Mitochondrial biogenesis fails in secondary biliary cirrhosis in rats leading to mitochondrial DNA depletion and deletions

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Submitted 28 May 2010; accepted in final form 8 March 2011

Mitochondrial biogenesis fails in secondary biliary cirrhosis in rats leading to mitochondrial DNA depletion and deletions. Our aim was to determine the molecular mechanisms that link long-term cholestasis to mitochondrial dysfunction. We studied a model of chronic cholestasis induced by bile duct ligation in rats. Key sensors and regulators of the energetic state and mitochondrial biogenesis, mitochondrial DNA (mtDNA)-to-nuclear DNA (nDNA) ratio (mtDNA/nDNA) relative copy number, mtDNA deletions, and indexes of apoptosis (BAX, BCL-2, and cleaved caspase 3) and cell proliferation (PCNA) were evaluated. Our results show that long-term cholestasis is associated with absence of activation of key sensors of the energetic state, evidenced by decreased SIRT1 and pyruvate dehydrogenase kinase levels and lack of AMPK activation. Key mitochondrial biogenesis regulators (PGC-1α and GABP-α) decreased and NRF-1 was not transcriptionally active. Mitochondrial transcription factor A (TFAM) protein levels increased transiently in liver mitochondria at 2 wk after bile duct ligation, but they dramatically decreased at 4 wk. Reduced TFAM levels at this stage were mirrored by a marked decrease (65%) in mtDNA/nDNA relative copy number. The blockade of mitochondrial biogenesis should not be ascribed to activation of apoptosis or inhibition of cell proliferation. Impaired mitochondrial turnover and loss of the DNA stabilizing effect of TFAM are likely the causative event involved in the genetic instability evidenced by accumulation of mtDNA deletions. In conclusion, the lack of stimulation of mitochondrial biogenesis leads to mtDNA severe depletion and deletions in long-term cholestasis. Hence, long-term cholestasis should be considered a secondary mitochondrial hepatopathy.

chronic cholestasis; PGC-1α; TFAM; mitochondrial DNA

CHOLESTASIS IS COMMON IN NUMEROUS chronic liver diseases and it is a prominent feature of primary biliary cirrhosis, primary sclerosing cholangitis, biliary atresia, and iatrogenic obstruction of bile ducts (25). Chronic cholestasis results in intracellular accumulation of toxic hydrophobic bile acids leading to hepatocyte death by apoptosis and necrosis, and eventually to liver fibrosis and cirrhosis (27, 38). A central feature of chronic cholestasis and biliary cirrhosis in the liver is mitochondrial dysfunction (19, 27), evidenced by a loss of mitochondrial membrane potential (38) and decreased activities of respiratory chain complexes and ATP production (10, 19). Mitochondrial dysfunction leads to decreased fatty acid oxidation and ketone body formation (20, 22, 23). Mitochondrial impairment is likely to be central in the progression of liver damage during chronic cholestasis because mitochondria play central roles in energy homeostasis, signaling, and apoptosis (13).

During chronic cholestasis the mitochondrial protein content increases despite the reduced activities of mitochondrial complexes (19). It has been reported that mitochondrial content per gram of liver increases up to 14 days of chronic cholestasis and decreases thereafter (9), suggesting that during chronic cholestasis mitochondrial biogenesis tries to maintain mitochondrial metabolism of the liver, but it seems not to be effective in the long term.

Mitochondrial biogenesis requires a complex interplay between nuclear and mitochondrial genomes (35). Peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) is a key transcriptional regulator of cellular energy metabolism that stimulates mitochondrial biogenesis through activation of nuclear respiratory factor-1 (45). PGC-1α together with nuclear respiratory factor 1 (NRF-1) as well as GA-binding protein-α (GABP-α, or nuclear respiratory factor-2) are the main transcriptional factors that regulate the coordinated expression of nuclear-encoded mitochondrial proteins (28, 35, 45), such as the mitochondrial transcription factor A (TFAM). TFAM is responsible for the maintenance of mtDNA copy number (17). TFAM is able to bind and wrap mtDNA, activating and regulating mitochondrial DNA (mtDNA) transcription and replication (24). Tiao et al. (42) recently studied mitochondrial biogenesis in the short term of experimental cholestasis and found that Pgc1-α gene expression, TFAM protein, and mtDNA copy number decreased in the liver within the first 72 h of cholestasis. Nevertheless, the regulation of mitochondrial biogenesis in the long-term cholestasis remains to be established.

Given the fine tuning in mitochondrial biogenesis to cope with changing metabolic conditions (13), we aimed to identify the status of the major mitochondrial regulatory pathways in long-term cholestasis to understand the molecular basis of the associated mitochondrial dysfunctions. Our results show a failure of mitochondrial biogenesis and genomic instability related to a marked decrease in TFAM.

MATERIALS AND METHODS

Animal experimentation. Adult male Wistar rats weighing 220–260 g were held in individual cages. Animals were distributed into two groups: a group that underwent bile duct ligation (BDL), and a sham-operated group pair-fed to BDL animals (sham). The total number of rats used was 30: 18 BDL rats and 12 control rats. Animals were euthanized under anesthesia at 14 and 28 days postsurgery. All
rats received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals (NIH publication 86-23 revised 1985). The study was approved by the Research Committee of the School of Medicine at the University of Valencia (Valencia, Spain).

Common bile duct ligation. BDL was performed as previously described (38). The correct induction of chronic cholestasis was assessed by the increase in total bilirubin concentration in plasma [6.3 ± 3.3 mg/dl in BDL vs. 0.5 ± 0.3 mg/dl in Sham]. Liver cirrhosis was confirmed by histological analysis at 4 wk after BDL (see supplemental material; the online version of this article contains supplemental data).

Isolation of mitochondria. Mitochondria were isolated by discontinuous Percoll gradient (as described in Ref. 31). With use of this method, citrate synthase activity increased 4.1 ± 0.2-fold (n = 4) in control livers and 3.8 ± 0.3-fold (n = 3) in cirrhotic livers upon mitochondrial isolation. The corresponding enrichment of mitochondrial isolation was also confirmed by Western blotting using antibodies specific for mitochondrial proteins. The recovery of mitochondria in terms of milligrams of mitochondrial pellet per milligrams of liver tissue was 1.34% in control rats and 0.65% in BDL rats. The appropriate quality of our isolated mitochondria relies on the respiratory ratio using succinate as substrate (see Table 1). Mitochondrial fraction was suspended in mitochondrial buffer before storage at −80°C.

Measurement of mitochondrial oxygen consumption and membrane potential. Freshly isolated mitochondria were assayed at 37°C for oxygen consumption via a Clark-type oxygen electrode (Hansatech Instruments, King’s Lynn, UK) equipped with Oxygraph Plus Version 1.01 and for membrane potential (∆ψ) via a tetraphenylphosphonium (TPP⁺) electrode (WPI Europe, Berlin, Germany). Membrane potential calculations were made by using a modified Nernst equation as previously reported (37).

Determination of metabolites. Lactate levels were measured in the liver by the method described by Gutmann and Wahlefeld (11). The hepatic ATP concentration was assessed by using a commercial bioluminescent assay kit (Enliten ATP assay kit, Promega, Madison, WI), according to the method of Yang et al. (47). ADP was measured after conversion to ATP with pyruvate kinase (PK) and phosphoenolpyruvate (PEP) and was determined by the luciferin-luciferase assay as the difference between the measurements with and without PK and PEP.

Nuclear protein extraction. Nuclear protein extracts were prepared from liver tissue by the method of Deryckere and Gannon (6). Protein measurement was performed by the Bradford assay.

Table 1.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward Primer (5'-3')</th>
<th>Reverse Primer (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atp5i</td>
<td>CCGCAAGGCGCTACAGTTAC</td>
<td>TTCCCTCCTGCTTTCTTC</td>
</tr>
<tr>
<td>Atp5b</td>
<td>CAGCATTTGCAAGGAGCAG</td>
<td>CAACTTCTGCCCCAGGCTT</td>
</tr>
<tr>
<td>Ppg</td>
<td>CATCTATCCAGTGCGTAC</td>
<td>GAGGGGCTTGAACAGGAG</td>
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<tr>
<td>Tfam</td>
<td>ACGAAAAGAGAGGCTGAG</td>
<td>CTTGGAATTCTCCCCGAGT</td>
</tr>
<tr>
<td>Nrf1</td>
<td>GCACTCCTTGGAAATGTTG</td>
<td>TGTGCGCAGCTGTTTCAG</td>
</tr>
<tr>
<td>Ppargc1a</td>
<td>AAGCCGCCAGGAGTAGAT</td>
<td>CGTTGTGCTTACGGGTTGA</td>
</tr>
<tr>
<td>Gabpa</td>
<td>TGACACGCGTGTGAAATTA</td>
<td>GATGCGGCGTACAGAAAGG</td>
</tr>
<tr>
<td>Pparc1</td>
<td>GATCTTTGCCTATTGGAA</td>
<td>AGGTCTTCTTCGGCTTAT</td>
</tr>
<tr>
<td>Thbs1</td>
<td>AAGGATTTGCGCGAAATTTA</td>
<td>CCGTTGCTGGCGCTTTTCC</td>
</tr>
<tr>
<td>Cys</td>
<td>CAGCGCTGCTGATTCTTCTAC</td>
<td>TCCCCACGGTTGATACCTTGT</td>
</tr>
<tr>
<td>Il1b</td>
<td>TACCGACCTCAGGGAAGG</td>
<td>GAGGGATTTGTTGCTTGT</td>
</tr>
<tr>
<td>Tnfα</td>
<td>TGCTGCTAGCCTTTTGCTT</td>
<td>CCGTTGAGAAGGAGCTCTT</td>
</tr>
<tr>
<td>Rplp0/Arbp</td>
<td>CGGAGCTGGTTGTGACATGTG</td>
<td>GCCCTTACGAGGAGGTTG</td>
</tr>
</tbody>
</table>

**Primer for gene expression**

**Table 2. ATP/ADP and mitochondrial function in liver from rats with long-term cholestasis**

<table>
<thead>
<tr>
<th>State 4</th>
<th>State 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>BDL</td>
</tr>
<tr>
<td>Glutamate/ADP</td>
<td></td>
</tr>
<tr>
<td>O₂ consumption</td>
<td>9.1 ± 3.6</td>
</tr>
<tr>
<td>RR with glutamate-ADP</td>
<td>6.4 ± 1.2</td>
</tr>
<tr>
<td>Succinate</td>
<td></td>
</tr>
<tr>
<td>O₂ consumption</td>
<td>34 ± 9</td>
</tr>
<tr>
<td>RR with succinate</td>
<td>4.1 ± 0.4</td>
</tr>
<tr>
<td>ATP/ADP</td>
<td>1.3 ± 0.2</td>
</tr>
</tbody>
</table>

**BDL, bile duct ligation; RR, respiratory ratio (state 3/state 4 oxygen consumption). The number of experiments was 3–6. Statistical significance is expressed as follows:** *P < 0.05; †P < 0.01 vs. Sham.

**Fig. 1.** Sensors and regulators of the energetic state in chronic cholestasis. A: hepatic lactate levels in liver from Sham (n = 4) and bile duct ligation (BDL; n = 7) rats. Values are expressed as means ± SD; **P < 0.01. B: Western blotting of pyruvate dehydrogenase kinase (PDK), phospho (p)-AMPK (Tyr172), AMPK1, and sirtuin-1 (SIRT1) (representative images) in liver from Sham and BDL rats (n = 3–4). α-Tubulin was used as loading reference.
Real-time RT-PCR. Liver tissue was homogenized in TRIzol reagent, and total RNA was isolated by the chloroform:phenol method (Invitrogen). RT was performed using the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems).

Quantitative PCR experiments were performed on a thermal cycler (7900HT, Applied Biosystems) by using PowerSYBR Green PCR Master Mix (Applied Biosystems). Results were normalized by use of Rplp0 as reference. The threshold cycle (Ct) was determined, and relative gene expression levels were subsequently calculated by the $2^{\Delta \Delta \text{Ct}}$ formula. Primers used for PCR experiments are shown in Table 1.

**Fig. 2.** Gene expression of mitochondrial biogenesis regulators in chronic cholestasis. Relative mRNA levels of Ppargc-1a, Pprc1, Nrf-1, and Gabp-a in liver from Sham (n = 4) and BDL rats (n = 4–7) studied by real-time RT-PCR. Rplp0 was used as a reference gene. Values are expressed as means ± SD. **P < 0.01.

**Real-time RT-PCR.** Liver tissue was homogenized in TRIzol reagent, and total RNA was isolated by the chloroform:phenol method (Invitrogen). RT was performed using the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems).

Quantitative PCR experiments were performed on a thermal cycler (7900HT, Applied Biosystems) by using PowerSYBR Green PCR Master Mix (Applied Biosystems). Results were normalized by use of Rplp0 as reference. The threshold cycle (Ct) was determined, and relative gene expression levels were subsequently calculated by the $2^{\Delta \Delta \text{Ct}}$ formula. Primers used for PCR experiments are shown in Table 1.

**Determination of the mtDNA/nDNA relative copy number.** Total DNA was extracted by a standard protocol (34). Equal amounts of DNA were amplified by using specific primers (Table 1). Results were analyzed comparatively (mitochondrial-to-nuclear DNA ratio, mtDNA/nDNA).

**Determination of mtDNA deletions.** Total DNA (400 ng) was amplified with Taq polymerase (Promega) by use of specific primers (Table 1). Products were resolved by agarose gel electrophoresis stained with ethidium bromide.

Western blotting. Immunoblotting was performed using the ECL system (Bio-Rad) and chemiluminescence was detected with a charge-coupled device camera (LAS-3000, Fujifilm) or film autoradiography (Kodak Biomax). Relative protein abundance was referred to total protein as determined by either α-tubulin or Ponceau S staining. Antibodies used in Western blotting were as follows: pyruvate dehydrogenase kinase (PDK; Santa Cruz, sc-28783), phospho-AMPK (Thr172) (Cell Signaling, no. 2531), AMPK1 (GenScript, no. A00664), sirtuin-1 (SIRT1; Upstate, 07-131), PGC-1α (Santa Cruz, sc-13067), NRF-1 (Santa Cruz, sc-23624), GABP-α (Santa Cruz, sc-28311), lamin A/C (Santa Cruz, sc-7293), phospho-AKT (S473) (Cell Signaling, no. 4058), AKT (GenScript, no. 5117), α-tubulin (Santa Cruz, sc-8035), heat shock protein 60 (HSP60; Stressgen, SPA-805), TFAM (Biovision, no. 3885), translocase of the outer mitochondrial membrane 20 kDa (TOM20; Santa Cruz, sc-11415), translocase of the outer mitochondrial membrane 70 kDa (TOM70; Santa Cruz, sc-26495), cytochrome c (Santa Cruz, sc-13156), GAPDH (Cell Signaling, no. 2128), PCNA (Santa Cruz, sc-56), phospho-p65 (S536) (Cell Signaling, no. 3033), p65 (Santa Cruz, sc-109), Bax (Biolegend, no. 62510), Bcl-2 (Cell Signaling, no. 2870), and caspase-3 (Cell Signaling, no. 9665).

**Statistical analysis.** All results are given as means ± SD. Significant differences were assessed by the one-way ANOVA statistic followed by a Tukey’s post hoc test.

**RESULTS**

**Mitochondrial function in long-term cholestasis.** Table 2 shows that oxygen consumption in liver mitochondria using glutamate/malate as complex I-linked substrates did not change...
in state 4 in long-term cholestasis, but in state 3 it was less than half in BDL rats than in control animals, leading to a 56% reduction in the respiratory ratio. In contrast, when succinate was used as complex II-linked substrate, oxygen consumption did not change significantly in liver mitochondria from BDL rats compared with those from control rats in both states 4 and 3. In agreement with our previous work (38), in the present study long-term cholestasis also caused a decrease in mitochondrial membrane potential (see Table 2). This impairment in mitochondrial function is associated with an increase in the flux through glycolysis, evidenced by the fourfold increase in hepatic lactate levels in BDL rats (see Fig. 1A). Hepatic ATP-to-ADP ratio tended to decrease, although not significantly, in BDL rats compared with Sham rats (see Table 2). The adaptive stimulation of glycolysis upon long-term cholestasis may account for maintenance of this ratio without significant changes.

Sensors and regulators of the energetic state in long-term cholestasis. PDK, phospho-AMPK (p-AMPK), and SIRT1 were measured as major sensors and regulators of the energetic status. PDK and SIRT1 protein levels were markedly decreased in BDL liver vs. controls \((P < 0.01)\), whereas p-AMPK was not significantly different between BDL and paired livers \((P > 0.05)\) (Fig. 1B).

Regulation of mitochondrial biogenesis at the nuclear level in long-term cholestasis. \(Ppargc-1\alpha\) mRNA expression was markedly decreased in BDL vs. controls \((P < 0.01)\); conversely, \(Pprc-1\) and \(Nrf-1\) gene expression was upregulated in BDL vs. controls \((P < 0.01)\) (Fig. 2). \(Gabp-\alpha/Nrf-2\) mRNA was not significantly different between BDL and controls.

In accordance with the mRNA expression, \(Pgc-1\alpha\) and \(GABP-\alpha\) protein levels were decreased in BDL livers relative to controls \((P < 0.01)\) (Fig. 3A), whereas \(NRF-1\) levels were higher in BDL liver than in controls \((P < 0.05)\). It is noteworthy that \(NRF-1\) suffered a molecular weight shift that could be due to a posttranslational modification. However, this modification does not seem to be glycosylation, nor phosphorylation since it could not reversed by alkaline phosphatase (results not shown). \(NRF-1\) is a shuttling nuclear/cytoplasmic protein, and hence its nuclear localization in BDL liver was investigated.

Similarly to the Sham liver, \(NRF-1\) was also present in the nuclear extracts from BDL liver (Fig. 3B). \(NRF-1\) phosphorylation and \(trans\)-activation of its target genes is promoted by phosphorylation of AKT at serine 473 \([p\-AKT(Ser473)]\) (41). Hence, we also studied \(p\-AKT(Ser473)\), which was decreased significantly in BDL liver vs. controls despite upregulation of AKT levels \((P < 0.01)\) (Fig. 3C).

Gene expression of nuclear-encoded mitochondrial proteins in long-term cholestasis. The transcription factors that control mitochondrial biogenesis regulate the gene expression of critical nuclear encoded mitochondrial proteins, such as polymerase \(\gamma\) (\(Polg\)), \(Tfam\), and cytochrome \(c\) (\(Cycs\)). The gene expression of these target genes was decreased in BDL rats with respect to controls (Fig. 4). The mRNA expression of \(Atp5b\) and \(Atp5i\), two subunits of the oxidative phosphorylation complexes, was also downregulated in liver from BDL rats, whereas gene expression of the transcription factor \(Tfb2m\) was not affected upon long-term cholestasis (Fig. 4).

**TFAM and other mitochondrial proteins during chronic cholestasis.** Protein levels of TFAM, a major regulator of mtDNA replication and transcription, increased at 2 wk after BDL, but they diminished markedly at 4 wk (Fig. 5A). Marked changes were also found at 4 wk in two transmembrane receptor proteins, TOM70 and TOM20, components of the translocase of the outer mitochondrial membrane (TOM). TOM70 levels were remarkably lower at 4 wk, whereas TOM20 increased at this stage (Fig. 5A). No significant changes were found for HSP60 and cytochrome \(c\).

**mtDNA/nDNA relative copy number and cell proliferation in long-term cholestasis.** The mtDNA/nDNA relative copy number did not change significantly at 2 wk, but it decreased dramatically, by 65% \((612\ vs.\ 1689)\), in BDL livers vs. controls \((P < 0.01)\) at 4 wk (Fig. 5B). PCNA levels, an index of cell proliferation, were upregulated both at 2 and 4 wk after BDL (see Fig. 5C). Therefore, the upregulation of TFAM at 2 wk seems to maintain mtDNA/nDNA ratio in presence of increased cell proliferation. However, the marked decrease in TFAM levels in the long term, i.e., at 4 wk, leads to a dramatic

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**Fig. 4.** Gene expression of nuclear-encoded mitochondrial proteins in chronic cholestasis. Relative mRNA levels of \(Polg\), \(Tfam\), and \(Tfb2m\) (top) and \(Cycs\), \(Atp5b\), \(Atp5i\) (bottom) in liver from Sham \((n = 4)\) and BDL \((n = 7)\) rats studied by real-time PCR. \(Rplp0\) was used as a reference gene. mtDNA, mitochondrial DNA; OXPHOS, oxidative phosphorylation. Values are expressed as means ± SD. *\(P < 0.05\) and **\(P < 0.01\).
Inflammatory status and evolution of apoptosis during chronic cholestasis. Figure 6 shows that chronic cholestasis leads to a chronic proinflammatory state, evidenced by upregulation of IL-1β and TNF-α mRNAs and phosphorylation of p65, the latter as an index of NF-κB activation, at both 2 and 4 wk. This chronic inflammation and the maintained upregulation of TNF-α were associated with a transient activation of apoptosis. Indeed, although apoptosis was significantly triggered at 2 wk of BDL, as evidenced by increased levels of cleaved caspase 3 and BAX, these indexes of apoptosis returned almost to normal levels at 4 wk (Fig. 7). Upregulation of BCL-2 without a parallel increase in BAX levels at 4 wk (Fig. 7) would explain, at least in part, the reduction of apoptosis in long-term cholestasis.

mtDNA deletions occur in long-term cholestasis. Since chronic TFAM deficiency might result in higher mtDNA instability, we investigated the appearance of large mtDNA mutations by PCR analysis. The results revealed that different mtDNA deletions may occur in the development of long-term cholestasis. Indeed, five of eight BDL livers exhibited extra bands in the PCR amplification, indicating the presence of deletions in the mitochondrial genome (Fig. 8) that were absent in the livers of control animals.

DISCUSSION

The adaptive response to energy demand fails in the liver in long-term cholestasis. Chronic cholestasis leads to severe mitochondrial dysfunction in rodents and humans, evidenced by loss of membrane potential as well as decreased activity of the respiratory chain complexes, fatty acid oxidation, and ketone body formation (10, 18–20, 22, 23, 38). In the present study long-term cholestasis caused a decrease in mitochondrial oxygen consumption likely to be ascribed to a loss in complex-I activity together with an adaptive stimulation of glycolysis. In addition, we found a striking lack of induction of metabolic sensors, PDK, AMPK, and SIRT1, in face of a metabolic stress. PDK inactivates via phosphorylation the pyruvate dehydrogenase complex that catalyzes the irreversible decarboxylation of pyruvate to acetyl coenzyme A. Hence, PDK controls the entry of pyruvate into the citric acid cycle and contributes to switch the energy source from glucose oxidation to fatty acid oxidation (44). PDK was downregulated in long-term cholestasis, releasing the brake for glucose oxidation and lipogenesis.
AMPK is activated by energetic stress (low ATP and high AMP) and acts to maintain cellular energy stores by switching off energy-consuming biosynthetic pathways and switching on ATP-generating pathways, such as fatty acid oxidation and mitochondrial biogenesis (3). Despite the metabolic stress associated with mitochondrial impairment, we did not find AMPK activation in liver of BDL rats.

The NAD$^+$-dependent type III deacetylase SIRT1 is another metabolic sensor that plays a key role in hepatic glucose and lipid homeostasis by inducing gluconeogenesis, fatty acid oxidation, and cholesterol degradation (32). Long-term cholestasis led to SIRT1 downregulation, suggesting lower capacity for fatty acid oxidation and cholesterol degradation.

Consequently, the downregulation of PDK4, AMPK, and SIRT1 shows that mitochondrial impairment and the corresponding energetic stress do not trigger an adaptive response in the liver in long-term cholestasis.

Decreased mitochondrial biogenesis occurs in the liver in long-term chronic cholestasis. The energetic deficiency should activate mitochondrial biogenesis through PGC-1α, NRF-1, and GABP-α/NRF2 (2, 18, 28, 30). PGC-1α is a key regulator of cellular energy metabolism with crucial roles in the control of mitochondrial biogenesis and antioxidants (30). Expression of Ppargc-1a gene is highly inducible according to the tissue energy demands, and accordingly it is rapidly upregulated in the liver upon starvation (30, 45). However, Ppargc-1a gene expression and PGC-1α protein levels were downregulated upon long-term chronic cholestasis, contributing not only to a reduced mitochondrial biogenesis but also to the mitochondrial oxidative stress that occurs in BDL rats (38). Further research is needed to elucidate the upstream mechanisms responsible for the downregulation of PGC1-α.

CREB is a major regulator of PGC1α mRNA expression (12), and changes in its expression and phosphorylation level might contribute to PGC1-α downregulation in long-term cholestasis.

PDK-4, the major PDK isoform in the liver, is under the control of PGC1-α, which downregulates glucose oxidation by inducing PDK4 gene expression (44). Consequently, the decrease in PGC1-α may explain the absence of PDK-4 upregulation.

Both energetic stress and oxidative stress activate NRF-1 through phosphorylation (2, 41), in the latter case via AKT/protein kinase B. Although NRF-1 was upregulated in the liver of BDL rats, it exhibited a posttranslational modification not related to phosphorylation, and its specific target gene Cycs was downregulated. Its coactivator PGC1-α was decreased and AKT was not phosphorylated in BDL liver. Hence, the NRF-1 pathway seems to be inactive or reduced upon long-term chronic cholestasis despite the energetic stress and oxidative stress that occur in this condition (10, 38). Nrf-1 upregulation might be mediated, at least in part, by the parallel increase in Pprc1. The upregulation of Pprc1 mRNA could be associated with the proliferative state present in long-term cholestasis, as described in other proliferative models (1).

GABP-α also senses cellular energy demands and regulates the transcriptional activity of COX nuclear subunits and TFAM (28). GABP-α/NRF-2 was largely decreased in the liver upon long-term cholestasis. Consequently, in biliary cirrhosis there is a general failure in the regulation of mitochondrial biogenesis to meet energetic and signaling demands.

The activity of Tfam rat promoter is mainly regulated by the coactivator PGC-1α together with the trans-acting elements NRF-1 and GABP-α/NRF-2 (45). GABP-α and PGC-1α were largely decreased in BDL rats, and NRF-1 was not transcriptionally active. Accordingly, Tfam transcript level was decreased in BDL liver. In addition, expression of nuclear tran-
scripts Cycs, Polg, Atp5i, and Atp5b that encode for mitochondrial proteins was downregulated in the liver upon long-term cholestasis. Similar findings were obtained with mitochondrial-encoded genes CoxI and Cyb (results not shown). The upregulation of the Pprc1, a related coactivator of PGC-1α, does not seem enough to maintain the expression of mitochondrial encoded genes.

Our experiments show a transient increase in mitochondrial TFAM levels at 2 wk after BDL, but a marked decrease of TFAM at 4 wk. Downregulation of Tfam expression in this latter stage would contribute to the low TFAM levels, which may be responsible, at least in part, for the mitochondrial dysfunction in long-term cholestasis. Indeed, TFAM gene knockdown led to reduced mitochondrial respiratory activity and ATP production (14).

The loss of mitochondrial TFAM in long-term cholestasis might also be ascribed to a failure in the import of matrix-targeted proteins. Indeed, mitochondrial membrane potential (Δψm), a driving force for the import of most matrix-directed mitochondrial proteins (29), is decreased in chronic cholestasis, as we reported previously (38) and confirm here. In BDL livers we also found a dramatic decrease in TOM70 levels, a transmembrane receptor protein that belongs to the TOM complex (5). In contrast, levels of TOM20, another transmembrane receptor, were increased in BDL mitochondria, probably as an adaptive compensatory response. Therefore, changes in the composition of the TOM complex might affect the import of matrix-targeted proteins.

Recently, Suliman et al. (39) have reported that S-nitrosylation of mitochondrial HSP60 regulates TFAM accumulation
in mitochondria in peritonitis. Although no significant changes in HSP60 levels were found in our experimental model of long-term cholestasis, the contribution of S-nitros(y)lated HSP60 to the decreased mitochondrial TFAM levels and its chaperone function remains to be investigated in this liver disease.

Since TFAM is a major regulator of mtDNA transcription and replication (17, 24), its remarkable fall together with downregulation of Polg expression may be directly responsible for the mtDNA depletion, 65% decrease in mtDNA/nDNA relative copy number, found in long-term cholestasis. According to our results, this blockade of mitochondrial biogenesis should not be ascribed to activation of apoptosis or inhibition of cell proliferation.

mtDNA deletions occur in the liver in long-term cholestasis. Deletions or rearrangement of mtDNA have been identified in mitochondrial hepatopathies, which may be associated with a severe reduction in the mtDNA copy number (26). In addition, TFAM is considered a histone-like protein that protects mtDNA and its fall might lead to mtDNA instability. Our study shows that long-term cholestasis leads to mtDNA deletions that are likely to be consequence of loss TFAM. As an alternative explanation, oxidative stress, which has been previously observed in BDL liver (38), could be responsible for oxidative damage to mtDNA and occurrence of mtDNA deletions (40). Somatic mtDNA mutations have been associated with hepatocarcinogenesis (48). Consequently, and taking into account these mtDNA mutations together with the severe mtDNA depletion, long-term cholestasis and biliary cirrhosis should be considered a secondary mitochondrial hepatopathy.

In conclusion, in long-term cholestasis we identified a lack of adaptive response to energetic stress that normally induces mitochondrial biogenesis. In addition, impaired mitochondrial turnover, severe mtDNA depletion, and increased mtDNA instability are likely to account for the reduction in mitochondrial ATP production and increased mitochondrial reactive oxygen species production that are characteristic of chronic cholestasis. Therefore, long-term cholestasis and biliary cirrhosis should be considered a secondary mitochondrial hepatopathy in which both the mitochondrial impairment and lack of stimulation of mitochondrial biogenesis may sustain and perpetuate liver damage.

GRANTS

This work was supported by Grants CSD-2007-00020, SAF2006-60963, and SAF2009-09500 from Ministerio de Ciencia e Innovación together with FEDER funds to J. Sastre and Grant Prometeo/2010/074 to J. Viña.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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