A Silybin-Phospholipid Complex Prevents Mitochondrial Dysfunction in a Rodent Model of Nonalcoholic Steatohepatitis

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ABSTRACT

Mitochondrial dysfunction and oxidative stress are determinant events in the pathogenesis of nonalcoholic steatohepatitis. Silybin has shown antioxidant, anti-inflammatory, and antifibrotic effects in chronic liver disease. We aimed to study the effect of the silybin-phospholipid complex (SILIPHOS) on liver redox balance and mitochondrial function in a dietary model of nonalcoholic steatohepatitis. To accomplish this, glutathione oxidation, mitochondrial oxygen uptake, proton leak, ATP homeostasis, and H2O2 production rate were evaluated in isolated liver mitochondria from rats fed a methionine- and choline-deficient (MCD) diet and the MCD diet plus SILIPHOS for 7 and 14 weeks. Oxidative proteins, hydroxynonenal (HNE)- and malondialdehyde (MDA)-protein adducts, and mitochondrial membrane lipid composition were also measured. Treatment with SILIPHOS limited glutathione depletion and mitochondrial H2O2 production. Moreover, SILIPHOS preserved mitochondrial bioenergetics and prevented mitochondrial proton leak and ATP reduction. Finally, SILIPHOS limited the formation of HNE- and MDA-protein adducts. In conclusion, SILIPHOS is effective in preventing severe oxidative stress and preserving hepatic mitochondrial bioenergetics in nonalcoholic steatohepatitis induced by the MCD diet. The modifications of mitochondrial membrane fatty acid composition induced by the MCD diet are partially prevented by SILIPHOS, conferring anti-inflammatory and antifibrotic effects. The increased vulnerability of lipid membranes to oxidative damage is limited by SILIPHOS through preserved mitochondrial function.

Nonalcoholic fatty liver disease (NAFLD) is a pathological condition characterized by histological features of alcoholic liver disease occurring in patients who do not abuse alcohol (Ludwig et al., 1980). Nonalcoholic steatohepatitis (NASH) refers to NAFLD patients who have evidence of inflammation on liver biopsy. It has been associated with a broad range of metabolic disorders such as obesity, type 2 diabetes mellitus, hyperlipidemia, rapid weight loss, and jejunosileal bypass surgery (Chitturi and Farrell, 2001). The clinical importance of NASH is related to its capacity to evolve in liver cirrhosis and cancer (Caldwell and Crespo, 2004).

The pathogenesis of NASH is not well understood: Day and James (1998) proposed a double-hit model, in which steatosis represents the first hit, making the liver vulnerable to a second hit, which leads to hepatic inflammation and fibrosis. Oxidative stress, lipid peroxidation, and cytokines (tumor necrosis factor-α, transforming growth factor-β, IL-1β, IL-6, and IL-8) are considered the best candidates for the role of second hit. Several recent studies have revised this model of pathogenesis, assigning to oxidative stress a central role and limiting steatosis to being an epiphenomenon of the injurious mechanism (Cortez-Pinto et al., 2006).

ABBREVIATIONS: NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; IL, interleukin; MCD, methionine-choline deficient; SILIPHOS, silybin-phospholipid complex; GSH, reduced glutathione; GSSG, oxidized glutathione; RCI, respiratory control index; Δψ, mitochondrial membrane potential; CTRL, controls, fed a standard diet; HNE, 4-hydroxy-2-nonenal; MDA, malondialdehyde; DNPH, dinitrophenylhydrazine; ANOVA, analysis of variance; H&E, hematoxylin and eosin; ROS, reactive oxygen species.
For the study of NASH pathogenesis several animal models are available, including rodents with genetic defects (ob/ob mice or Fa/Fa rats) or those fed a methionine- and choline-deficient (MCD) diet. The ob/ob mouse is insulin-resistant and develops hepatic steatosis; however, it does not develop significant advanced liver disease and thus potential mechanisms derived from this model may not be applicable to human NASH. The widely used model, MCD diet, induces a nutritional deficiency without insulin resistance, causing steatosis (mainly dependent on a defect in hepatic triacylglycerols secretion), inflammation, and fibrosis (Rinella and Green, 2004) and impairing liver redox balance similarly to effects in human patients (Begriche et al., 2006).

Oxidative stress in NASH is closely related to mitochondrial dysfunction (Caldwell et al., 1999; Perez-Carreras et al., 2003; Serviddio et al., 2008a). We have recently determined that during NASH progression oversupply of free fatty acids induces an increase in mitochondrial H2O2 production that in turn oxidizes mitochondrial membranes and regulates activity of uncoupling protein 2 and carnitine palmitoyl transferase 1 (Serviddio et al., 2008c). Mitochondria play a key role in hepatocyte metabolism, being the site of β-oxidation and oxidative phosphorylation. We and others have shown that mitochondrial abnormalities are closely related to the pathogenesis of NAFLD, which raises the possibility that NAFLD is a mitochondrial disease (Sanyal et al., 2001; Serviddio et al., 2008b).

At present, there is no proven therapy for NASH, and pharmacotherapy is an area of active research (Clark and Diehl, 2003; Begriche et al., 2006). The introduction of drugs directly able to reduce oxidative stress, in association with clinical strategies directed to lowering lipid accumulation, would be important in the treatment of these disorders.

Silybin (2R,3R,3,5,7-trihydroxy-2-[(2R,3R)-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-2,3-dihydrobenzo[b][1,4]-dioxin-6-yl]chroman-4-one) is the major constituent of milk thistle extract, which has shown hepatoprotective effects in several in vitro and animal models (Ferenci et al., 1989; Muriel et al., 1992; Wellington and Jarvis, 2001). In addition, it has been recently demonstrated that silybin inhibits the production of proinflammatory and profibrogenic factors (Trappoliere et al., 2009). Similar to many other flavonoids, silybin is poorly absorbed when it is administered orally; however, when conjugated with phospholipids the amount of silybin reaching the liver is more than 6 times greater than that from nonconjugated silybin (Morazzoni et al., 1993).

The silybin-phospholipid complex exhibited hepatoprotective and antifibrotic effects in a rodent model of fibrosis (Di Sario et al., 2005); it has been also tested in a study in an Italian cohort of patients with NAFLD, showing a significant improvement in liver enzyme levels and indexes of liver fibrosis (Loguerchio et al., 2007). However, the mechanisms of protection have not been completely defined.

Several findings indicate that oxidative stress in NASH liver is closely related to mitochondrial dysfunction. We have recently defined a complex mechanism of mitochondrial impairment accounting for progression of NASH (Serviddio et al., 2008a). In the present study we investigated the effect of the silybin-phospholipid complex on hepatic redox balance and mitochondrial bioenergetics, uncoupling, and membrane lipid composition using the MCD diet model of NASH. Rats were sacrificed after 7 weeks of diet, because we had demonstrated previously that at this time significant mitochondrial dysfunction occurred (Serviddio et al., 2008a,b). Furthermore, to study the effect in advanced disease, rats were also sacrificed after 14 weeks of diet.

Materials and Methods

Chemicals and Reagents. Silybin complexed with phospholipids (SILIPHOS) was kindly provided by IBI-Lorenzini Pharmaceutical (Rome, Italy). Amplex Red was purchased from Invitrogen (Carlsbad, CA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Animals and Experimental Design. Male Wistar rats (Harlan, San Pietro al Natisone, Italy) weighing 350 to 400 g were caged individually in a temperature- and light-controlled environment with free access to food and water. All rats received care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996), as well as with Italian laws on animal experimentation. Animals were randomly divided into three groups: 1) controls, fed a standard diet (CTRL); 2) rats fed a high-fat MCD diet; and 3) rats fed the MCD diet supplemented with silybin (0.4 g/kg MCD diet) complexed with phospholipids (SILIPHOS). Diets were purchased from Mucedola s.r.l. (Settimo Milanese, Italy), and their compositions have been reported previously (Teramoto et al., 1993). Five rats for each group were sacrificed after 7 and 14 weeks of diet. Steatohepatitis was evaluated by serum alanine aminotransferase (ALT) activity (Sigma-Aldrich) and histological analysis according to standardized criteria (Kleiner et al., 2005).

Glutathione Assay. Hepatic levels of reduced (GSH) and oxidized glutathione (GSSG) were measured by high-performance liquid chromatography according to the method of Viña as described previously (Serviddio et al., 2004).

Mitochondrial Isolation and Oxygenic Measurements. Rat livers were rapidly processed for mitochondrial isolation as reported previously (Serviddio et al., 2007). Mitochondria protein concentration was determined using the Lowry micromethod kit (Sigma-Aldrich).

Freshly prepared mitochondria were assayed for oxygen consumption at 37°C in a thermostatically controlled oxygen 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 measured and calculated as previously (Serviddio et al., 2007). In a group of experiments, increased concentrations of the uncoupler carbonyl cyanide m-chlorophenylhydrazone were added into the medium during the state 4 respiration, and the recovery of the maximal electron transport capability (maximal expiration rate) was allowed (Heytler, 1979).

Determination of Mitochondrial Inner Membrane Potential and Proton Leak Analysis. Freshly prepared mitochondria were assayed for mitochondrial membrane potential (ΔΨm) 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 reported previously (Serviddio et al., 2007). The determination of membrane potential dependence of the proton leak activity in isolated mitochondria is based on the protocol described by Porter and Brand (1993).

F$_{F0}$F$_{1}$ ATPase (ATP Synthase) Activity and Hepatic ATP Content. F$_{F0}$F$_{1}$ATPase activity was measured as ATPase after ATP hydrolysis with an ATP-regenerating system coupled to NADPH oxidation (supplemental data) (Barrientos, 2002). The hepatic ATP concentration was assessed by bioluminescence (ENLITEN ATP-As-
say Kit; Promega, Madison, WI) according to the method of Yang et al. (2002).

Measurement of Mitochondrial \( \text{H}_2\text{O}_2 \) Production. The rate of peroxide production was determined in isolated liver mitochondria by a modification of the method of Barja (1999). Glutamate and malate or succinate in the presence of rotenone were used as oxidative substrates to investigate the rate of peroxide production from complex I–III or complex II–III, respectively (supplemental data).

Liver Tissue and Mitochondrial 4-Hydroxy-2-nonenal- and Malondialdehyde-Protein Adducts. Fluorescent adducts formed between 4-hydroxy-2-nonenal (HNE) or malondialdehyde (MDA) with tissue or mitochondrial proteins were monitored spectrofluorometrically as reported previously (supplemental data) (Massarenti et al., 2004).

Analysis of Mitochondrial Lipid Composition. To analyze fatty acids, liver mitochondria were saponified with ethanolic KOH for 2 h at 90°C. Fatty acids were extracted (Muci et al., 1992), and their corresponding methyl esters were prepared by trans-esterification with 17% methanolic boron trifluoride (BF$_3$) at 65°C for 30 min. Fatty acid methyl esters were then analyzed by gas-liquid chromatography (Giudetti et al., 2006). Peak identification was performed by using known standards, and relative quantification was automatically performed by peak integration.

Western Blot Analysis of Hepatic Oxidized Proteins. Analysis of oxidized proteins was performed by Western blot in liver homogenates using an Oxyblot kit (Millipore Bioscience Research Reagents, Temecula, CA). The same amounts of liver proteins (~35 \( \mu \text{g} \)) were reacted with dinitrophenylhydrazine (DNPH) for 20 min, followed by neutralization with a solution containing glycerol and 2-mercaptoethanol, resolved in 12.5% SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, blocked with nonfat milk, and incubated with a rabbit anti-DNPH antibody (1:150) at 4°C overnight. After washing, the membrane was incubated with the secondary antibody (1:300) conjugated to horseradish peroxidase and detected by a chemiluminescence detection kit (Cell Signaling Technology Inc., Danvers, MA). Reactive bands were visualized by the enhanced chemiluminescence method on a VersaDoc Image System (Bio-Rad Laboratories, Hercules, CA). Band density was determined with TotalLab software.

**Fig. 1.** A, serum ALT levels in rats fed the CTRL diet, the MCD diet, and the MCD plus SILIPHOS diet at 7 and 14 weeks. Data are expressed as means ± S.E.M. of five experiments for each group. Statistical differences were assessed using ANOVA and the Tukey-Kramer multicomparison test. B, histological analysis of liver specimens stained with H&E from the same groups. Liver of MCD rats at 7 weeks shows steatohepatitis characterized by panacinar steatosis predominantly as macrovesicular fat, together with lobular inflammation as evidenced by the presence of neutrophils and monocytes (H&E; original magnification, 100×). Rats treated with SILIPHOS at 7 weeks exhibited macrovesicular liver steatosis with minimal neutrophil recruitment (H&E; original magnification, 100×). At 14 weeks, MCD rats presented moderate-severe steatohepatitis consisting of diffuse lobular and portal inflammatory infiltrate with the presence of microgranulomas, whereas the SILIPHOS group showed neither portal inflammation nor microgranulomas (H&E; original magnification, 100×).
**Statistical Analysis.** Data are expressed as mean ± S.E.M. Because the data were not paired, differences among means were analyzed using one-way ANOVA after Gaussian distribution evaluation by a Kolmogorov-Smirnov test. The Tukey-Kramer multiple comparison test for all pairs of columns was applied as a post hoc test. In all instances, $P < 0.05$ was taken as the lowest level of significance. GraphPad Prism 4 for Windows (GraphPad Software Inc., San Diego, CA) was used to perform all of the statistical analysis.

**TABLE 1**
Liver glutathione levels in CTRL rats and rats fed the MCD diet or the MCD plus SILIPHOS diet at 7 and 14 weeks
Data are expressed as means ± S.D.M. of five experiments for each group. Statistical differences were assessed using ANOVA and the Tukey-Kramer multicomparison test.

<table>
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<th>CTRL</th>
<th>MCD</th>
<th>SILIPHOS</th>
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<tr>
<td><strong>GSSG (nmol/g liver tissue)</strong></td>
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<tr>
<td>7th Week</td>
<td>$168.7 \pm 27.8$</td>
<td>$252.8 \pm 59.3^*$</td>
<td>$181.5 \pm 8.8^{**}$</td>
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<tr>
<td>14th Week</td>
<td>$157.6 \pm 57.4$</td>
<td>$349.3 \pm 93.2^*$</td>
<td>$162.5 \pm 66.7^{**}$</td>
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<tr>
<td><strong>GSH (µmol/g liver tissue)</strong></td>
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<tr>
<td>7th Week</td>
<td>$11.52 \pm 0.65$</td>
<td>$9.10 \pm 0.60^*$</td>
<td>$8.73 \pm 1.38^*$</td>
</tr>
<tr>
<td>14th Week</td>
<td>$14.76 \pm 5.24$</td>
<td>$7.76 \pm 2.66^*$</td>
<td>$7.47 \pm 1.92^*$</td>
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<tr>
<td><strong>GSSG/GSH (%)</strong></td>
<td></td>
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<tr>
<td>7th Week</td>
<td>$1.46 \pm 0.42$</td>
<td>$2.77 \pm 0.70^*$</td>
<td>$2.08 \pm 0.63$</td>
</tr>
<tr>
<td>14th Week</td>
<td>$1.07 \pm 0.61$</td>
<td>$4.50 \pm 2.38^*$</td>
<td>$1.86 \pm 0.35^{**}$</td>
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* $P < 0.05$ versus CTRL group.
** $P < 0.05$ versus MCD group.

**Pyruvate – Malate**

**Fig. 2.** Peroxide production by rat liver mitochondria isolated from rats fed the CTRL diet, the MCD diet, and the MCD plus SILIPHOS diet at 7 and 14 weeks. Incubations were performed using pyruvate-malate (A) or succinate (B) as substrates. Data are expressed as means ± S.E.M. of five experiments for each group. Statistical differences were assessed using ANOVA and the Tukey-Kramer multicomparison test.
Results

Effects of SILIPHOS on Liver Function and Histology during the Progression of NASH Induced by the MCD Diet

The MCD diet resulted in increased serum ALT levels after both 7 and 14 weeks compared with those of controls; the increase was limited in animals treated with SILIPHOS (Fig. 1A). Histological analysis of liver specimens stained with hematoxylin and eosin (H&E) from controls and rats fed the MCD diet or the MCD diet plus SILIPHOS for 7 and 14 weeks is shown reported in Fig. 1B. Rats fed the MCD diet showed mild inflammation after 7 weeks, which progressed to moderate-severe inflammation after 14 weeks; rats treated with SILIPHOS exhibited a minimal inflammation response after 7 weeks, developing mild steatohepatitis after 14 weeks.

Effects of SILIPHOS on Redox Balance during NASH Development

GSH and GSSG were measured in liver from MCD, SILIPHOS, and CTRL rats at 7 and 14 weeks of diet. GSH was significantly reduced in NASH rats compared with CTRL rats, but no difference was observed between MCD and SILIPHOS livers. In contrast, SILIPHOS significantly reduced the level of hepatic GSSG compared with that in MCD rats. The GSSG/GSH ratio was calculated and is reported as the expression of hepatic GSSG compared with that in MCD rats. The GSSG/GSH ratio was significantly reduced in NASH rats compared with CTRL rats, indicating intact oxidative phosphorylation from complex I. The rate of succinate oxidation was also not affected by the MCD diet or by SILIPHOS treatment (Table 2).

The rate of hydroperoxide production was measured in liver mitochondria using pyruvate plus malate or succinate as complex I–III and complex II–III linked substrates, respectively, and results are shown in Fig. 2. SILIPHOS significantly reduced mitochondrial H₂O₂ synthesis compared with that in MCD rats.

Effects of SILIPHOS on Mitochondrial Bioenergetics

Mitochondrial Respiratory Chain Activity. Mitochondrial respiratory chain activity was measured in isolated liver mitochondria in all animal groups. Even though some authors pointed out the limitations of this approach because a loss of metabolites may occur during mitochondria isolation, it is now considered the optimal approach to evaluate the maximum capacity of the oxidative phosphorylation system (Fontaine et al., 1995). We determined oxidative phosphorylation capacity using polarography by measuring oxygen consumption in the presence of an oxidative substrate (glutamate-malate or succinate) and ADP, constituting state 3 of oxygen consumption. State 4 represents the oxygen consumed by mitochondria independent of ADP phosphorylation, whereas the state 3/state 4 ratio is defined as the RCI. Although liver mitochondria from the MCD group exhibited a reduction in both ADP-dependent (state 3) and ADP-independent (state 4) respiration using glutamate-malate as an oxidative substrate, SILIPHOS restored the respiration rates (Table 2). Consequently, SILIPHOS prevented the decrease in the RCI using glutamate-malate as the substrate (Table 2) as observed in MCD rats, indicating intact oxidative phosphorylation from complex I. The rate of succinate oxidation was also not affected by the MCD diet or by SILIPHOS treatment (Table 2).

The ADP/O ratio is an index of coupling between phosphorylation activity and mitochondrial respiration. SILIPHOS prevented the reduction of the ADP/O ratio observed in the

### TABLE 2

Respiratory activities and membrane potential in mitochondria isolated from rats fed the CTRL diet, the MCD diet, and the MCD plus SILIPHOS diet at 7 and 14 weeks. Glutamate-malate or succinate was used as the oxidative substrate. Data are expressed as means ± S.D.M. of five experiments for each group. Statistical differences were assessed using ANOVA and the Tukey-Kramer multicomparison test.

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<th>CTRL</th>
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<th>SILIPHOS</th>
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<tr>
<td><strong>Glutamate-malate</strong></td>
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<tr>
<td>State 3 (nmol O₂/min/mg protein)</td>
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<tr>
<td>7th Week</td>
<td>88.67 ± 12.28</td>
<td>59.28 ± 9.45*</td>
<td>77.94 ± 10.06**</td>
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<tr>
<td>14th Week</td>
<td>90.47 ± 13.97</td>
<td>51.73 ± 11.47***</td>
<td>74.53 ± 9.61**</td>
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<tr>
<td>State 4 (nmol O₂/min/mg protein)</td>
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<tr>
<td>7th Week</td>
<td>26.15 ± 5.01</td>
<td>18.02 ± 3.68*</td>
<td>20.1 ± 4.6</td>
</tr>
<tr>
<td>14th Week</td>
<td>25.07 ± 5.32</td>
<td>15.83 ± 5.83*</td>
<td>20.01 ± 5.46</td>
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<tr>
<td>Respiratory control index</td>
<td>4.39 ± 0.34</td>
<td>3.12 ± 0.37*</td>
<td>3.98 ± 1.13</td>
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<tr>
<td>7th Week</td>
<td>3.71 ± 0.44</td>
<td>3.01 ± 0.19*</td>
<td>3.76 ± 0.83</td>
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<tr>
<td>Membrane potential (mV)</td>
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<tr>
<td>7th Week</td>
<td>175.45 ± 8.11</td>
<td>152.06 ± 4.51*</td>
<td>171.15 ± 6.99**</td>
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<td>14th Week</td>
<td>179.41 ± 7.55</td>
<td>149.22 ± 6.54*</td>
<td>173.76 ± 4.98*</td>
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<tr>
<td><strong>Succinate</strong></td>
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<tr>
<td>State 3 (nmol O₂/min/mg protein)</td>
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<tr>
<td>7th Week</td>
<td>172.68 ± 27.54</td>
<td>148.2 ± 24.76</td>
<td>145.64 ± 22.46</td>
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<td>14th Week</td>
<td>168.92 ± 28.27</td>
<td>142.76 ± 20.80</td>
<td>143.50 ± 21.27</td>
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<tr>
<td>State 4 (nmol O₂/min/mg protein)</td>
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<tr>
<td>7th Week</td>
<td>58.3 ± 12.58</td>
<td>42.7 ± 15.95</td>
<td>47.31 ± 20.39</td>
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<tr>
<td>14th Week</td>
<td>55.37 ± 13.10</td>
<td>49.96 ± 6.1</td>
<td>51.65 ± 17.41</td>
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<tr>
<td>Respiratory control index</td>
<td>3.12 ± 0.39</td>
<td>3.46 ± 1.17</td>
<td>3.29 ± 0.47</td>
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<td>7th Week</td>
<td>3.06 ± 0.84</td>
<td>2.90 ± 0.69</td>
<td>2.99 ± 0.49</td>
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<tr>
<td>Membrane potential (mV)</td>
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<tr>
<td>7th Week</td>
<td>193.62 ± 5.91</td>
<td>170.26 ± 3.90*</td>
<td>201.07 ± 5.86**</td>
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<tr>
<td>14th Week</td>
<td>189.56 ± 2.91</td>
<td>159.08 ± 10.40*</td>
<td>168.27 ± 5.73*</td>
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* P < 0.05 versus CTRL group.

** P < 0.05 versus MCD group.

*** P < 0.01 versus CTRL group.
MCD group using both glutamate-malate and succinate as substrates (Fig. 3A).

The addition of an uncoupler (carbonyl cyanide m-chlorophenylhydrazone) to mitochondria allows analysis of the integrity of the mitochondrial respiratory chain. Compared with CTRL rats, mitochondria from MCD rats showed a uncoupled/state 3 ratio that was significantly lower at 7 and 14 weeks of the diet, with both glutamate-malate and succinate as substrates, suggesting a loss of functional integrity of the electron transport; SILIPHOS was able to prevent this effect at 7 weeks but not at 14 weeks (Fig. 3B).

**Mitochondrial Membrane Potential.** ΔΨ is the major component of the proton motive force produced by the mitochondrial respiratory chain for ATP synthesis. The MCD diet induced a significant reduction in ΔΨ from both complex I and II that was completely prevented by treatment with SILIPHOS at 7 weeks. However, at 14 weeks ΔΨ was significantly reduced in treated rats compared with CTRL rats (Table 2).

**Mitochondrial Proton Leak Analysis.** The inner mitochondrial membrane is almost completely impermeable to protons, and the electrochemical gradient generated by proton pumping of mitochondrial respiratory chain into intermembrane space is burned by complex V to synthesize ATP (Kadenbach, 2003). We have demonstrated previously that during NASH progression uncoupling is generated by activation of uncoupling protein 2, which dissipated the proton motive force as a proton leak (Serviddio et al., 2008a) with a detrimental effect on ATP homeostasis. We determined the membrane potential dependence of the proton leak in mitochondria-oxidizing glutamate-malate or succinate in the presence of rotenone as an inhibitor of complex I and oligomycin as an inhibitor of complex V (ATPase). Under these conditions, O2 uptake is completely dependent on the proton leak across the inner membrane. Mitochondria from CTRL rats exhibited the standard relationship between membrane potential and proton leak (Fig. 4): at low membrane potential a slight proton leak occurred. When the membrane potential reached approximately 150 mV, there was a consistent rise in the proton leak. Mitochondria from the MCD group showed a linear relationship between membrane potential and proton leak, suggesting that these mitochondria have an increased

![Fig. 3. A, ADP/O in liver mitochondria isolated from rats fed the CTRL diet, the MCD diet, and the MCD plus SILIPHOS diet at 7 and 14 weeks, using glutamate-malate or succinate as substrates. Data are expressed as means ± S.E.M. of five experiments for each group. Statistical differences were assessed using ANOVA and the Tukey-Kramer multicomparison test. B, uncoupled state/state 3 (the ratio between oxygen uptake in the uncoupled state and state 3) in liver mitochondria isolated from rats fed the CTRL diet, the MCD diet, and the MCD plus SILIPHOS diet at 7 and 14 weeks, using glutamate-malate or succinate as substrates. Data are expressed as means ± S.E.M. of five experiments for each group. Statistical differences were assessed using ANOVA and the Tukey-Kramer multicomparison test.](image-url)
rate of proton leak, which partially dissipates their mitochondrial membrane potential when the rate of electron transport is suppressed. Our data showed that treatment with SILIPHOS limited the proton leak compared with that in MCD rats at 7 and 14 weeks (Fig. 4).

SILIPHOS Improves Hepatic ATP Homeostasis in NASH

Because the mitochondrial proton leak uncouples substrate oxidation from ATP synthesis and induces energy depletion, we aimed to study whether SILIPHOS exerts beneficial effects on ATP homeostasis. Hepatic ATP content was measured together with the analysis of the activity of complex V. SILIPHOS limited ATP reduction compared with the MCD diet, at both 7 and 14 weeks without affecting complex V activity (Table 3).

Effects of MCD Diet-Induced NASH on Lipids Composition of Hepatic Mitochondrial Membranes

Membrane composition may influence many aspects of mitochondrial function, including enzyme activity, coupling of respiration, and susceptibility to oxidative damage (Hulbert, 1978; McMurchie et al., 1984). We analyzed lipid composition of membranes in mitochondria from MCD and SILIPHOS livers. As shown in Table 4, in NASH induced by the MCD diet we observed a reduction in the ratio of saturated/unsaturated fatty acids. In particular, we observed an increase in unsaturated fatty acids such as oleic, linoleic, γ- and α-lin-

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**TABLE 3**

Enzymatic activity of mitochondrial F<sub>0</sub>F<sub>1</sub>-ATPase and hepatic ATP content in CTRL rats and rats fed the MCD diet or the MCD plus SILIPHOS diet at 7 and 14 weeks

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<th>ATPase Enzymatic Activity</th>
<th>Hepatic ATP Content</th>
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<tr>
<td></td>
<td>n mol ATP/min/mg protein</td>
<td>n mol ATP/g liver</td>
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<td>7th Week</td>
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<td></td>
</tr>
<tr>
<td>CTRL</td>
<td>184.85 ± 20.51</td>
<td>9.49 ± 1.82</td>
</tr>
<tr>
<td>MCD</td>
<td>161.56 ± 58.14</td>
<td>3.08 ± 0.67*</td>
</tr>
<tr>
<td>SILIPHOS</td>
<td>151.48 ± 23.11</td>
<td>5.64 ± 1.43*</td>
</tr>
<tr>
<td>14th Week</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTRL</td>
<td>179.45 ± 26.42</td>
<td>9.36 ± 1.65</td>
</tr>
<tr>
<td>MCD</td>
<td>167.78 ± 58.26</td>
<td>3.57 ± 1.16*</td>
</tr>
<tr>
<td>SILIPHOS</td>
<td>154.81 ± 29.22</td>
<td>4.94 ± 1.43*</td>
</tr>
</tbody>
</table>

* P < 0.01 versus CTRL group.
** P < 0.05 versus MCD group.
analyses are shown in Fig. 6.

against oxidative damage, carbonyl groups on oxidized pro-

zinc, were significantly different in liver homogenates and mitochon-

drosomal oxidative stress in the development of liver damage

associated with reactive oxygen species (ROS) production

serving mitochondrial RCI and ATP content.

Bioenergetics in NASH: in fact, mitochondrial function anal-

lyzed by monitoring respiratory activity (Carini et al., 1992;

Higher bioavailability than nonconjugated silybin, with a spe-

Effect of SILIPHOS on End Products of Lipid Peroxidation

and Mitochondrial Protein Oxidation

Free radicals may oxidize polyunsaturated fatty acyl groups

of membrane phospholipids producing various alde-

hydes such as HNE and MDA, reactive mediators of free-

radical damage (Esterbauer et al., 1991). We reported a sig-

nificant increase in mitochondrial HNE-protein adducts
during NAS progression, as an expression of mitochondrial

oxidative stress and HNE production (Serviddio et al.,

2008a). The levels of HNE- and MDA-protein adducts were

significantly different in liver homogenates and mitochon-

dria of rats treated with SILIPHOS only at 14 weeks of diet (Fig. 5).

To better characterize the protection exerted by SILIPHOS

gainst oxidative damage, carbonyl groups on oxidized pro-
teins from MCD and SILIPHOS liver homogenates were
derivatized with DNPH and revealed by using an anti-dinitro-
phenyl antibody by Western blot (Oxyblot). Representative

analyses are shown in Fig. 6.

TABLE 4

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>CTRL</th>
<th>MCD</th>
<th>SILIPHOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free Fatty Acid</td>
<td></td>
<td>Week 7</td>
<td>Week 14</td>
</tr>
<tr>
<td>Myristic (C14:0)</td>
<td>0.89±0.07</td>
<td>0.40±0.18*</td>
<td>0.49±0.07*</td>
</tr>
<tr>
<td>Palmitic (C16:0)</td>
<td>22.36±1.07</td>
<td>21.85±2.47</td>
<td>20.67±0.35</td>
</tr>
<tr>
<td>Palmitoleic (C16:1)</td>
<td>2.05±0.27</td>
<td>0.34±0.08*</td>
<td>0.47±0.11*</td>
</tr>
<tr>
<td>Stearic (C18:0)</td>
<td>16.58±2.28</td>
<td>5.44±1.07*</td>
<td>5.27±0.5*</td>
</tr>
<tr>
<td>Oleic (C18:1)</td>
<td>15.32±1.34</td>
<td>16.91±1.41</td>
<td>21.77±0.64**,***</td>
</tr>
<tr>
<td>Linoleic (C18:2)</td>
<td>20.19±1.42</td>
<td>38.75±1.72*</td>
<td>38.83±0.87*</td>
</tr>
<tr>
<td>γ-Linolenic (C18:3, n-6)</td>
<td>0.0</td>
<td>1.90±0.12*</td>
<td>1.57±0.11**,***</td>
</tr>
<tr>
<td>Δ5-Linolenic (C18:3, n-3)</td>
<td>0.0</td>
<td>0.00±0.02*</td>
<td>0.37±0.06**,***</td>
</tr>
<tr>
<td>Arachidonic (C20:4)</td>
<td>18.54±1.57</td>
<td>7.10±0.74*</td>
<td>10.50±0.43***</td>
</tr>
<tr>
<td>Eicosapentaenoic (C20:5)</td>
<td>1.72±0.48</td>
<td>0.74±0.19*</td>
<td>0.57±0.15*</td>
</tr>
<tr>
<td>Docosapentaenoic (C22:5)</td>
<td>1.12±0.07</td>
<td>1.76±0.21*</td>
<td>1.77±0.29*</td>
</tr>
<tr>
<td>Docosahexaenoic (C22:6)</td>
<td>1.40±0.50</td>
<td>2.20±0.15*</td>
<td>1.73±0.3</td>
</tr>
<tr>
<td>Zat</td>
<td>39.88±2.80</td>
<td>28.93±2.57*</td>
<td>26.42±0.73*</td>
</tr>
<tr>
<td>Σmonounsat</td>
<td>17.29±2.52</td>
<td>18.90±3.78</td>
<td>22.23±0.58*</td>
</tr>
<tr>
<td>Σpolyunsat</td>
<td>42.90±3.13</td>
<td>50.30±1.72*</td>
<td>51.37±0.66*</td>
</tr>
<tr>
<td>Zat/Σunsat</td>
<td>0.66±0.07</td>
<td>0.40±0.08*</td>
<td>0.36±0.01*</td>
</tr>
<tr>
<td>Rate 18:0/18:1</td>
<td>0.74±0.03</td>
<td>0.27±0.05*</td>
<td>0.25±0.04*</td>
</tr>
<tr>
<td>Rate 18:1/18:1</td>
<td>0.13±0.03</td>
<td>0.02±0.005*</td>
<td>0.02±0.005*</td>
</tr>
</tbody>
</table>

sat, saturated fatty acids; monounsat, monounsaturated fatty acids; polyunsat, polyunsaturated fatty acids; unsat, unsaturated fatty acids.

* P < 0.05 versus CTRL group.

** P < 0.05 versus MCD group at 7 weeks.

*** P < 0.05 versus MCD group at 14 weeks.

* P < 0.05 versus MCD plus SILIPHOS group at 7 weeks.

Discussion

Silybin has been shown to exert hepatoprotective and an-
tioxidant effects. It can act as a radical scavenger by increas-
ing the levels of superoxide dismutase and glutathione per-

oxidase and has been reported to reduce malondialdehyde

and 4-hydroxy-2-nonenal in liver (Schumann et al., 2003; Di

Sario et al., 2005; Loguerchio et al., 2007; Polyak et al., 2007;

Trappoliere et al., 2009). However, despite its global use, the

molecular mechanism for the potential therapeutic effects of

silybin is not fully defined. In the present study we demon-

strate that a silybin-phospholipid complex improves mito-

dochondria bioenergetics, prevents uncoupling, and limits ox-

diative stress in a well characterized rodent model of

nonalcoholic steatohepatitis.

We supplemented a MCD diet with a complex of silybin

plus phospholipoids (SILIPHOS), which has demonstrated

higher bioavailability than nonconjugated silybin, with a spe-

specific protective effect on the hepatocyte (Carini et al., 1992;

Morazzoni et al., 1993). The present study provides evidence

that treatment with SILIPHOS preserves mitochondrial

bioenergetics in NASH: in fact, mitochondrial function analy-

sis reveals that respiratory activity is maintained in treated

rats and, more importantly, SILIPHOS prevents hepatic

ATP reduction occurring in NASH. The increase in hepatic

energetic status during NASH is crucial for maintaining

proper liver viability and function. Our data suggest that

silybin protects rat liver during NASH development by pre-

serving mitochondrial RCI and ATP content.

The impairment of mitochondrial function in NASH is

associated with reactive oxygen species (ROS) production

and oxidative stress, pointing out the importance of mito-
.chompochondrial oxidative stress in the development of liver damage

(Hensley et al., 2000; Perez-Carreras et al., 2003; Serviddio

et al., 2008a). Our data show that SILIPHOS reduces hepatic

oleinic, and docosapentaenoic acid, which are more suscepti-

ble to peroxidation (Poli and Schaur, 2000). We also observed

a marked reduction in saturated fatty acids (myristic and

stearic). Treatment with SILIPHOS did not produce any

changes in the lipid modifications induced by the MCD diet

in liver mitochondrial membranes. In addition, in the MCD

group we observed a decrease in

tric acids (n-3) in terms of eicosapentaenoic and docosahexaenoic

acids that was partially prevented by SILIPHOS. Arachi-

donic acid, which exerts anti-inflammatory and metabolic

effects, was also measured and was significantly reduced in

MCD rats but not in the SILIPHOS group compared with

CTRL rats.
GSH oxidation; moreover, it decreases mitochondria H₂O₂ production.

During NASH development, the increase in substrate supply exceeds the energy requirements and increases the mitochondrial redox pressure on the respiratory chain and the rate of ROS production, which in turn produce mitochondrial protein oxidation and HNE release. HNE may act as a positive signal on UCP2 that uncouples substrate oxidation from ATP synthesis, reducing the redox pressure on the respiratory chain and ROS formation, but also decreases ATP content (Serviddio et al., 2008a). The present study shows that SILIPHOS limits mitochondrial proton leak even though, with the progression of the disease, it determines moderate mitochondrial uncoupling. This result together with a progressive decrease in the ADP/O and uncoupled state/state 3 ratio observed in the SILIPHOS group, indicates a significant delay rather than a prevention in the onset of these parameters of mitochondrial function. This result is in good agreement with the effect observed in humans, in whom SILIPHOS has been demonstrated to be effective when administered early in NAFLD development (Loguercio et al., 2007).

SILIPHOS limits the reduction of liver ATP content without any effect on the specific activity of ATPase. Such results can be explained by the reported limitation of the mitochondrial proton leak, which increases the efficiency of oxidative phosphorylation activity in the presence of free fatty acid overload.

Lipid composition of the mitochondrial membranes influences proton leak across the inner mitochondrial membrane (Porter et al., 1996); proton conductance correlates positively with the amount of polyunsaturated fatty acids (Brand et al., 2003). Our data show that the MCD diet influences the lipid composition of mitochondrial membranes, particularly in terms of polyunsaturated fatty acid content. This augmentation is mostly dependent on the increased amount of linoleic,
induced by the MCD diet, conferring anti-inflammatory and antifibrotic effects. Although SILIPHOS does not reduce the susceptibility to peroxidation of lipid membranes, its protective effects on mitochondrial function limit the oxidative damage observed in NASH. Hence, this study encourages further preclinical and clinical studies using SILIPHOS to prove its efficacy in the early treatment of NASH.

Acknowledgments

We thank Nazzareno Capitanio for valuable suggestions and Esther De Simone for technical assistance.

References


Esterbauer H, Schaur RJ, and Zollner H (1991) Chemistry and biochemistry of highly reactive and may be considered as second toxic mes-

Fig. 6. Representative Oxyblot analysis of liver homogenates from CTRL, MCD (NASH), and MCD plus SILIPHOS (SILI) rats at 7 and 14 weeks (w).

linolenic, and docosapentaenoic acids, which have been asso-

ciated with increased mitochondrial proton conductance (Brand et al., 2003). However, the composition of the MCD diet, which is rich in polyunsaturated fatty acids, may influence the composition of membranes (Lee et al., 2007).

The increase in polyunsaturated acids (especially docosa-

References


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