Free Radical Biology and Medicine ∎ (∎∎∎∎) ∎∎∎–∎∎∎

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Silybin exerts antioxidant effects and induces mitochondrial biogenesis in liver of rat with secondary biliary cirrhosis

Gaetano Serviddio [a], Francesco Bellanti [a], Eleonora Stanca [b], Paola Lunetti [b], Maria Blonda [a], Rosanna Tamborra [a], Luisa Siculella [b], Gianluigi Vendemiale [a], Loredana Capobianco [a,*,1], Anna Maria Giudetti [a,*,1]

[a] Centro CURE, Institute of Internal Medicine, Department of Medical and Surgical Sciences, University of Foggia, Foggia, Italy
[b] Laboratory of Biochemistry and Molecular Biology, Department of Biological and Environmental Sciences and Technologies, University of Salento, 73100 Lecce, Italy

ARTICLE INFO

Article history:
Received 24 February 2014
Received in revised form
30 April 2014
Accepted 1 May 2014

Keywords:
Citrate carrier
Lipogenic enzymes
Mitochondrial biogenesis
Peroxisome proliferator-activated receptor coactivator-1α
Secondary biliary cirrhosis
Free radicals

ABSTRACT

The accumulation of toxic hydrophobic bile acids in hepatocytes, observed during chronic cholestasis, induces substantial modification in the redox state and in mitochondrial functions. Recent reports have suggested a significant role of impaired lipid metabolism in the progression of chronic cholestasis. In this work we report that changes observed in the expression of the lipogenic enzymes acetyl-CoA carboxylase and fatty acid synthase were associated with a decrease in the activity of citrate carrier (CIC), a protein of the inner mitochondrial membrane closely related to hepatic lipogenesis. We also verified that the impairment of citrate transport was dependent on modification of the phospholipid composition of the mitochondrial membrane and on cardiolipin oxidation. Silybin, an extract of silymarin with antioxidant and anti-inflammatory properties, prevented mitochondrial reactive oxygen species (ROS) production, cardiolipin oxidation, and CIC failure in cirrhotic livers but did not affect the expression of lipogenic enzymes. Moreover, supplementation of silybin was also associated with mitochondrial biogenesis. In conclusion, we demonstrate that chronic cholestasis induces cardiolipin oxidation that in turn impairs mitochondrial function and further promotes ROS production. The capacity of silybin to limit mitochondrial failure is part of its hepatoprotective property.

Cholestasis is the intrahepatic accumulation of potentially toxic bile acids that occurs in several chronic liver diseases as an effect of obstruction or destruction of bile ducts [1].

High concentrations of hydrophobic bile acids have been reported to induce necrosis in primary hepatocytes [1]. We have previously reported that accumulation of hydrophobic biliary acids promotes mitochondrial production of reactive oxygen species (ROS)[2] that, in turn, may induce cell death [1]. Owing to their detergent effect, bile acids also affect mitochondrial membrane structure and function. In a model of secondary biliary cirrhosis we have found a dramatic loss in mitochondrial cardiolipin content and membrane potential due to the impairment of mitochondrial complex I and II activity [2]. Moreover, in the same model, we have also demonstrated a severe depletion and deletions of mitochondrial DNA (mtDNA) due to decreased mitochondrial turnover [3].

Cholestasis can also affect lipid absorption and metabolism. Accumulation of bile acids in hepatocytes affects several pathways involved in lipoprotein metabolism as well as lipoprotein secretion, with a final decrease in high-density lipoprotein level and appearance of an aberrant lipoprotein X in plasma [4]. Ursodeoxycholic acid improves liver injury also in *Abcb4*$^{-/-}$ mice, pointing out the role of lipid metabolism in the progression of cholangiopathies and biliary fibrosis [5].

Very recently, we showed that supplementation of silybin to a standard diet significantly reduces necrosis, inflammation, and fibrosis in an animal model of chronic cholestasis and that such positive effect is correlated with a potential regulatory role of silybin in the phospholipid remodeling pathway [6].

The citrate carrier (CIC) is a nuclear-encoded protein located in the inner mitochondrial membrane that plays an important role in hepatic lipogenesis. In fact, by transporting acetyl-CoA (in the form of citrate) from the mitochondria to the cytosol it provides a

Abbreviations: ACC, acetyl-CoA carboxylase; BDL, biliary duct ligation; CIC, citrate carrier; CS, citrate synthase; DNPH, dinitrophenylhydrazine; FAS, fatty acid synthase; PE, phosphatidylethanolamine; PC, phosphatidylcholine; mtDNA, mitochondrial DNA; PGC-1α, peroxisome proliferator-activated receptor coactivator-1α; PAF, platelet-activating factor; ROS, reactive oxygen species; SIL, silybin; SREBP-1c, sterol regulatory element-binding protein-1c

* Corresponding authors. Fax: +39 832 298626.
E-mail addresses: anna.giudetti@unisalento.it (L. Capobianco), loredana.capobianco@unisalento.it (A.M. Giudetti).
[1] These authors are joint senior authors.

http://dx.doi.org/10.1016/j.freeradbiomed.2014.05.002
0891-5849/

carbon source for fatty acid and cholesterol synthesis [7]. It has been demonstrated that CIC activity is enhanced in hyperthyroidism and cancer and is significantly reduced during starvation, in diabetic rats, and in rats fed a polyunsaturated fatty acid-enriched diet [7]. Moreover, molecular studies suggest that CIC activity is mainly regulated by transcriptional and posttranscriptional mechanisms [7].

In this study we aimed to verify the role of mitochondrial dysfunction and CIC in the alteration of lipid metabolism observed in biliary cirrhosis. We also aimed to verify the capacity of silybin to limit the impairment of mitochondrial function and to restore lipid metabolism.

Materials and methods

Materials

A Bio-Rad protein assay kit was purchased (Bio-Rad Laboratories, Hercules, CA, USA); Amberlite XAD-2, Pipes, Triton X-100, Triton X-114, Sephadex G-75, 1,2,3-benzenetricarboxylic acid (1,2,3-BTA), cardiolipin, and primers for real-time PCR were from Sigma; [1,5-^{14}C]citrate was from Healthcare and egg yolk phospholipids were from Fluka. Acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), sterol regulatory element-binding protein-1c (SREBP-1c), succinate dehydrogenase subunit a (Sdha), and α-tubulin antibodies were from Millipore. All other reagents were of analytical grade.

Animal model

Male Wistar rats, weighing 200–250 g, supplied by Harlan Italy Srl (S. Pietro al Natisone, UD), were housed at 22 ± 1 °C with a 12/12-h light/dark cycle and 30–40% humidity. The animals were maintained and sacrificed according to the Italian Official Statement No. 116/92. Rats were divided into three groups: a group subjected to bile duct ligation (BDL), a group of control rats subjected to the entire surgical procedure except for the ligation (sham), and finally a group of BDL rats treated with silybin (BDL + SIL). The duct ligation was performed as previously described [1]. Animals were sacrificed after 28 days by anesthetic overdose. Rats were fed the standard diet (4RF21; Mucedola Srl, Milan, Italy) or the standard diet supplemented with silybin (0.4 g/kg MCD diet) complexed with phospholipids.

Serum bilirubin level was verified at the end of the protocol and found to be 7.3 ± 3.3 and 0.5 ± 0.3 mg/dl in BDL and sham groups, respectively. The characteristics of liver cirrhosis were demonstrated as in [1,6].

Measurement of CIC activity in intact mitochondria and in proteoliposomes

Rat liver mitochondria were prepared by differential centrifugation as previously reported [8]. Mitochondrial protein concentration was determined by the Bradford method [9] with bovine serum albumin as a standard.

CIC activity was assayed in freshly isolated liver mitochondria loaded with malate as described in [8]. Reconstitution of CIC activity in proteoliposomes and CIC activity assay in the reconstituted system were as follows: rat liver mitochondria (10–15 mg proteins) were solubilized with a buffer containing 3% Triton X-100 (w/v), 20 mM Na_2SO_4, 1 mM EDTA, 10 mM Pipes, pH 7.0, at a final concentration of about 10 mg protein/ml. After incubation for 10 min at 2 °C, the mixture was centrifuged at 25,000 *g* for 20 min at 2 °C thereby obtaining a supernatant, referred to as the mitochondrial extract. The mitochondrial extract was reconstituted by cyclic removal of detergent [10]. The reconstitution mixture consisted of protein solution (50 μl, 0.09 mg), 10% Triton X-114 (75 ml), 10% phospholipids (egg lecithin) as sonicated liposomes (100 ml), 10 mM citrate, cardiolipin (0.6 mg), 20 mM Pipes, pH 7.0, and water (final volume, 700 ml). The mixture was recycled 13 times through an Amberlite column. All operations were performed at 4 °C, except for the passages through Amberlite, which were carried out at room temperature. To measure the citrate transport, external substrate was removed from the proteoliposomes on Sephadex G-75 columns preequilibrated with buffer A (50 mM NaCl and 10 mM Pipes, pH 7.0). Transport at 25 °C was initiated by the addition of 0.15 mM [1,5-^{14}C]citrate to the eluted proteoliposomes and terminated by the "inhibitor-stop" method with the addition of 20 mM 1,2,3-BTA [11]. In controls, the inhibitors were added simultaneously to the labeled substrate. Finally, the external radioactivity was removed by Sephadex G-75 and radioactivity in the liposomes was measured. Transport activity was calculated by subtracting the control values from the experimental values [10].

Analysis of mitochondrial membrane phospholipids and oxidized cardiolipin

Total lipids were extracted from mitochondria (10 mg protein) as previously reported [12]. The extracts were dried under a flow of N_2 and resuspended in a proper volume of $CHCl_3$. Analyses of phospholipids was carried out as described previously [12]. Phospholipids were quantified by determining inorganic phosphate. Peroxidized cardiolipin was identified by HPLC, as described in [13,14], with UV detection at 233 nm indicative of conjugated dienes [12]. Bovine heart cardiolipin, autoxidized overnight in a thin film at 37 °C, was used as standard [15].

Mitochondrial oxygraphic measurements

Freshly prepared mitochondria were assayed for oxygen consumption at 37 °C in a thermostatically controlled oxygraph apparatus equipped with a Clark electrode (Hansatech Instruments Ltd., Norfolk, UK). Oxygen uptake in State 3 and State 4 and the respiratory control index (RCI) were calculated as previously reported [16] using glutamate/malate or succinate as oxidative substrates.

Complex I activity measurement

NADH:coenzyme Q oxidoreductase (complex I) activity was assayed spectrophotometrically as in [17] by measuring the decrease in NADH absorbance at 340 nm. Reaction medium (1.0 ml) was supplemented with 60 μM decylubiquinone, 0.1 μg antimycin A, 1 mM KCN, and 0.5 mg of mitochondrial proteins. The reaction was initiated by 100 μM NADH and 2 μM rotenone was added after 2 min. Enzyme activity was determined as the difference in absorbance in the absence and in the presence of rotenone.

Determination of mitochondrial membrane potential and proton leak

Freshly prepared mitochondria were assayed for mitochondrial membrane potential (ΔΨ) at 37 °C in the presence of 5 mM glutamate plus 5 mM malate or 5 mM succinate plus 2 μM rotenone and 5 μM oligomycin by a Clarke and a tetraphenylphosphonium electrode (WPI, Berlin, Germany). Membrane potential calculations were made using a modified Nernst equation as previously reported [16]. The determination of membrane potential dependence of the proton leak activity in isolated mitochondria is based on the protocol described by Porter and Brand [18]. Briefly, mitochondria were incubated with succinate–rotenone

Please cite this article as: Serviddio, G; et al. Silybin exerts antioxidant effects and induces mitochondrial biogenesis in liver of rat with secondary biliary cirrhosis. *Free Radic. Biol. Med.* (2014), http://dx.doi.org/10.1016/j.freeradbiomed.2014.05.002