The Role of Mitochondria in Cholestatic Liver Injury

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There is increasing evidence that the integrity of antioxidant defenses is of vital importance in extrahepatic cholestasis, particularly with regard to the functioning of the liver’s mitochondria. Although the mechanisms by which cholestasis causes oxidant/antioxidant imbalance in mitochondria are poorly understood, hepatic injury caused by cholestasis may be due to oxidative stress from the mitochondria. The injury has been observed in experimental models of cholestasis, especially in a model of biliary cholestasis established in rats with bile duct ligation (BDL). In the BDL rat model, the mitochondrial DNA copy number is changed and apoptosis is activated in the liver. In addition, Peroxisome Proliferator-activated Receptor-Coactivator-1α and transcriptional factor A are impaired. Compared to sham-operated rats, glutathione activity is decreased after BDL. Peroxidation of the mitochondrial phospholipids may cause cell necrosis and the level of a by-product of this peroxidation, malondialdehyde, may contribute to cell death after BDL. The disturbance of the oxidant-antioxidant balance, especially in mitochondria, may be responsible for cholestatic liver injury in cholestasis rats. This review describes recent development in the pathogenesis of cholestasis from the viewpoint of mitochondrial biogenesis and suggests possible directions for future study. (Chang Gung Med J 2009; 32:346-53)

Key words: cholestasis, antioxidant enzymes, mitochondria

Cholestasis constitutes one of the most common and severe manifestations of acquired or inherited liver disease.¹,² It is characterized by an accumulation of hepatotoxic substances.³,⁴ The main cause of hepatotoxicity involves alterations to various important cell functions, such as mitochondrial energy production.⁵ There is increasing evidence that the integrity of the antioxidant defenses is of vital importance during extrahepatic cholestasis, particularly with regards to the functioning of the liver mitochondria.⁶,⁷ The role of mitochondrial defects in severe liver disease cannot be overlooked. For instance, mitochondrial fatty acid oxidation disorders can lead to hepatic failure and cause sudden death.⁸

Our previous results have shown that oxidative stress and the influence of mitochondria is most important during the early stage of cholestasis.⁹ The antioxidant capacity of hepatocytes seems to be able to deal with the increased levels of reactive oxygen species (ROS) over the first few days⁰ and even up to 2-4 weeks after bile-duct ligation (BDL).¹¹ Thus, timing is the most important factor in the management of early cholestasis.

Early recognition and diagnostic evaluation of the cholestatic infant are therefore essential to the successfully treatment of liver disease.¹² The underlying biochemical alterations leading to liver cell injury may be triggered by the cholestatic process...
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Mitochondrial biogenesis in cholestasis

The synthesis of all mitochondrial proteins is reduced in BDL rats.\(^{11,14}\) The reason for the decrease in the activity of the enzyme complexes of the respiratory chain is primarily because the proteins encoded by the nucleus are increased. This makes cause a decrease in the endogenous synthesis of mitochondrial proteins.\(^{14}\) The decrease in mitochondrial protein synthesis may result from impaired translation of mitochondrial mRNA.\(^{14}\) Recently, cumulative evidence has supported the concept that peroxisome proliferator-activated receptor gamma coactivator-1\(\alpha\) (PGC-1\(\alpha\)) may be a major regulator of mitochondrial biogenesis.\(^{15}\) PGC-1\(\alpha\), acting as a coactivator and binds to the corresponding nuclear genes to help the translation of a series of nuclear DNA-encoded respiratory enzymes, mitochondrial transcription factor A (Tfam) and mitochondrial transcription factor B (mTFB).\(^{15}\) The activation of the nuclear gene transcriptional by these signals triggers the mitochondrial biogenesis process. The process first triggers the expression of the polypeptides and proteins involved in transcription and replication of mtDNA.\(^{16}\) These factors then are imported into the mitochondrial matrix to activate further processes.\(^{15}\) PGC-1\(\alpha\) is a key modulator of hepatic gluconeogenesis and is a central target of the insulin-cAMP axis in the liver.\(^16\) Once PGC-1\(\alpha\) is activated, it powerfully induces and coordinates gene expression stimulating mitochondrial oxidative metabolism,\(^{17}\) and mitochondrial content.\(^{18}\) The mitochondrial numbers and the expression of PGC-1\(\alpha\) are decreased during cholestasis. The mechanism by which PGC-1\(\alpha\) decreases in cholestasis rats may be because the PGC-1\(\alpha\) encoded by the nucleus is disturbed and this results in a decrease in glucogenesis.\(^{14}\)

Tfam binds the promoters within the D-loop region of the mtDNA and stimulates transcription from the mtDNA templates.\(^{14}\) It is a key regulator involved in mtDNA transcription and replication. Some other specific enzymes, like mTFB, also participate in the complex process of mtDNA transcription and replication, and act as key initiators.\(^{14}\) Regulation of mtDNA transcription seems to be an essential step in the regulation of mitochondrial biogenesis. An increase in endogenous mitochondrial protein synthesis is usually associated with increased transcription of mtDNA.\(^{14}\) On the other hand, a decrease in mitochondrial protein synthesis can result from an impaired translation of mitochondrial mRNA.\(^{14}\) Recent studies have suggested that the existence of a regulated cascade of proteins encoded by the nucleus, such as nuclear respiratory factors and mitochondrial transcription factors, which mediate the interaction between the nucleus and the mtDNA.\(^{19,20}\) These factors may therefore provide a link between the cell’s energy requirement and the mitochondrial content of a cell or an organ.\(^{14}\) From a kinetic standpoint, it is evident that a decrease in mitochondria content cannot be achieved by a decrease in biogenesis alone, but also should involve an increase in degradation.\(^{14}\) Regulation of mtDNA transcription seems to be an essential step in the regulation of mitochondrial biogenesis.\(^{31}\) In contrast to the above, higher concentrations of Tfam have an inhibitory effect on mitochondrial biogenesis.\(^{22}\) Levels of Tfam strongly influence the copy number of mtDNA, but the actual role of Tfam in the process of transcription regulation still remains unclear. This is because copy number control and transcription regulation cannot be dissected from each other in vivo.\(^{22}\) Our unpublished observations showed that Tfam mRNA and protein decrease in hours immediately after BDL, which means that nuclear to mito-
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The mitochondrial content per cell is considered to be controlled primarily by biogenesis. In the compensation stage, mitochondrial content is increased and in the damaged stage, it is decreased as a result of regulation of oxidative stress. A reduced activity of the enzyme complexes of the respiratory chain has been shown to be associated with mitochondrial proliferation in experimental animals. Mitochondrial proliferation and an increased mtDNA copy number, are part of the mechanism that maintains mitochondria volume in a cell or tissue. A decrease in mitochondrial function is associated with an increase in the mitochondrial volume fraction per hepatocyte in BDL rats. The increased mitochondrial volume may represent a strategy to maintain hepatic energy metabolism in rats with secondary biliary cirrhosis. The increase of mtDNA copy number in cells is suggested to be a result of a feedback response that compensates for defective mitochondria. Persistent oxidative stress in mitochondria leads to a decline in mitochondrial respiratory function with decreased mtDNA copy number. However, it remains unclear as to how the copy number of mtDNA and the abundance of mitochondria are regulated under various different physiological and developmental conditions. Furthermore, little is known about the change in liver mtDNA copy number after BDL in rats. Our observations show that mtDNA copy number is significantly decreased three days after BDL. Thus, it appears that mitochondrial damage becomes more severe after 72 hours.

Oxidative and nitrosative stress in cholestasis

Lipid peroxidation and oxidative mechanisms have been investigated in liver mitochondria from BDL rats and correlated with the activity of the enzyme complexes that make up the electron transport chain. The deleterious accumulation of lipid peroxides is correlated with a marked impairment of the soluble antioxidant defense mechanism. Removal of lipid peroxides was ineffective in liver damaged rats four weeks after BDL. Malondialdehyde (MDA) is an end-product of lipid peroxidation and hepatic MDA levels increased/doubled after BDL compared to sham-operated rats. Lipid peroxidation of the hepatic mitochondria correlates with the severity of cholestatic injury. Under these conditions, a restricted electron transfer rate leads to increased mitochondrial production of O2− and H2O2. Chronic mitochondrial oxidative stress will be the second stage during the development of liver disease.

In human studies, biliary obstructed patients show significantly higher levels of MDA compared to control patients. The consequent oxidative stress is highlighted by the enhanced concentrations of MDA in the bile of these patients. Application of biliary drainage, which gives rise to a rapid decrease of MDA levels, has been reported. This suggests that hepatic oxidative alterations during cholestasis are closely dependent on cholestasis itself as a consequence of toxic hydrophobic bile acid accumulation. These are also thought to have a stimulatory effect in cholestatic conditions on the expression of canalicular membrane transporter proteins in hepatocyte and to intracellular impairment of protein metabolism.

Mitochondrial oxidative and nitrosative stress play a critical role in triggering the acetaldehyde-induced mitochondrial membrane permeability transition that induces cell death. Nitric oxide (NO) produced by mtNOS can generate peroxynitrite, which induces oxidative and/or nitrosative stress. This releases cytochrome c from mitochondria in addition to inactivating susceptible mitochondrial enzymes. These functions indicate a pro-apoptotic role for mtNOS. Thus, an excessive production of NO and its derivative peroxynitrite contribute to a coexisting MnSOD deficiency in the mitochondria and this leads to liver cell necrosis in cholestatic animals. As previously reported, liver cell injury appears to involve the failure of intra-mitochondrial dismutation and enhanced nitrosative stress during the first 24 hours after BDL.

Glutathione (GSH) is known to play an important role in the mechanisms governing bile formation. Bile salt, independent of bile flow, is related to the rate of GSH secretion. GSH is critical to maintaining the mitochondrion’s functional integrity against oxidative stress. In BDL rats, it has been shown that the hepatic concentration of reduced
GSH progressively decreases with ongoing cholestasis.\(^1\) Mitochondrial GSH content decreases in BDL rats by 20% to 33% from day 7 after surgery.\(^{23}\) Alptekin et al. reported that hepatic GSH levels are elevated 14 days after BDL and are decreased 21 days after BDL, with this decrease being due to lowered \(r\)-glutamylcysteine synthetase.\(^{4,35}\) It has also been reported that the decreased secretion of GSH in cholestasis is affected by thiol-dependent bile flow\(^{36}\) or is mediated by pro-inflammatory cytokines induction.\(^{37}\) Patients with cholestatic liver disease are usually undernourished because of their toxic status and digestive problems. This may result in a reduced availability of amino acid precursors for GSH synthesis and/or impairment of the trans-sulfuration pathway.\(^3\)

It has been important to evaluate oxidation, together with the antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx), in liver from BDL-rats.\(^{11}\) Since hepatocytes contain a relatively high density of mitochondria compared with other cells, a continuous synthesis of ATP is most important. Disorders affecting mitochondrial oxidative phosphorylation (OXPHOS) and hepatocellular metabolism directly influence fatty acid oxidation, which results in impaired bile flow, cell death, and fibrogenesis.\(^{15}\) Oxidative repair enzymes repair oxidative-damaged DNA.\(^{38}\) Superoxide is rapidly converted to \(\text{H}_2\text{O}_2\) by mitochondrial SOD, unless \(\text{H}_2\text{O}_2\) is removed by the action of GPx.\(^{36}\) GPx reduces hydroperoxides as well as \(\text{H}_2\text{O}_2\) while oxidizing two molecules of GSH.\(^{39}\) The presence of GPx is thus of great importance because the molecule scavenges \(\text{H}_2\text{O}_2\) and protects the organelles against \(\text{H}_2\text{O}_2\)’s damaging effects.\(^{36}\) GPx is downregulated 14 days after BDL.\(^{35}\) This indicates that the GSH-dependent defense system in the mitochondria is defective in BDL-rats.\(^{25}\) In Enign et al.’s study, the cytoplasm antioxidant enzyme activities and GSH content were sufficient to detoxify lipid peroxides during the first 24 hours of extrahepatic cholestasis.\(^8\) The scavenging by \(\text{H}_2\text{O}_2\) protects organelles against damaging effects but this decreases as the cholestasis progresses. As a result of this, early treatment is vital for maximum effectiveness in such cases.

**The pathogenesis of mitochondria in cholestasis and hepatocyte apoptosis**

The speedy development of progressive organ failure in patients with extrahepatic biliary obstruction is most likely related to their reduced detoxification capacity, including antioxidants in the liver, and is also associated with increased retention of toxic hydrophobic bile acids.\(^{5,23}\) Direct assessment of the activities of the enzyme complexes of the electron transport chain reveals the presence of decreased complex I and complex III activity in the presence of deoxycholate,chenodeoxycholate or lithocholate.\(^5\) A higher chenodeoxycholate concentration (300 umol/L) also inhibits complex IV, but has no effect on complex II.\(^{20}\) The activity of complexes II and III of the electron transport chain is decreased in BDL rats from days 7 to 28 after surgery, and the activity of the complex IV is reduced from days 14 to 28.\(^{25}\) Though a different mechanism, these results are in agreement with the concept that cholestasis is associated with an increased production of reactive radical species\(^{40}\) and oxidative products,\(^{15}\) which are then responsible for several intracellular derangements.\(^3\)

Different concentrations of ursodeoxycholate have a different effect on liver protection against induced mitochondrial toxicity.\(^{41}\) Ursodeoxycholate (100 umol/L) significantly decreased the incorporation of chenodeoxycholate into mitochondrial membranes.\(^{41}\) However, 300 umol/L ursodeoxycholate in combination with chenodeoxycholate exhibited greater toxicity on the functioning of the electron transport chain.\(^{41}\) It is proposed that ursodeoxycholate’s effect is due to binding to the pro-apoptotic protein Bax, which prevents Bax translocation from the cytosol to mitochondria and the initiation of the critical apoptosis pathways.\(^{42}\) In cholestasis, toxic bile acids induce hepatocyte injury by apoptosis, necrosis, and eventually biliary fibrosis and cirrhosis.\(^{27}\)

Changes in mitochondrial biogenesis are usually thought to be responsible for alterations in the cellular mitochondrial content\(^43\) and the half-life of liver mitochondria is 3 to 10 days.\(^{44}\) Mitochondrial oxidative stress contributes to oxidative damage and necrotic cell death.\(^{45}\) The induction of a mitochondrial membrane permeability transition by hydrophobic bile salts can be a critical step in hepatocyte death by either necrosis or apoptosis.\(^{46}\) The mechanism of apoptosis is mediated by activation of caspases, a family of cysteine proteases.\(^{45-47}\) Once activated, caspase 9 cleaves caspases 3 and 7, resulting in their activation and this leads to the subsequent stereotypic apoptotic events.\(^{48}\) Nitrosylated caspase-9, which
is able to target caspase-3, is located predominantly in the mitochondrial fraction of cells.\(^{(49)}\) Mannick et al/ has suggested the possibility that S-nitrosylation is a general mechanism by which mitochondrial caspase activity is controlled.\(^{(49)}\) It is also been found that mitochondria are essential to the execution of hepatocyte apoptosis through both the intrinsic pathway and the Bid-dependent extrinsic pathway.\(^{(6)}\) NF-κB activation has also been demonstrated in nuclear extracts by the electrophoretic mobility gel shift assay from 3-day BDL mice but not in controls.\(^{(23)}\) Immunohistochemical studies of NF-κB have demonstrated nuclear localization in the hepatocytes of BDL mice, which is consistent with NF-κB activation in this liver cell type.\(^{(49)}\) The activation of caspases and NF-κB is needed for this hepatocyte apoptosis mechanism.

**Prolonged cholestasis (Fig. 1)**

Whether oxidative stress is the cause or an effect of cholestasis still has no definite answer. Nonetheless, we do know that mitochondria play a key role in the initiation and progression of liver disease during which the mitochondria's primary products, superoxide radicals, H₂O₂ and other downstream ROS up-regulate the expression of proinflammatory cytokines and trigger cell death.\(^{(11,28)}\) Thus, it would seem that a vicious cycle of mitochondrial function disturbance leading to more severe cholestasis is greatly involved in pathogenesis. A progressive impairment of hepatic mitochondrial respiratory function, hepatic mitochondrial electron transport enzyme activity and oxidative phosphorylation function have been observed in BDL rats at 2 and 4 weeks after ligation.\(^{(13)}\) This disturbance of the oxidant-antioxidant balance may be responsible for cholestatic liver injury.\(^{(35)}\) The activity levels of mitochondrial enzymes are either unchanged (ATPase, cytochrome c oxidase) or decreased in 2 to 5 week BDL rats.\(^{(14)}\) In line with this finding, GSH level has been found to decrease due to a lowered level of glutamylcysteine synthetase.\(^{(80)}\) Thus, in comparison with control rats, mitochondrial metabolism is impaired in perfused livers from BDL rats.\(^{(24)}\) In Singh et al.’s study, there was a shift in the pro-oxidant/antioxidant balance in favor of lipid peroxidation with MDA levels increasing at an early stage among BDL rats.\(^{(4)}\) The initiation of collagen synthesis occurs in the portal tract of the obstructed bile duct.\(^{(26)}\) This process is associated with enzymatic antioxidant system impairment in the hepatic mitochondria of BDL rats.\(^{(26)}\) In this context, hepatic glycogen store recovery is rapid after the relief of biliary obstruction\(^{(45)}\) but the activity of complex II and V of the respiratory chain do not recovered.\(^{(6)}\) The possible pathogenesis of this cholestasis is outlined in Figure 1. In the future, studies of important mitochondrial biomarkers in terms of DNA or pro-

**Fig. 1** The pathogenesis of mitochondrial biogenesis during cholestasis. Abbreviations used: ATP: adenosine triphosphate; GPx: glutathione peroxidase; GSH: Glutathione; MDA: Malondialdehyde; MnSOD: Mn-superoxide dismutase; mtDNA: mitochondrial DNA; PGC-1α: peroxisome proliferator-activated receptor gamma coactivator-1α; ROS: reactive oxygen species; Tfam: transcriptional factor A.
In conclusion, disturbance of the oxidant-antioxidant balance, especially in the mitochondria, may be responsible for cholestatic liver injury in human cholestasis and in BDL rats. Extra-hepatic cholestasis is associated with a decreased hepatic mitochondrial content, with an increased level of lipid peroxidation and with apoptosis. Free radicals and lipid peroxides are generated and these entities participate in the pathogenesis of cholestatic liver injury.

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粒線體在膽汁鬱積性肝傷害的角色
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越來越多證據顯示，肝臟內粒線體的抗氧化功能，在膽汁鬱積是相當重要的保護機制，而肝臟的傷害原因可能來自於粒線體中過多的氧化壓力；但是它們的相関機轉仍不是很清楚，目前有很多不同肝臟傷害的動物實驗模式已經在進行，其中鼠肝纖維的技術是比較成熟而且比較常被應用在研究當中。實鼠肝纖維的動物實驗，顯示肝細胞的凋亡被探討的相關機轉及粒線體細胞核轉錄因子 (Tfam) 會受到影響。另外一些粒線體相關酵素例如 Glutathione peroxidase 活性、cytochrome c 活性、adenosine triphosphatase 活性、Mn-superoxide dismutase 活性，在實驗鼠肝纖維實驗中都明顯的被加以調節，其中粒線體脂質的氧化作用是造成細胞壞死的原因，而它的代謝產物 malondialdehyde 是細胞凋亡的最主要產物，所以干擾氧化及抗氧化的平衡是造成膽汁鬱積肝臟傷害的重要原因，我們這篇文章將描述最近在膽汁鬱積中，粒線體生成因的病理上作一回顧性探討，以使做為未來進一步深入研究的方向。(長庚醫誌 2009;32:346-53)

關鍵詞：膽汁鬱積，抗氧化酵素，粒線體