in this chronic disease. In particular, the contribution of inflammation and apoptosis as well as the lack of stimulation of mitochondrial biogenesis, the marked mitochondrial DNA depletion and occurrence of mitochondrial DNA mutations to the progression of this severe disease have been highlighted. All these findings certainly support the consideration of long term cholestasis as a secondary mitochondrial hepatopathy.

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Abbreviations: A1/Bfl, BCL2-related protein A1, ALT, alanine aminotransferase, ARE, antioxidant response element, BAK, and/or BCL2-antagonist/killer 1 (BAK), BAX, BCL2-associated X protein (BAX), BCL-2, B-cell CLL/lymphoma 2, BDL, bile duct ligation, BID, BH3 interacting domain death agonist, CDCA, chenodeoxycholic acid, cFLIP/cFLAR, CASP8 and FADD-like apoptosis regulator, cIAP2, baculoviral IAP repeat containing 3, c-Maf, c-avian musculoaponeurotic fibrosarcoma, Cu/ZnSOD, copper/zinc superoxide dismutase, Cycs, cytochrome c, somatic, DCA, deoxycholic acid, DISC, death initiating signaling complex, DR, death receptor, ERK1/2 mitogen-activated protein kinase 1/2, ERR- a, estrogen related receptor a, ETC, electron transport chain, FADD, Fas (TNFRSF6)-associated via death domain, FAS/APO1/CD95, TNF receptor superfamily, member 6, FasL, Fas ligand, GABP-a, GA binding protein transcription factor, alpha subunit 60kDa, GADD45beta, DNA-damage-inducible protein beta, GCDC, glycochenodeoxycholate, GSH, reduced glutathione, GSSG, oxidized glutathione, Hepatitis C Virus, HCV, HSC, hepatic stellate cells, Hsp60, heat shock

protein 60 kDa, iNOS, inducible nitric oxide synthase, IκB, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha, JNK, mitogen-activated protein kinase 8 and 9, LCA, litocholic acid, LDL, low density lipoprotein, Lpr, lymphoproliferation, MafG, V-maf musculoaponeurotic fibrosarcoma oncogene homolog G, Mdr2, multidrug resistance 2, MEK1/2, mitogen-activated protein kinase kinase 1/2, MnSOD, manganese superoxide dismutase, MPT, mitochondrial permeability transition, Mrp2, multidrug resistance-associated protein 2, mtDNA, mitochondrial DNA, nDNA, nuclear DNA, NF-κB, nuclear factor-kappa B, NRF-1, nuclear respiratory factor 1, Nrf2, nuclear factor, erythroid derived 2, like 2, NRF-2, nuclear respiratory factor 2, PBC, primary biliary cirrhosis, P:O ratio, relationship between ATP synthesis and oxygen consumption, PBDL, partial bile duct ligation, PGC-1α, peroxisome proliferator activated receptor γ coactivator 1α, PGC-1β, peroxisome proliferator activated receptor γ coactivator 1β, PI3K, phosphatidylinositol 3-kinase, PKA, protein kinase A, PKC, protein kinase C, POLG, polymerase gamma, mitochondrial, PRC, PGC-1α -related coactivator, PTPase, protein tyrosin phosphatase, RCI, respiratory control index, RIP, receptor (TNFRSF)-interacting serine-threonine kinase 1, ROS, reactive oxygen species, SMAC/DIABLO, second mitochondria-derived activator of caspases, TBARS, thiobarbituric acid reacting substances, tBid, truncated Bid, TCDCA, taurochenodeoxycholic acid, TDCA, taurodeoxycholic acid, TFAM, mitochondrial transcription factor A, TFB1M, mitochondrial transcription factor B1 (TFB1M), TFB2M, mitochondrial transcription factor B2 (TFB2M), TGF-β, transforming growth factor b, TNF-α, tumor necrosis factor a, TOM, translocase of the outer membrane, TRADD, TNFRSF1A-associated via death domain (TRADD), TRAIL, tumor necrosis factor-related apoptosis-inducing ligand, TRAIL/APO2L, TNF (ligand) superfamily, member 10, TRAIL-R2, TRAIL receptor 2, TUDCA, tauroursodeoxycholic acid, UCP-2, uncoupling protein 2, UDCA, ursodeoxycholic acid, XIAP, X-chromosome linked inhibitor of apoptosis, Ψ_m, mitochondrial membrane potential.

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