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Mitochondrial Complex I Subunits Are Decreased in Murine Nonalcoholic Fatty Liver Disease: Implication of Peroxynitrite

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We investigate the cause of the low activity of mitochondrial complex I found in ob/ob mice with nonalcoholic fatty liver disease. In mitochondrial proteins from ob/ob mice, we assessed complex I activity, fully assembled complex I, and its subunits, oxygen consumption, gene expression of complex I subunits, and oxidative damage to DNA. In mitochondrial proteins from the liver of ob/ob mice, complex I activity, fully assembly of this complex and complex I subunits were markedly reduced. Likewise, gene expression of mitochondrial DNA-encoded subunits was significantly decreased in obese mice, but not nuclear DNA-encoded subunits. Treatment of obese mice with uric acid, anti-TNFα antibody or a mimic of manganese superoxide dismutase normalized all these abnormalities. "In vitro" addition of peroxynitrite to mitochondrial proteins from wild-type mice reproduced the abnormalities found in ob/ob mice (decreased complex I activity, the amount of fully assembled complex I, and its subunits, and mitochondrial oxygen consumption). Low activity of complex I found in ob/ob mice can be ascribed to a reduced amount of fully assembled complex, which may be attributed to degradation and reduced synthesis of its subunits by peroxynitrite. Exposure of mitochondrial proteins from normal mice to peroxynitrite reproduced the proteomic abnormalities present in ob/ob mice.

Keywords: Mitochondrial Respiratory Chain • Prohibitin • Tyrosine-nitrated proteins • Nitric oxide • Oxidative phosphorylation

Introduction

Nonalcoholic fatty liver disease (NAFLD) represents a spectrum of liver disease extending from pure fatty liver through nonalcoholic steatohepatitis (NASH) to cirrhosis that occurs in patients who do not consume significant amounts of alcohol.[1] NAFLD is likely the most frequent histological finding in patients with abnormal liver function tests in the Western World States.[2] Although the pathogenesis of NAFLD remains undefined, some investigators have proposed a "double hit" theory in the development of NASH. While the "first hit" involves the accumulation of fat in the liver, the "second hit" includes oxidative stress resulting in lipid peroxidation, stellate cell activation, and fibrogenesis.[3] Mitochondrial dysfunction might play a crucial role in the induction of both "hits", because mitochondria are involved in the β-oxidation of free fatty acids and are the most important cellular source of reactive oxygen species (ROS).[4]

In a previous study, we have reported that activity of the mitochondrial respiratory chain (MRC) is decreased in liver tissue of patients with NASH.[5] More recently, we found the same dysfunction in the liver of ob/ob mice with NAFLD and suggested that this mitochondrial defect may be due to the 3-tyrosine nitration of mitochondrial proteins by the peroxynitrite anion. We found that treatment of these animals with uric acid, a peroxynitrite scavenger, decreased 3-tyrosine nitrated proteins and improved the activity of MRC complexes. In this study, we proposed that increased peroxynitrite formation may be due to an elevated tumor necrosis factor alpha (TNFα) and inducible nitric oxide synthase (iNOS) activity in the liver. Thus, TNFα levels, iNOS protein expression, and 3-tyrosine nitrated proteins were markedly increased in the liver of ob/ob mice and treatment of these animals with anti-TNFα antibody has similar effects as those obtained with uric acid, including the decreased 3-tyrosine nitrated mitochondrial proteins and the resolution of liver lesions.[6]

Complex I (NADH:ubiquinone oxidoreductase) is the first and the largest of the five multiprotein complexes that constitute the MRC involved in the oxidative phosphorylation.[7−9] This complex is formed by at least 45 subunits, of which seven are encoded by the mitochondrial genome and the remaining 38 by the nuclear genome.[8,10,11] Defects in complex I have been implicated in a number of human diseases, including Leber's hereditary optic neuropathy, mitochondrial encephalopathy lactic acidosis stroke-like episodes, and Leigh syndrome. However, structural organization of this complex has never been reported in NAFLD. In this study, we analyzed the

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complex I activity in the liver from wild-type and ob/ob mice and investigated gene expression and amount of complex I subunits in liver mitochondria from these animals. In addition, we examined the effect of treatment of obese mice with uric acid, anti-TNFα, or manganese(III) tetrakis (5,10,15,20 benzoic acid) porphyrin (MnTBAP) on the activity and assembly/stability process of complex I. Finally, we studied the "in vitro" effects of peroxynitrite on mitochondrial oxygen consumption and complex I activity and conformation. We found that complex I activity, fully assembly of this complex, complex I subunits, and mitochondrial DNA-encoded subunits were markedly reduced in mitochondrial proteins from ob/ob mice. Treatment of obese mice with uric acid, anti-TNFα antibody, or MnTBAP normalized all these abnormalities. "In vitro" addition of peroxynitrite to mitochondrial proteins from wild-type mice reproduced the abnormalities found in ob/ob mice.

Material and Methods

All procedures were carried out in accordance with the Spanish Guidelines for the Care and Use of Laboratory Animals. The 6-week-old male C57BL/6J wild-type mice and the 6-week-old obese C57BL/6J $Lep^{(-/-)}$ male mice (ob/ob) were purchased from Charles River Laboratory (Charles River Laboratories España, SA, Santa Perpetua de la Mogada, Spain). Animals were housed at constant room temperature (23 °C) (3 mice per cage) under 12 h light/dark cycles with *ad libitum* access to water and standard laboratory mouse chow. Forty-six mice were distributed in six groups: group I (wild-type) included 12 C57BL/6J mice. Group II (ob/ob) was formed by 16 obese ob/ob mice treated with 200 μL of 0.8% saline solution. Group III (MnTBAP) was composed of six ob/ob mice treated with 10 mg/(kg/day) of MnTBAP (Calbiochem, San Diego, CA), a mimic of manganese superoxide dismutase. Group IV (IgG1) was formed by six ob/ob mice treated with 10 mg/kg IgG1 immunoglobulin (Sigma-Aldrich Quimica SA, Tres Cantos, Spain) three times per week (tiw). This group of animals was used as control of anti-TNFα treated mice. Group V (anti-TNF) consisted of six ob/ob mice treated with 10 mg/(kg/tiw) anti-TNFα antibody (*Remicade*, Schering Plough, Leiden, Holland). Group VI consisted of six ob/ob mice treated with 20 mg/day uric acid (Sigma-Aldrich Quimica SA, Tres Cantos, Spain). Uric acid was used as a suspension of 20 mg in 200 μL of 0.8% saline solution. Saline, MnTBAP, IgG1 immunoglobulin, and anti-TNFα solutions and UA suspension were administered intraperitoneally (i.p.) for 12 weeks. After treatment, animals were anesthetized and killed and the liver was rapidly harvested for further analysis. A portion of the liver tissue was placed in a 10% formaldehyde solution and routinely processed for histological assessment.

In-Gel Activity Assays. To measure mitochondrial complex I in-gel activity, MRC complexes were separated using one-dimensional blue native polyacrylamide gel electrophoresis (BN-PAGE) as described by Schägger[12] and modified by Nijtmans et al.[13]

MRC Activity Assays. Frozen liver tissues (50−70 mg) were homogenized with 15 vol of 20 mmol/L KP buffer, pH 7.4, and centrifuged at 800*g* for 10 min. The activity of complex I of the mitochondrial respiratory chain and citrate synthase (CS) was measured using a DU-650 spectrophotometer (Beckman Instruments, Palo Alto, CA) as described elsewhere.[5] Activities were expressed as nanomoles of substrate used per minute per milligram of protein. Enzyme assays were performed in triplicate.

Measurement of Oxygen Consumption. Mitochondrial respiration was measured polarographically at 37 °C with a computer-controlled Clark-type electrode (Oxygraph, Hansatech Instruments Ltd., Norfolk, U.K.) as previously described.[14] The respiratory control index was calculated as state 3/state 4 oxygen consumption ratio.

Assessment of Full Assembly of MRC Complexes. Mitochondrial proteins were isolated using one-dimensional BN-PAGE as described above. Prohibitin complex (molecular weight, 1.2 MDa) was separated using a gradient gel of 3−12% acrylamide (NativePAGE 3−12% Bis-Tris Gel, Invitrogen, Carlsbad, CA). Following electrophoresis, proteins were transferred to a polyvinyl difluoride membrane (0.45-μm pore size) (Immobilon-P transfer membrane, Millipore Co., Bedford, MA) according to Triepels.[15] Western blotting of these proteins was performed using primary antibodies against complex I subunit 39 (NDUFA9), complex II subunit 70 (SDHA), complex III subunit core 2 (UQCRC2), and complex IV subunit COX I (MTCO1) on blocking buffer for 2 h (Molecular Probes, Inc., Eugene, OR). After washing, blot was incubated for 1 h with peroxidase-conjugated anti-mouse antibody as a secondary antibody, prepared at a 1:5000 dilution (Molecular Probes, Inc., Eugene, OR). Immunoreactive material was visualized by chemiluminescence (ECL, Western Blotting Detection, GE Healthcare, Madrid, Spain) according to the manufacturer's instructions. The blot was finally exposed to Hyperfilm MP (Amersham, GE Healthcare, Madrid, Spain). ECL signals were quantified using the ImageJ image analysis software.[16]

Second-Dimension Electrophoresis for Assessing Complex Subunits. For second-dimension BN/SDS-PAGE, lane containing mitochondrial complexes was excised from the one-dimension gel, placed on a glass plate, and incubated with a dissociating solution containing 1% SDS and 1% 2-mercaptoethanol, for 1 h at room temperature. After removal of the dissociating solution, a second glass plate was assembled and the second-dimension SDS−polyacrylamide gel was poured between both glass plates and below the first-dimension strip. After polymerization, the stacking gel was poured around the strip and between both gels. Mitochondrial proteins from wild-type mice and ob/ob mice were run simultaneously and in the same condition in the second dimension. Following electrophoresis, proteins were transferred to a polyvinyl difluoride membrane. Western blotting was performed using primary antibodies against complex I subunits 39 kDa (NDUFA9), 30 kDa (NDUFS3), 17 kDa (NDUFB6), 15 kDa (NDUFA6), complex II subunit 70 kDa (SDHA), complex IV subunit COX I (MTCO1), complex V subunit alpha (ATP5A1) (Molecular Probes, Inc., Eugene, OR), ND1, ND2, ND4, and ND4L (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) on blocking buffer for 2 h. After washing, blots were treated as indicated above.

Measurement of 8-Hydroxy-2′-deoxyguanosine (8-OHdG) in Nuclear and Mitochondrial DNA. Nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) were isolated from 100 mg of liver using genomic and mitochondrial DNA isolation kits according to the manufacturer's protocol (BioVision Research Products, Mountain View, CA). Oxidative damage to nDNA and mtDNA was determined by measuring 8-OHdG using a competitive enzyme immune assay following the manufacturer's indications (8-Hydroxy-2-deoxy-guanosine EIA Kit, Cayman Chemical Co., Ann Arbor, MI).

Quantitative Real-Time Polymerase Chain Reaction. Total RNA was extracted from the liver of control and ob/ob mice using the TRI-Reagent (Sigma-Aldrich, Steinheim, Germany)

Table 1. Primers Used in Quantitative Real-Time PCR

NDUFA9	Sense:	5′-CAT TAC TGC AGA GCC ACT-3′
	Antisense:	5′-ATC AGA CGA AGG TGC ATG AT-3′
NDUFB6	Sense:	5′-ATA ACT TTT TGC GGG ACG GG-3′
	Antisense:	5′-CAG GAA AAT CTC TCA TTG GTG-3′
NDUFS3	Sense:	5′-AGG AAC ATG GCG GCG GCT GC-3′
	Antisense:	5′-ATT TCA GCC ACA TAC TCT CC-3′
ND1	Sense:	5′- TGC ACC TAC CCT ATC ACT C-3′
	Antisense:	5′- ATT GTT TGG GCT ACG GCT C-3′
ND2	Sense:	5′- ATG AGT AGG CCT GGA ATT C-3′
	Antisense:	5′-ATC AGA AGT GGA ATG GGG C-3′
ND4	Sense:	5′-ATA ATT ATA ACT AGC TCA ATC TGC-3′
	Antisense:	5′-TCG TAG TTG GAG TTT GCT AG-3′
ND4L	Sense:	5′-CTC ACC ATA GCC TTC TCA C-3′
	Antisense:	5′-CGT AAT CTG TTC CGT ACG TG-3′
ND6	Sense:	5′-TGT ATG AGG TTG ATG ATG TTG G-3′
	Antisense:	5′-CCG CAA ACA AAG ATC ACC C-3′
SDHA	Sense:	5′-CAT ACT GTT GCA GCA CAG G-3′
	Antisense	5′-CC ACC AAA TGC ACG CTG ATA-3′
β-Actin	Sense:	5′-ATG GAT GAC GAT ATC GCT G-3′
	Antisense:	5′- GTT GGT AAC AAT GCC ATG TTC-3′

according to the manufacturer's instructions. RNA was treated with DNase I to remove DNA contamination (Sigma-Aldrich, Steinheim, Germany). cDNA was generated from 1 μg of sample RNA using First Strand cDNA Synthesis Kit for RT-PCR (Roche Applied Science, Indianapolis, IN) at 25 °C, 5 min; 42 °C, 60 min; 95 °C, 5 min, and 4 °C, 5 min. Quantitative real-time PCR was performed on a Light Cycler 1.0 (Roche Applied Science) in 20 μL with 50 ng of cDNA, 0.5 μM primers, and 2 μL of FastStart DNA Master SYBR Green I (Roche Applied Science, Mannheim, Germany). Data from the real-time, quantitative PCR were analyzed following the $2^{-\Delta\Delta C}{}_T$ method as described by Livak et al.[17] Sequence of primers used in these experiments are shown in Table 1. Expression of complex I subunits was normalized to that corresponding β-actin. The amplification conditions were 45 cycles of denaturation at 95 °C for 10 s, annealing at 57 °C for 5 s, and extension at 72 °C for 20 s.[18] The correct size and purity of the amplified products was verified by agarose gel electrophoresis.

Peroxynitrite Modification of Mitochondrial Proteins. For peroxynitrite modification of mitochondrial proteins, we followed the procedure described by Murray et al.[19] Briefly, peroxynitrite (110 mM) and degraded peroxynitrite (Upstate Biotechnology, Lake Placid, NY) were diluted in 0.3 M sodium hydroxide. The concentration of peroxynitrite was determined by titration measuring absorbance at 302 nm (extinction coefficient 1670 M^{-1} cm^{-1}). Mitochondria at 5 mg/mL in 25 mM potassium phosphate, pH 7.2, 5 mM $MgCl_2$ were placed on ice. Fifty micromoles peroxynitrite was deposited on the side of the tube above the mitochondria; vortexing results in rapid mixing of the mitochondrial membranes and peroxynitrite. After a few seconds, pH of the peroxynitrite solution decays in the neutral pH of the buffer. This procedure was repeated a number of times until exposing mitochondrial proteins to a total of 50−2800 μM peroxynitrite. 3-Tyrosine nitration of mitochondrial proteins was checked by Western blot using anti-3-nitrotyrosine antibody.

Statistical Analysis. Statistical analysis was aided by the SPSS Statistical Software for Windows, version 9 (SPSS, Inc., Chicago, IL). The unpaired *t* test was used to assess the significance of differences between means. All results were expressed as mean ± SD. *P*-values <0.05 were considered significant.

Results

1. Histology. As compared with the liver of lean mice (Supporting Figure A), the liver of ob/ob mice showed a marked accumulation of fat droplets in approximately 60% of hepatocytes (Supporting Figure B−D). Four of these mice exhibited grade 1 (5−33%) steatosis, 5 mice had grade 2 (33−66%) steatosis, and the remaining 7 mice had grade 3 (>66%) steatosis. In 15% of hepatocytes, fat was seen mainly as macrovesicular droplets, whereas, in the remaining 45% of hepatocytes, fat caused microvesicular steatosis (Supporting Figure B and C). Biochemical analysis of hepatic triglyceride content revealed that the amount of triglyceride was significantly increased in ob/ob mice (lean mice, 8.63 ± 4.2 mg/g liver; ob/ob mice, 62.7 ± 11.6 mg/g liver ($P < 0.001$). Focal areas of ballooning degeneration and collections of mononuclear cells and neutrophils were also present in the parenchyma (Supporting figure D). Treatment of ob/ob mice with MnTBAP (Supporting figure E and F), uric acid (Supporting figure G and H), or anti-TNFα (Supporting figure I and J) for 3 months led to an almost resolution of hepatic steatosis and inflammation. Triglyceride concentrations in liver tissue in ob/ob mice treated with MnTBAP, uric acid, or anti-TNFα were 22.1 ± 7.4 ($p <$ 0.01 vs wild-type and $p < 0.001$ vs untreated ob/ob mice), 23.6 ± 4.2 ($0 < 0.001$ vs wild-type and untreated ob/ob mice), and 13.5 ± 6.8 mg/g liver (NS vs wild-type mice; $p < 0.001$ vs untreated ob/ob mice), respectively.

1.1. Activity of Complex I Is Reduced in ob/ob Mice. As Figure 1A shows, liver mitochondria from ob/ob mice displayed a strikingly reduced complex I in-gel activity (lane ob) when compared with control levels (lane C57). However, activity of this complex returned to control levels when ob/ob animals were treated for 12 weeks with 20 mg/(kg/day) uric acid, 10 mg/(kg/day) MnTBAP, or 10 mg/(kg/tiw) anti-TNF, but not with 10 mg/(kg/tiw) unspecific IgG1.

We also measured the activity of the complex I in the liver tissue from wild-type and ob/ob mice by a spectrophotometric method. The activity of this complex was decreased from 53.1 ± 2.1 [(nmol·min^{-1}·mg protein^{-1}/nmol·min^{-1}·mg protein^{-1}CS) × 100] (complex I/CS) in wild-type mice to 31.6 ± 2.3 complex I/CS ($p < 0.001$) (59.5 ± 5.2%) in ob/ob mice (Figure 1B). Specific activities of CS were 1193 ± 259 nmol/(min/mg protein) for wild-type mice and 1163 ± 233 nmol/(min/mg protein) for ob/ob mice, indicating no proliferation of mitochondria in ob/ob mice. There were no significant differences between both values. Treatment of ob/ob mice with uric acid, MnTBAP, or with anti-TNF antibody, i.p. for 12 weeks normalized the activity of MRC complex I (Figure 1B).

1.2. Fully Assembled Complex I Is Decreased in the Liver from ob/ob Mice. First-dimension BN-PAGE system illustrates that fully assembled complex I was markedly diminished in ob/ob mice as compared with wild-type mice, which agrees with the low complex I activity found in these obese mice (Figure 1C). Likewise, the amount of fully assembled complexes II, III, and IV was also reduced. However, treatment of ob/ob mice with uric acid, MnTBAP, or anti-TNFα antibody, i.p. for 12 weeks normalized the amount of these complexes in mitochondrial preparations of these animals. Treatment of ob/ob mice with IgG1 immunoglobulin, as control of anti-TNFα-treated mice, did not cause any significant effect on the amount of fully assembled complex I.

1.3. Amount of Complex I Subunits Is Markedly Reduced in ob/ob Mice. To study in more detail how assembly or

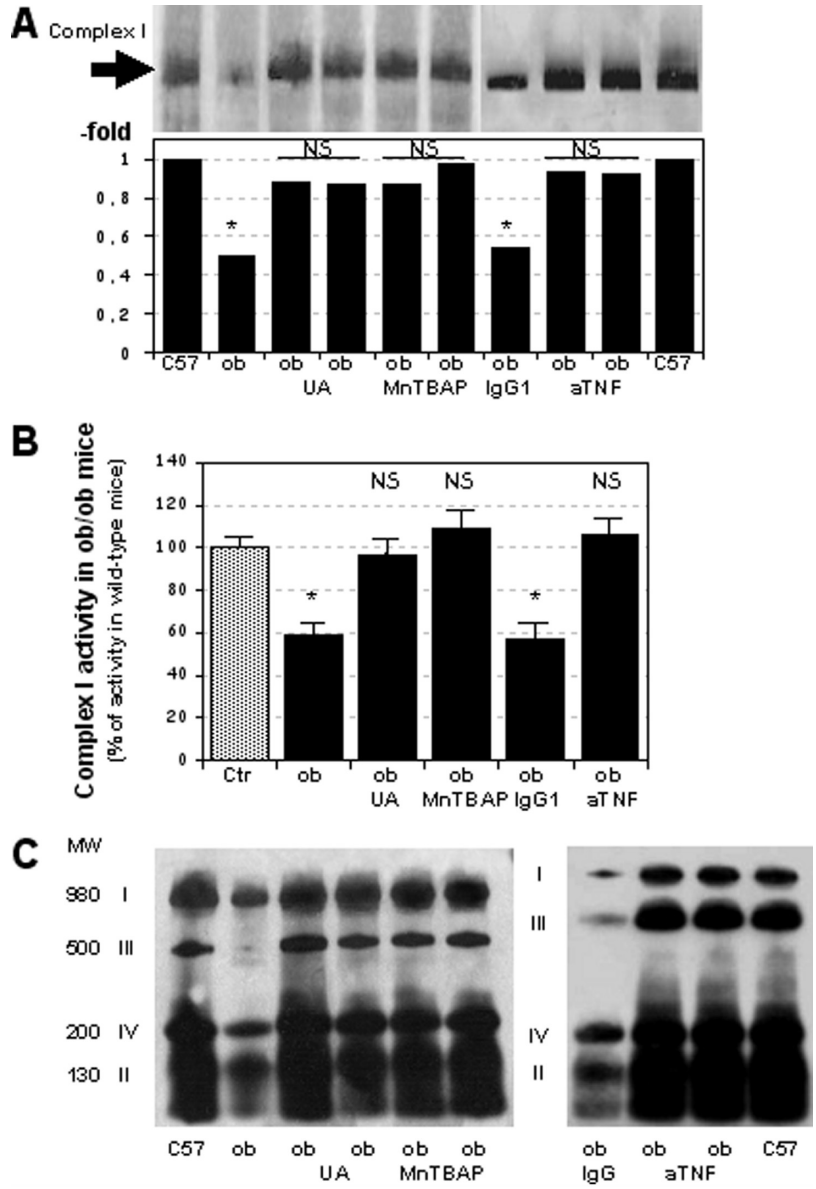

Figure 1. Activity and fully assembled mitochondrial complex I are decreased in ob/ob mice. (a) Complex I in-gel activity is decreased in ob/ob mice. Mitochondrial proteins were separated on a 5−15% blue native-polyacrylamide gel and complex I activity was measured by incubating this gel with a solution containing nitrotetrazolium blue. Lanes C57, control mice; lane ob, ob/ob mice treated with saline solution; lanes ob/UA, ob/ob mice treated with uric acid; lanes ob/MnTBAP, ob/ob mice treated with MnTBAP; lane ob/IgG1, ob/ob mice treated with IgG1 immunoglobulin; lanes ob αTNF, ob/ob mice treated with anti-TNF antibody. Saline, MnTBAP, IgG1 immunoglobulin, and anti-TNF solutions and UA suspension were administered intraperitoneally for 12 weeks. (B) Activity of the mitochondrial complex I in the liver from ob/ob mice. Enzyme activity of the complex I was measured by spectrophotometry as described in Material and Methods in the liver from wild-type and ob/ob mice, as well as in ob/ob mice treated with uric acid, MnTBAP, or anti-TNFα as indicated in panel A. Results are expressed as percentage of activity in wild-type mice. $^{**}p < 0.01$; $^{***}p < 0.001$. (C) BN-PAGE analysis of mitochondrial respiratory chain complexes in wild-type and ob/ob mice. Mitochondrial proteins were analyzed as described in Material and Methods. Western blot analysis was performed using antibody against complex I subunit NDUFA9, complex II subunit 70, complex III subunit core 2 protein, and complex IV subunit COX 1. Lanes C57, ob, ob/UA, ob/MnTBAP, ob/IgG1, and ob/αTNF represent experimental condition as indicate in panel A.

stability of complex I was affected, this complex was resolved by second-dimension SDS-PAGE. Complex I nDNA-encoded subunits 39, 30, 17, and 15 kDa, and mtDNA-encoded subunits ND1, ND2, ND4, and ND4L were detected using specific antibodies. Using this procedure, the most striking finding was the decrease in the amount of all complex I subunits in ob/ob mice (Figure 2A). This reduction was particularly marked in mtDNA-encoded subunits. Thus, in ob/ob mice, while the amount of nDNA-encoded subunits was decreased to 56 ± 10% of control values, mtDNA-encoded subunits were reduced to only 17 ± 1% of the amount found in control mice ($P < 0.01$). Likewise, nDNA-encoded subunits 70 kDa (complex II), Core-1 (complex III), and alpha (complex V) were decreased to about 50% in obese mice. On the contrary, mtDNA-encoded COX-I subunit (Complex IV) was decreased to 18% in these animals (Figure 2A). In none of the subunits tested, an accumulation of low-molecular-weight subcomplexes was recognized, suggesting that impaired assembly of complex I was not the cause of the reduced amount of this complex found in ob/ob mice. Treatment of ob/ob mice with uric acid, MnTBAP, or anti-TNFα

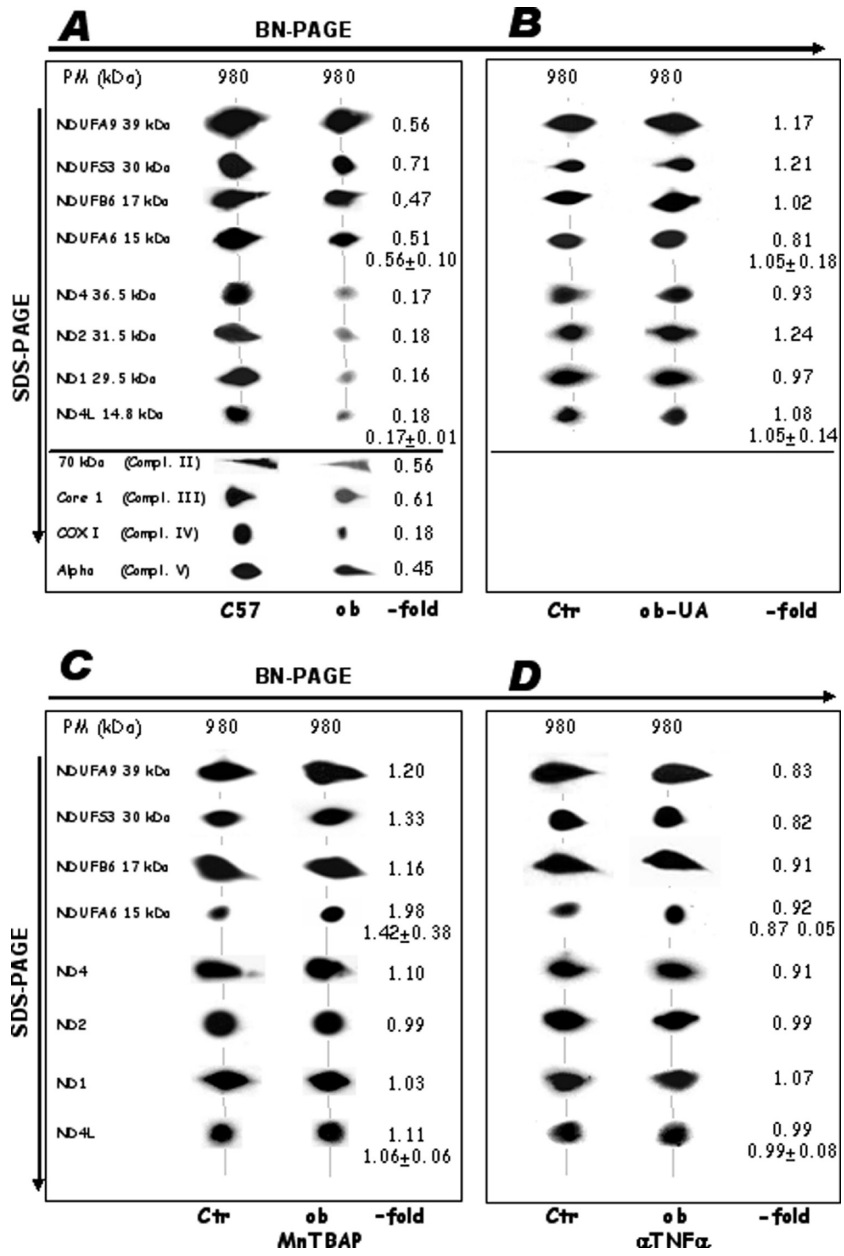

Figure 2. Identification of mitochondrial complex I subunits in wild-type and ob/ob mice. Mitochondrial proteins (30 μg) extracted from wild-type and ob/ob mice were separated in the first dimension using BN-PAGE and in the second dimension using SDS-PAGE. Gels containing proteins from control and ob/ob mice were run in parallel in the same conditions. The presence of individual complex I subunits was identified by immunoblotting using antibodies against the complex I subunits 39-kDa (NDUFA9), 30-kDa (NDUFS3), 17-kDa (NDUFB6), and 15-kDa (NDUFA6), ND4 36.5 kDa, ND2 31.5 kDa, ND1 29.5 kDa, and ND4L 14.8 kDa. Likewise, subunits 70 kDa (complex II), Core 1 (complex III), COX-I (complex IV), and alpha (complex V) were identified using appropriated antibodies. Vertical lines indicate the positions of complex I (980 kDa) subunits. (A) Wild-type and ob/ob mice treated with 200 μL 0.8% saline solution; (B) wild-type and uric acid (UA)-treated ob/ob mice; (C) wild-type mice and ob/ob mice treated with MnTBAP; (D) wild-type mice and ob/ob mice treated with anti-TNFα antibody. Saline, MnTBAP, and anti-TNFα solutions and UA suspension were administered intraperitoneally for 12 weeks. Fold, amount of subunit in ob/ob mice divided by the amount of the same subunit in lean mice.

for 12 weeks increased the protein content of all complex I subunits, frequently over control levels (Figure 2B−D).

1.4. Oxidative Damage to mtDNA. The 8-OHdG content in nDNA was identical in ob/ob and control mice (36.32 ± 1.1 vs 36.2 ± 1.2 pg/(mL/μg DNA)). However, as Figure 3A shows, 8-OHdG was significantly increased in mtDNA isolated from both groups of mice compared with the content found in nDNA (control, 71.0 ± 4.1; ob/ob, 204.0 ± 45.0 pg/(mL/μg DNA), $p <$ 0.001). This increase was particularly prominent in mtDNA obtained from ob/ob mice ($p < 0.001$).

1.5. Mitochondrial Gene Transcription Is Decreased in the Liver from ob/ob Mice. To determinate gene expression of complex I subunits, we examined the steady-state levels of nDNA-encoded (*NDUFA9*, *NDUFB6*, *NDUFS3*) mRNA and mtDNA-encoded (*ND1*, *ND2*, *ND4*, *ND4L*, *ND6*) mRNA in the liver from control and ob/ob mice. In addition, we also measured gene expression of complex II subunit 70 (SDHA), an nDNA-encoded protein. This study revealed that gene expression of nDNA-encoded subunits was similar in ob/ob and wild-type mice (Figure 3B), whereas expression of mtDNA-

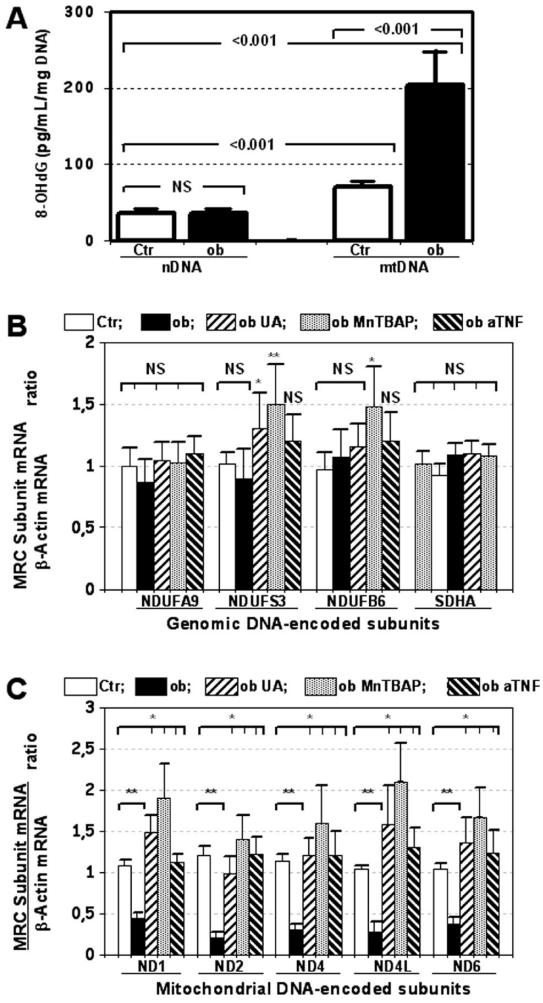

Figure 3. Mitochondrial DNA is oxidized and mitochondrial gene expression is decreased in ob/ob mice. (A) 8-Hydroxy-2′-deoxyguanosine is increased in mitochondrial DNA from ob/ob mice. 8-Hydroxy-2′-deoxyguanosine (8-OHdG) content was measured in nuclear (nDNA) and mitochondrial DNA (mtDNA) from 10 wild-type and 12 ob/ob mice as described in Material and Methods. (B) Nuclear gene expression of representative complex I subunits. mRNA of complex I subunits and complex II subunit 70 kDa was analyzed by quantitative real-time PCR as described in Material and Methods. (C) Mitochondrial gene expression of representative complex I subunits. The subunit/β-actin mRNA ratio was measured in the liver from five control (Ctr) and six ob/ob (ob) mice. *$p < 0.01$ as compared with control mice. NS, not significant.

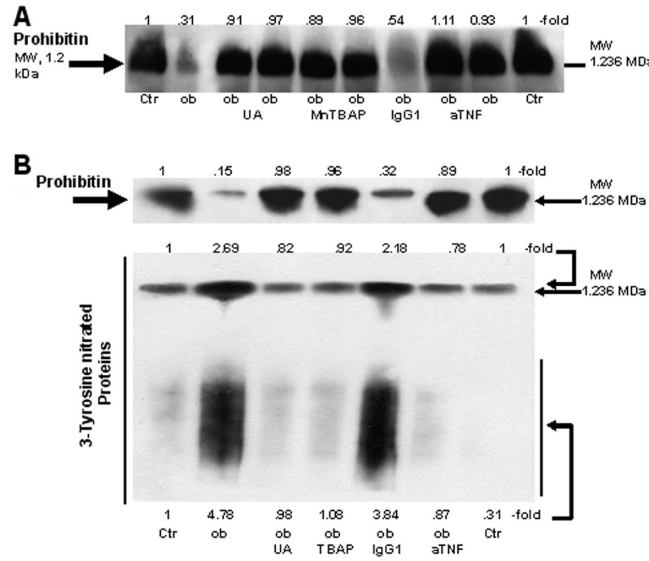

Figure 4. Prohibitin is decreased and 3-tyrosine nitrated proteins are increased in liver mitochondria from ob/ob mice. (A) Identification of prohibitin in liver mitochondria from control and ob/ob mice. Mitochondria were isolated from the liver of wild-type and ob/ob mice and 30 μg of mitochondrial proteins was analyzed as described in Material and Methods. Western blot analysis was performed using antibody against prohibitin. Lanes Ctr, control, lean mice; lane ob, ob/ob mice treated with 200 μL of 0.8% saline solution; lanes ob/UA, ob/ob mice treated with 20 mg/day uric acid; lanes ob/MnTBAP, ob/ob mice treated with 10 mg/(kg/day) MnTBAP; lane ob/IgG1, ob/ob mice treated with 10 mg/(kg/tiw) IgG1 immunoglobulin; lanes ob/TNFα, ob/ob mice treated with 10 mg/(kg/tiw) anti-TNF antibody. Saline, MnTBAP, IgG1 immunoglobulin, and anti-TNFα solutions and UA suspension were administered intraperitoneally for 12 weeks. (B) 3-Tyrosine nitrated proteins in liver mitochondria from wild-type and ob/ob mice. Membrane was first probed with prohibitin antibody, and after removing this antibody, it was probed with specific antibody against 3-nitrotyrosine. Lanes as indicated in panel A.

encoded subunits was reduced to 37 ± 9% in the obese animals. Treatment of ob/ob mice with uric acid, MnTBAP, or anti-TNFα for 12 weeks increased gene expression of mtDNA encoded subunits significantly, occasionally over control levels (Figure 3C).

1.6. Prohibitin Levels Are Reduced in Mitochondrial Membrane from ob/ob Mice. Since prohibitin is a protein complex that protects mitochondrial complexes from degradation, we investigated whether this protein is decreased in mitochondria from obese mice. As Figure 4 shows, immunoreactive prohibitin was clearly decreased in protein extracts obtained from ob/ob mice. However, the amount of this protein returned to the control level in animals treated with uric acid, MnTBAP, or anti-TNFα antibody. To know whether this protein is 3-tyrosine nitrated, we blotted the same membrane with anti-3-nitrotyrosine antibody and demonstrated that prohibitin was nitrated in all groups of animals, but in ob/ob mice and in IgG1 treated obese mice, this nitration was particularly marked. Moreover, in untreated ob/ob mice and in mice treated with IgG1, a long trace of lower molecular weight tyrosine nitrated proteins was identified (Figure 4B).

1.7. Peroxynitrite Reproduces Complex I Defects Found in ob/ob Mice. "In vitro" incubation of 30 μg of mitochondrial proteins from wild-type mice with 0−2800 μM peroxynitrite induced 3-tyrosine nitration of mitochondrial proteins (Figure 5A), and decreased CS (Figure 5B) and complex I enzymatic activity (Figure 5C) in a dose-dependent manner. As this anion also inhibited CS activity (Figure 5B), we normalized complex I activity by the activity of CS prior to the addition of peroxynitrite. Likewise, complex I in-gel activity decreased to 93%, 75%, and 5% of basal levels when mitochondrial proteins were exposed to 800, 1600, or 2800 μM peroxynitrite, respectively (Figure 5D). Finally, Figure 5E shows that increasing dose of peroxynitrite resulted in a progressive decrease in mitochondrial oxygen consumption.

To analyze the effects of peroxynitrite on complex I assembly, mitochondrial proteins from wild-type mice incubated with 0−400 μM peroxynitrite were separated by BN-PAGE. Figure 6A shows that incubation of mitochondrial proteins with this anion resulted in the reduction of the amount of fully as-

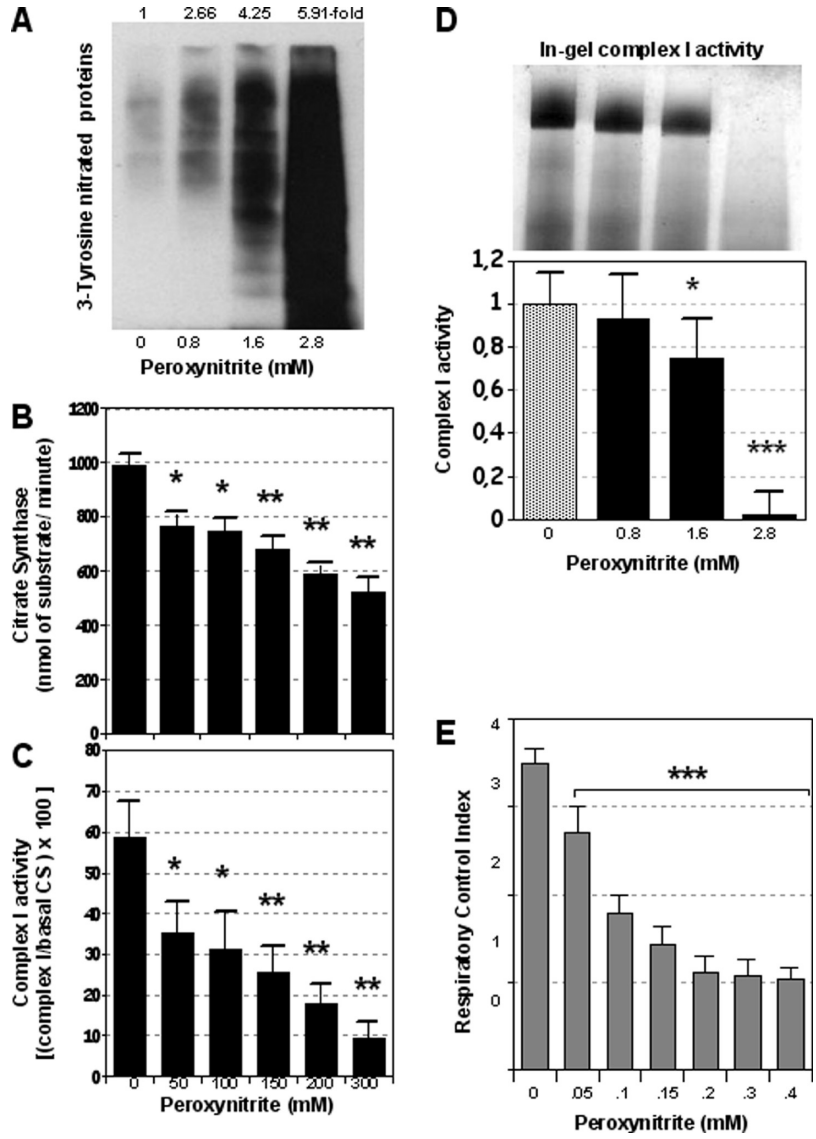

Figure 5. "In vitro" effects of peroxynitrite on enzymatic activity of complex I of the mitochondrial respiratory chain. (A) Mitochondrial proteins (25 μg) extracted from lean mice were exposed to 800−2800 μM peroxynitrite as described in Material and Methods. 3-Tyrosine nitrated proteins were analyzed by Western blotting using anti-3-nitrotyrosine antibody. (B) Mitochondrial protein from wild-type mice were treated with 50−300 μM peroxynitrite and citrate synthase (B) and complex I (C) activities were measured by spectrophotometry. Complex I activity was normalized to the citrate synthase (CS) activity in mitochondrial preparation prior to peroxynitrite addition. (D) Effect of 800−2800 μM peroxynitrite on in-gel complex I activity. (E) Mitochondria isolated from wild-type mice were treated with 50−400 μM peroxynitrite and oxygen consumption was measured using a Clark-type electrode. Data are expressed as respiratory control index, calculated as the state 3/state 4 oxygen consumption ratio.

sembled complex I in a dose-dependent manner and in the formation of a second band close to the main band, which intensity increased progressively with the dose of peroxynitrite. These bands were followed by a lower molecular weight trace extended until the electrophoretic front line. To determine the effect of peroxynitrite on subunit composition of the complex I, we analyzed subunits 39, 30, 17, 15 kDa, ND1, ND2, ND4, and ND4L using two-dimensional BN/SDS-PAGE in mitochondrial proteins from wild-type mice exposed to 300 μM peroxynitrite. Using this method, we confirmed that peroxynitrite decreased the amount of individual complex I subunits, some of them to an almost undetectable spot. Moreover, the main spot caused by 39 kDa subunit displayed a lower molecular weight than 39 kDa subunit from untreated proteins and was followed by a long tail extended from about 980 kDa to the electrophoretic front line (Figure 6B).

Discussion

Assessment of complex I activity of the MRC in the liver from ob/ob mice using in-gel activity assay and spectrophotometry demonstrates that this activity is markedly reduced in these animals as compared with control mice (Figure 1). These results concur with those we previously found in NASH patients and in ob/ob mice when this activity was measured by spectrophotometry.[5,6] As in our previous study, treatment of obese animals with antiTNFα antibody, MnTBAP, or uric acid normalized complex I activity supporting the view that liver TNFα, oxidative stress, and peroxynitrite radicals may play a key role in the inactivation of this enzymatic complex. In the present study, we also show that fully assembled complexes I, II, III, and IV are decreased in liver mitochondria from ob/ob mice when

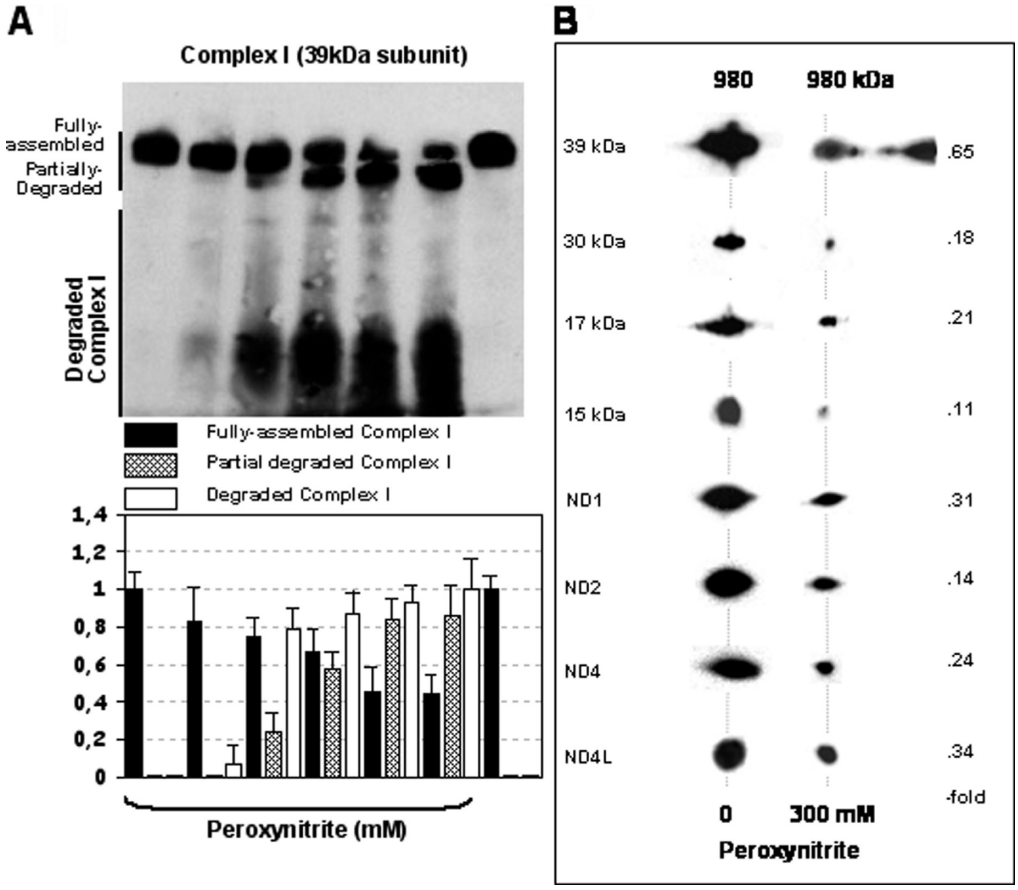

Figure 6. Peroxynitrite degrades and decreases the amount of fully assembled complex I of the mitochondrial respiratory chain in lean mice. (A) Liver mitochondria (30 μg of protein) isolated from wild-type mice were incubated with 0, 50, 100, 200, 300, 400 μM peroxynitrite or 400 μM degraded peroxynitrite (DPN) and analyzed by one-dimensional BN-PAGE. Membrane was probed with antibody against 39 kDa complex I subunit. (B) The lane corresponding to the sample exposed to 300 μM peroxynitrite was cut out of the first-dimension gel and the strip was analyzed in a second-dimension BN-SDS-PAGE. Western blot analysis was performed using specific antibodies against complex I subunit 39, 30, 17, and 15 kDa, as well as against ND1, ND2, ND4, and ND4L.

compared to wild-type mice, which is in keeping with the low activity of these complexes found in these animals.[6]

Using two-dimensional BN/SDS-PAGE, we demonstrated that the amount of all complex I subunits tested were strikingly decreased in liver mitochondria from ob/ob mice. This decrease was particularly marked in mtDNA-encoded subunits (Figure 2A).

The low amount of complex I subunits may be ascribed to a reduced synthesis of the subunits of this complex, to a defect in its assembly/stability, or to a combination of both, that is, to a defect in the synthesis combined with a reduced stability of these polypeptides. Complex I of the MRC is composed of 45 different peptides, 38 of them are encoded by the nDNA and the remaining seven by the mtDNA. Our study clearly shows that gene expression of mtDNA-encoded complex I polypeptides was decreased to about 30% of control levels (Figure 3B), while expression of nDNA-encoded subunits was similar in control and ob/ob mice (Figure 3C). Therefore, a reduced synthesis of complex I subunits can explain the low amount of mtDNA-encoded polypeptides found in ob/ob mice, but not the reduced amount of nDNA-encoded subunits. These results concur with those reported by Haque et al.[20] who showed that the expression of mtDNA-encoded COX-II subunit, but not nDNA-encoded COX-IV subunit, was significantly decreased in NASH patients. This difference might be ascribed to the oxidative damage of the mtDNA. Determination of the level of 8-OHdG, a reliable marker of oxidative DNA damage,[21] demonstrated that 8-OHdG was significantly increased in mtDNA, but not in nDNA, from ob/ob mice as compared with wild-type mice (Figure 3A). mtDNA is located in the mitochondrial matrix close to the MRC where most reactive oxygen substances (ROS) are generated; therefore, mtDNA is particularly prone to oxidative damage compared to nDNA.[22] Moreover, mtDNA lack of protective histones and mitochondrial DNA repair systems appears to be less efficient. Accumulation of mtDNA lesions may decrease the synthesis of mtDNA-encoded respiratory chain polypeptides and in turn may cause more oxidative damage to mtDNA.[23] Our study shows that this reduced gene expression of mtDNA-encoded subunits can be reversed not only by treating obese mice with the antioxidant MnTBAP, but also by treating this animals with the peroxynitrite scavenger uric acid. This suggests that peroxynitrite may also be involved in the inhibition of mitochondrial gene expression. Consistent with this notion, a number of authors have demonstrated that exposure of cells to peroxynitrite leads to a decline in mtRNA transcripts.[24,25] In fact, peroxynitrite can cause damage to DNA bases by deamination and nitration of guanine residues.[26,27]

Defects in the assembly process can be recognized by the finding of low molecular-weight intermediates in the two-dimensional BN/SDS-PAGE. As shown in Figure 2A, accumulation of these intermediates was not detected in any ob/ob mice,

suggesting that a defect in the assembly of complex I subunits was not the cause of the reduced amount of this complex found in obese mice. The third option by which complex I subunits are decreased in ob/ob mice is an increased degradation of damaged complex I subunits. This mechanism may explain not only the low amount of mtDNA-encoded subunits found in ob/ob mice, but also the reduction in subunits encoded by the nDNA, whose synthesis is normal. The fact that mtDNA-encoded polypeptides are decreased significantly more that nDNA-encoded subunits might be ascribed to a combination of both mechanisms, the low synthesis and the enhanced degradation of these subunits. A number of factors have been recognized to be implicated in the stabilization of complex I, including prohibitin,[28] complex III,[29,30] NDUFAF1,[31] and B17.2.[32] Prohibitin is a member of a highly conserved protein family with a variety of cellular roles.[33] It is anchored to the inner mitochondrial membrane, where it binds directly to newly synthesized mitochondrial proteins, including complex I subunits,[28] and stabilizes them against degradation by membrane-bound AAA metalloproteases.[34−36] Deletion of prohibitin genes caused accelerated degradation of nonassembled membrane proteins by AAA protease,[35] while overexpression of this protein was found to stabilize non-native polypeptides against degradation.[36] Because of its role in the prevention of complex degradation, we measured the levels of prohibitin in mitochondrial protein extracts of control and ob/ob mice. As Figure 4A shows, these levels were markedly reduced in ob/ob mice as compared with control mice. These results are consistent with those reported by others in obese patients and in ob/ob mice.[37] Therefore, absence of prohibitin might favor the degradation of complex I components and, consequently, the reduced amount of complex I subunits found in obese mice. The reason for these low levels of prohibitin in ob/ob mice is not known. However, considering that its levels returned to the control level when ob/ob mice were treated with uric acid, MnTBAP, or anti-TNFα, we believe that this defect, like this found in complex I subunits, may also be related with the oxidative stress and with the increased formation of peroxynitrite anion in obese mice. Prohibitin has been identified as a molecular target of nitration.[38] In the present study, we show that this protein is 3-tyrosine nitrated in mitochondrial membranes from obese mice (Figure 4B).

Complex III is another factor that may stabilize complex I.[29,30] Our study shows that the signal originated by complex III (Figure 1C) was also markedly reduced and, in fact, was limited to a tiny band at 500 kDa molecular weight. Treatment of ob/ob mice with uric acid, MnTBAP, or anti-TNFα antibody also resulted in the recovery of the levels of complex III subunits existing in wild-type mice (Figure 2). All these results suggest that reduction in the amount of prohibitin and complex I and III subunits may be a consequence of the oxidative stress or peroxynitrite aggression.

In a previous study, we hypothesized that MRC dysfunction and liver lesions found in ob/ob mice, and likely in NASH patients as well, are due to the tyrosine nitration of mitochondrial proteins by peroxynitrite or a peroxynitrite-derivate radical. Increased hepatic TNFα and iNOS expression might enhance peroxynitrite formation and inhibition of MRC complexes.[6] In the current study, we present evidence showing that "in vitro" treatment of mitochondrial proteins isolated from wild-type mice with peroxynitrite leads not only to the 3-tyrosine nitration of these proteins, but also to the loss of the enzymatic activity, the drop of mitochondrial respiration, the reduction in the amount of mitochondrial complexes, and to the degradation of their subunits. Thus, treatment of mitochondrial proteins from wild-type mice with peroxynitrite led to the formation of a double band followed by a long track extended to the front line (Figure 6) when incubated with antibodies against 39 kDa subunit from complex I. While the double band is likely the result of the partition of the fully assembled complex I, the track following the main bands is originated by fragments resulting from degradation of this complex.

These results concur with those reported by others showing that nitration of mitochondrial proteins is associated with a decreased in their catalytic activity.[39−41] Murray et al. demonstrated that "in vitro" incubation of mitochondrial preparation with 1600 μM peroxynitite resulted in an inhibition of complex I activity by about 50%.[19] Likewise, we found that administration of uric acid, a natural scavenger of peroxynitrite anion,[42,43] to ob/ob mice decreased strikingly 3-tyrosine nitrated mitochondrial proteins and normalized activity of complex I.[6] Peroxynitrite is produced by the reaction of nitric oxide (NO) with superoxide anion (O_2^-) and there is a number of evidence showing that NO and superoxide anion formation is increased in the liver of ob/ob mice and NASH patients. Thus, Laurent et al. showed that the concentrations of nitrites and nitrates were significantly increased in liver homogenates of ob/ob mice,[44] we demonstrated that iNOS expression is upregulated in hepatocytes and liver cell mitochondria of these animals,[6] and evidence of oxidative stress has been reported in patients with NASH[45] and obese mice.[6] At least three mechanisms have been proposed in the NO inhibition of complex I: S-nitrosation, tyrosine nitration, and damage to FeS centers. However, none of these mechanisms have been definitively shown to mediate inhibition of this mitochondrial complex.[46] In this study, we show that peroxynitrite induces the loss of complex I subunits, as well as prohibitin, likely by degradation of these proteins. In these experimental conditions, defects in the synthesis or in the assembly of complex I as cause of its decreased amount in the mitochondrial membranes can be excluded.

In conclusion, the present study shows that the low activity of the complex I of the MRC found in ob/ob mice can be attributed to the reduced amount of all components of this enzymatic complex. This defect can be ascribed partially to the reduced synthesis of some polypeptides and mainly to the degradation of these components induced by peroxynitrite radicals. Neutralization of peroxynitrite might be a critical step in the management of NAFLD in ob/ob mice and, eventually, in human.

Abbreviations: BN-PAGE, blue native polyacrylamide gel electrophoresis; CS, citrate synthase; iNOS, inducible nitric oxide synthase; MnTBAP, manganese(III) tetrakis (5,10,15,20 benzoic acid) porphyrin; MRC, mitochondrial respiratory chain; mtDNA, mitochondrial DNA; NASH, nonalcoholic steatohepatitis; NAFLD, nonalcoholic fatty liver disease; nDNA, nuclear DNA; 8-OHdG, 8-hydroxy-2′-deoxyguanosine.

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Supporting Information Available: Effect of manganese [III] tetrakis (5,10,15,20 benzoic acid) porphyrin (MnTBAP), uric acid, and antitumor necrosis factor alpha (TNFα)

on liver histology of ob/ob mice. This material is available free of charge via the Internet at http://pubs.acs.org.

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