In vitro treatment of HepG2 cells with saturated fatty acids reproduces mitochondrial dysfunction found in nonalcoholic steatohepatitis

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ABSTRACT
Activity of the oxidative phosphorylation system (OXPHOS) is decreased in humans and mice with nonalcoholic steatohepatitis. Nitro-oxidative stress seems to be involved in its pathogenesis. The aim of this study was to determine whether fatty acids are implicated in the pathogenesis of this mitochondrial defect. In HepG2 cells, we analyzed the effect of saturated (palmitic and stearic acids) and monounsaturated (oleic acid) fatty acids on: OXPHOS activity; levels of protein expression of OXPHOS complexes and their subunits; gene expression and half-life of OXPHOS complexes; nitro-oxidative stress; and NADPH oxidase gene expression and activity. We also studied the effects of inhibiting or silencing NADPH oxidase on the palmitic-acid-induced nitro-oxidative stress and subsequent OXPHOS inhibition. Exposure of cultured HepG2 cells to saturated fatty acids resulted in a significant decrease in the OXPHOS activity. This effect was prevented in the presence of a mimic of manganese superoxide dismutase. Palmitic acid reduced the amount of both fully-assembled OXPHOS complexes and of complex subunits. This reduction was due mainly to an accelerated degradation of these subunits, which was associated with a 3-tyrosine nitration of mitochondrial proteins. Pretreatment of cells with uric acid, an antiperoxynitrite agent, prevented protein degradation induced by palmitic acid. A reduced gene expression also contributed to decrease mitochondrial DNA (mtDNA)-encoded subunits. Saturated fatty acids induced oxidative stress and caused mtDNA oxidative damage. This effect was prevented by inhibiting NADPH oxidase. These acids activated NADPH oxidase gene expression and increased NADPH oxidase activity. Silencing this oxidase abrogated totally the inhibitory effect of palmitic acid on OXPHOS complex activity. We conclude that saturated fatty acids caused nitro-oxidative stress, reduced OXPHOS complex half-life and activity, and decreased gene expression of mtDNA-encoded subunits. These effects were mediated by activation of NADPH oxidase. That is, these acids reproduced mitochondrial dysfunction found in humans and animals with nonalcoholic steatohepatitis.

KEY WORDS: Mitochondrial respiratory chain, Nonalcoholic steatohepatitis, NADPH oxidase, Oxidative phosphorylation, Proteomic, Nitro-oxidative stress, OXPHOS

INTRODUCTION
Nonalcoholic fatty liver disease (NAFLD) represents a spectrum of liver diseases extending from pure fatty liver through nonalcoholic steatohepatitis (NASH) to cirrhosis and hepatocarcinoma that occurs in individuals who do not consume a significant amount of alcohol (Matteoni et al., 1999). Although the pathogenesis of NAFLD remains undefined, the so-called ‘two hits’ model of pathogenesis has been proposed (Day and James, 1998). Whereas the ‘first hit’ involves the accumulation of fat in the liver, the ‘second hit’ includes oxidative stress resulting in inflammation, stellate cell activation, fibrogenesis and progression of NAFLD to NASH (Chitturi and Farrell, 2001). 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 source of reactive oxygen species (ROS) (Fromenty et al., 2004). In previous studies, we have shown that oxidative phosphorylation (OXPHOS) is defective in individuals with NASH (Pérez-Carreras et al., 2003), in ob/ob mice with NAFLD (García-Ruiz et al., 2006) and in mice on a high-fat diet (García-Ruiz et al., 2014). We also demonstrated that this mitochondrial dysfunction can be prevented by treating mice with antioxidants and antiperoxynitrates, such as melatonin or uric acid, indicating that oxidative and nitrosative stress might play a crucial role in the pathogenesis of this defect. However, the cause of this stress remains unclear. Potential sources of nitro-oxidative stress are multiple, including cytochrome P450-2E1 (CYP2E1) (Weltman et al., 1998), nicotinamide adenine dinucleotide phosphate-oxidase or NADPH (nicotinamide adenine dinucleotide phosphate) oxidase (NADPHox) (De Minicis et al., 2006), mitochondrial electron transport chain (Fridovich, 2004) and xanthine oxidase (XDh) (Spiekermann et al., 2003). CYP2E1, a member of the oxido-reductase cytochrome family, can oxidize a variety of small molecules, including fatty acids (Caro and Cederbaum, 2004), to produce superoxide anions, a very potent reactive oxygen species (ROS). Activity and expression of this enzyme is increased in the liver of humans and animals with NAFLD (Weltman et al., 1998), and this increase correlates with the severity of NAFLD. NADPHox is a multiprotein complex found in all types of liver cells, including hepatocytes, that reduces molecular oxygen to superoxide and hydrogen peroxide (De Minicis et al., 2006). In a previous study, we have shown that mice with diet-induced NASH have elevated NADPHox gene expression and activity (García-Ruiz et al., 2014), and other authors have found the same changes in mice fed a methionine-choline-deficient diet (Greene et al., 2014). A number of factors can induce NADPHox activity, including free fatty acids (Hatanaka et al., 2013) and TNFα (Mohammed et al., 2013), among others. Considering that fatty acids are increased in the liver of obese mice (García-Ruiz et al., 2014), it might be possible that these acids are responsible for the
increased NADPHox activity, the oxidative stress and eventually for the OXPHOS dysfunction found in individuals with NASH and in obese mice. OXPHOS dysfunction, in turn, might create a vicious cycle that would contribute to increase the oxidative stress.

The aims of this study were to determine whether fatty acids are implicated in the pathogenesis of this mitochondrial defect and to know the role played by NADPHox in the generation of this dysfunction.

RESULTS
Saturated fatty acids decreased OXPHOS enzyme activity

Treatment of HepG2 cells with 200 μM oleic acid, a monounsaturated fatty acid, did not significantly alter activity of OXPHOS complexes. However, treatment of these cells with the same doses of palmitic or stearic acids, two saturated fatty acids, decreased mitochondrial enzyme activity, owing to a decreased amount of OXPHOS complex subunits. These effects are mediated by fatty-acid-induced nitro-oxidative stress and by NADPH oxidase. The use of antioxidants or antiperoxynitrites can prevent these changes. Therefore, treatment with these agents or with inhibitors of the NADPH oxidase, as well as strategies for reducing hepatic free fatty acid concentration, might be useful in preventing the progression of NAFLD in humans.
shows, palmitic and stearic acids decreased cellular ATP from 9.86±0.33 nmol/mg protein to 5.16±0.3 or 4.67±0.4 nmol/mg protein (P<0.01), respectively. Treatment of cells with 200 μM oleic acid did not affect cellular ATP. Likewise, the ATP:ADP ratio was also significantly decreased in cells treated with palmitic or stearic acids, but not in those treated with oleic acid (Fig. 1C).

**Palmitic acid decreased fully-assembled OXPHOS complexes and complex subunits**
The first-dimension BN-PAGE system illustrates that fully-assembled OXPHOS complexes decreased in a dose-dependent manner in HepG2 cells treated with increasing doses of palmitic acid for 24 hours (Fig. 1D). To study how mitochondrial OXPHOS complex subunits were affected by palmitic acid, these complexes were resolved by second-dimension SDS-PAGE and subunits were detected using specific antibodies. Employing this procedure, the most striking finding was a fall in the amount of all studied complex subunits in cells treated with palmitic acid (Fig. 1E). No significant differences were observed whether subunits were encoded by genomic or mitochondrial DNA.

**Palmitic acid decreased gene expression of mitochondrial DNA (mtDNA)-encoded OXPHOS subunits**
Because a decrease in these subunits might be due to a diminished synthesis or to an accelerated degradation, we measured gene expression of some representative subunits of these complexes. We found that expression of genomic DNA (nDNA)-encoded subunits was normal in cells treated with 200 μM palmitic acid for 24 hours, and that preincubation with an antioxidant, such as MnTBAP, did not increase significantly the levels of these subunits (Fig. 2A). By contrast, gene expression of mtDNA-encoded subunits declined significantly in HepG2 cells treated with this fatty acid (Fig. 2B).

Pretreatment of cells with MnTBAP increased gene expression of these subunits over the levels in control cells (Fig. 2B). In contrast, gene expression of mtDNA-encoded subunits declined not increase significantly the levels of these subunits (Fig. 2A). By contrast, gene expression of mtDNA-encoded subunits declined significantly in HepG2 cells treated with this fatty acid (Fig. 2B).

**Palmitic acid accelerated degradation of OXPHOS complexes**
In order to know whether saturated fatty acids caused degradation of complex proteins, confluent HepG2 cells were cultured in the absence or presence of 200 μM palmitic acid for 24 hours. After this time, gene transcription was inhibited by adding 5 μM actinomycin D. At 3, 6, 12 and 24 hours after addition of actinomycin D, fully-assembled OXPHOS complexes were analyzed by BN-PAGE. As Fig. 3 shows, palmitic acid decreased the half-life of OXPHOS complexes to about 18.8±6.6% of controls. This effect was associated with an increased amount of 3-tyrosine-nitrated proteins (Fig. 4A, C). Treatment of cells with 1 mM uric acid, a scavenger of peroxynitrite, prevented both the 3-tyrosine nitration of mitochondrial proteins (Fig. 4D) and the shortening of OXPHOS-complex half-life caused by palmitic acid (Fig. 3). Moreover, palmitic acid increased iNOS gene and protein expression (Fig. 4E, F).

**Saturated fatty acids induce oxidative stress**
Because nitro-oxidative stress seemed to be involved in the effects of saturated fatty acids on OXPHOS complexes, we wanted to know whether fatty acids are able to induce oxidative stress. As Fig. 5A shows, treatment of HepG2 cells with 200 μM palmitic or stearic acids for 24 hours led to a marked increase in the cellular levels of TBARS (thiobarbituric-acid-reacting substances), an index of oxidative stress. By contrast, treatment of cells with 200 μM oleic acid did not modify these levels.

In order to identify the oxidative system responsible for this stress, we treated HepG2 cells with 200 μM palmitic acid in the presence and absence of 4 mM MnTBAP, a mimic of superoxide dismutase, 0.3 mM allopurinol, 10 μM VAS2870 {1,3-benzoazol-
results, we silenced MnTBAP. By contrast, inhibiting XDH did not avoid the effects of palmitic acid (Fig. 5D) shows, the palmitic-acid-induced inhibition of OXPHOS complex activity was totally blocked by inhibiting NADPHox with VAS2870. In cells incubated with chlormethiazole, the effects of palmitic acid were partially reverted, whereas allopurinol pretreatment did not modify activity of these complexes.

To confirm that NADPHox mediated the effects of palmitic acid on OXPHOS complexes, we measured OXPHOS activity in cells with silenced RAC1, a component of the NADPHox complex. As Fig. 6A shows, the inhibitory effect of palmitic acid on the OXPHOS activity was not observed in the absence of NADPHox activity. This effect of palmitic acid persisted, but was less pronounced, in cells with silenced CYP2E1. However, in these cells, treatment with palmitic acid also increased NADPHox activity although less markedly (Fig. 6B). Finally, this effect persisted in cells with silenced XDH (Fig. 6C).

Saturated free fatty acids increased NADPHox activity and gene expression in cultured HepG2 cells

Because NADPHox seemed to play a crucial role in the pathogenesis of oxidative stress caused by saturated fatty acids, we wanted to know whether these acids are able to activate this enzyme complex. Thus, we measured NADPHox activity in HepG2 cells treated with 200 μM palmitic, stearic or oleic acids. Although treatment of these cells with increasing doses of monounsaturated oleic acid did not modify significantly NADPHox activity, treatment with the same doses of palmitic or stearic acids led to a significant dose-dependent increase in this activity (Fig. 7A). Likewise, time-response curves demonstrate that palmitic acid increased NADPHox activity in a time-dependent fashion and that this effect was maximal by treating cells for 6 hours (Fig. 7B). Similarly, treatment of cultured HepG2 cells with 200 μM of either palmitic or stearic acids elevated significantly p22phox, p47phox, RAC1, NOX2 and NOX4 gene expression (Fig. 7C). By contrast, the same amount of oleic acid did not modify the expression of these genes significantly. Palmitic acid did not increase XDH gene expression but increased slightly CYP2E1 gene expression (Fig. 7D). Moreover, saturated fatty acids not only upregulated gene expression of NADPHox components but also induced phosphorylation of p47phox, one component of the NADPHox. This effect was not observed with oleic acid (Fig. 7E).

**DISCUSSION**

In the present study, we show for the first time that saturated fatty acids, but not the monounsaturated oleic acid, decreased markedly the activity of all OXPHOS complexes in HepG2 cells (Fig. 1A). As a result of this effect, ATP content (Fig. 1B) and ATP:ADP ratio were also significantly reduced in cells exposed to saturated fatty acid (Fig. 1C). Although we are not aware of other studies where the effects of fatty acids on the OXPHOS activity have been studied, some authors have reported that palmitic acid decreased cellular ATP content in muscle cells (Lambertucci et al., 2012; Hirabara et al., 2010). Our findings allow us to suggest that fatty acids, concentrations of which are increased in plasma and liver of individuals with NASH (Allard et al., 2008) and obese mice (García-Ruiz et al., 2014), could be responsible for the lowering of OXPHOS enzyme activity observed in these individuals and animals (García-Ruiz et al., 2006; García-Ruiz et al., 2010; Solís-Muñoz et al., 2011). These effects of saturated fatty acids were blocked by the use of an antioxidant (Fig. 1A), indicating that oxidative stress might be implicated in these effects.
The present study also provides an explanation for the low activity of OXPHOS enzyme complexes in cells exposed to saturated fatty acids, because complex subunits and the amount of fully assembled complexes were markedly reduced in mitochondria of treated cells (Fig. 1D,E). This low amount of complex subunits might be caused by a reduced synthesis of these...
Our study shows that gene expression of mtDNA-encoded subunits the synthesis of mtDNA-encoded OXPHOS polypeptides. In fact, mtDNA, because accumulation of mtDNA lesions might decrease of proteins might be attributable to the oxidative damage of the in nDNA-encoded subunits. These differences between both groups found in cells exposed to palmitic acid, but not the reduced amount least partially, the low amount of mtDNA-encoded polypeptide. Therefore, a reduced synthesis of OXPHOS subunits can explain, at nDNA-encoded subunits was not affected by this acid (Fig. 2A,B).

Therefore, mitochondrially generated ROS might subsequently lead to more mtDNA mutations and trigger a vicious cycle in which mitochondrial dysfunction produces larger amounts of ROS which in turn can induce further oxidative damage to mitochondrial function.

Our study also shows for the first time that palmitic acid accelerates degradation of OXPHOS complexes, reducing half-lives of fully-assembled complexes by about sixfold (Fig. 3). This effect seems to be dependent on the nitrosative stress, because pretreating cells with uric acid, a natural scavenger of peroxynitrite anion (Whiteman et al., 2002), prevented degradation of these complexes (Fig. 3). Moreover, mitochondrial proteins were 3-tyrosine nitrated in palmitic-acid-treated cells (Fig. 4C), and this acid increased iNOS gene and protein expression (Fig. 4E,F). In a previous study, we showed that ‘in vitro’ peroxynitrite caused not only nitration of mitochondrial proteins but also increased their degradation, decreased OXPHOS enzyme activity, reduced the amount of fully assembled OXPHOS complexes and reduced the amount of individual complex subunits (García-Ruiz et al., 2010). Also, Murray et al. demonstrated that ‘in vitro’ incubation of mitochondrial proteins with peroxynitrite inhibited complex I activity (Murray et al., 2003). This accelerated degradation of mitochondrial proteins justifies not only the low amount of mtDNA-encoded subunits, but also the decrease in nDNA-encoded subunits whose synthesis was normal. Peroxynitrite is produced by the reaction of nitric oxide (NO) with superoxide anion (O2–). A number of studies have shown that NO and superoxide anion formation is increased in the liver of individuals with NASH (Laurent et al., 2004; Sanyal et al., 2001) and obese mice (García-Ruiz et al., 2006).

Our study clearly shows that saturated fatty acids, but not the monounsaturated oleic acid, induced oxidative stress (Fig. 5A) and that this stress was totally abrogated by inhibiting NADPHox with VAS2872, a specific inhibitor of this oxidase (Fig. 5B), or by silencing RAC1, a component of NADPHox (Fig. 5C). We also show that palmitic and stearic acids, concentrations of which are significantly increased in the liver of obese mice (Wang et al., 2011), are able to markedly elevate NADPHox activity in HepG2 cells (Fig. 7A,B). Therefore, the oxidative stress found in HepG2 cells exposed to saturated fatty acids might be mediated mainly by the activation of NADPHox. This oxidase is a multiprotein complex composed of membrane-bound components (p22phox, Nox family) and cytosolic components (p47phox, p67phox, p40phox, Rac1/2) (De Minicis et al., 2006). Following stimulation, the cytosolic proteins become phosphorylated and are transferred to the membrane, where they bind to the membrane-bound components, increasing NADPHox activity and reducing molecular oxygen to generate superoxide and hydrogen peroxide. In HepG2 cells, our study shows that the increase in NADPHox activity was due to an upregulation of NADPHox-component gene expression (Fig. 7C) and to an enhanced phosphorylation of p47phox (Fig. 7E). The effects of palmitic acid on gene expression of other oxidative systems, such as

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**Fig. 6.** The inhibitory effect of palmitic acid on OXPHOS activity was totally abrogated in the absence of NADPHox activity. In HepG2 cells, RAC1 (RAC) (A), CYP2E1 (CYP) (B) or XDH (C) gene expression was silenced using appropriate siRNAs and activity of OXPHOS and NADPHox was measured in the presence and absence of 200 μM palmitic acid. **P<0.01; ***P<0.001 versus untreated control cells (siRNA Ct). CS, citrate synthase. In the top right-hand corner of each panel are northern blots showing cDNA levels for RAC1 (A), CYP (B) and XDH (C) after knocking down expression of these genes, respectively.

subunits, by their increased degradation or by a combination of both defects.

Our study shows that palmitic acid significantly decreased gene expression of mtDNA-encoded polypeptides, whereas expression of nDNA-encoded subunits was not affected by this acid (Fig. 2A,B). Therefore, a reduced synthesis of OXPHOS subunits can explain, at least partially, the low amount of mtDNA-encoded polypeptide found in cells exposed to palmitic acid, but not the reduced amount in nDNA-encoded subunits. These differences between both groups of proteins might be attributable to the oxidative damage of the mtDNA, because accumulation of mtDNA lesions might decrease the synthesis of mtDNA-encoded OXPHOS polypeptides. In fact, our study shows that gene expression of mtDNA-encoded subunits increased markedly by pretreating cells with an analog of superoxide synthase. Moreover, determination of the levels of 8-OHdG, a marker for oxidative DNA damage (Kasai 1997), demonstrated that 8-OHdG was significantly increased in mtDNA, but not in nDNA, of palmitic-acid-treated cells (Fig. 2C). mtDNA is particularly prone to suffer oxidative damage (Yakes and Van Houten, 1997) because it is not covered by protective histones and other DNA-associated proteins, allowing direct exposure to ROS. Moreover, mitochondrial DNA repair systems seem to be less efficient than those for nuclear DNA (Druzhyna et al., 2008; Gao et al., 2004). Finally, mtDNA is located near to the damaged OXPHOS chain, another major source of ROS. Therefore, mitochondrially generated ROS might subsequently lead to more mtDNA mutations and trigger a vicious cycle in which mitochondrial dysfunction produces larger amounts of ROS which in turn can induce further oxidative damage to mitochondrial function.
CYP2E1 or XDH, were less marked or even absent (Fig. 7D). There is no information about the effect of fatty acids on NADPHox activity in HepG2 cells. Nevertheless, a number of authors have shown that palmitate generated ROS via NADPHox in several cell lines (Han et al., 2012; Lambertucci et al., 2008).

NADPHox-dependent oxidative stress seems to be responsible for the depression in OXPHOS activity caused by palmitic acid, given that this effect did not occur in the presence of VAS2872 (Fig. 5D) or in cells silenced NADPHox (RAC1) (Fig. 6). We have very little information about the effects of NADPHox on OXPHOS function. Nox4, one member of the Nox family located in the mitochondrial inner membrane, has been shown to inhibit activity of complex I of OXPHOS and to decrease the concentration of complex I subunits (Koziel et al., 2013). Gene expression of this factor is upregulated not only by transforming growth factor-β (Carmona-Cuenca et al., 2006), whose concentration is increased in the liver tissue of obese mice (García-Ruiz et al., 2014), but also by palmitic acid in HepG2 cells (Fig. 7C).

Other oxidative systems that could also contribute to the oxidative stress caused by saturated fatty acids are XDH and CYP2E1. The latter cytochrome is induced in humans and animals with NASH (Weltman et al., 1998; Lieber et al., 2004). Our study shows that inhibition or silencing CYP2E1 reduced oxidative stress (Fig. 5B,C) and prevented partially the effect of palmitic acid on OXPHOS complexes (Fig. 5D; Fig. 6), indicating that this cytochrome is also involved in the pathogenesis of oxidative stress caused by this fatty acid. However, these effects might be due to the cross-talk existing between CYP2E1 and NADPHox (Ekström and Ingelman-Sundberg, 1989). In fact, our study shows that palmitic-acid-induced oxidative stress caused by this fatty acid was analyzed by RT-PCR as described in the Materials and Methods. The mRNA:GAPDH mRNA ratio was measured in control cells and in cells treated with fatty acids for 24 hours. ***P<0.001 as compared with control cells. Results represent mean values ± s.d. of one representative experiment performed in quadruplicate. (D) The effect of 200 μM palmitic acid on RAC1 (NADPHox), XDH and CYP2E1 gene expression in the absence and presence of 4 mM MnTBAP. ***P<0.001. **P<0.01 as compared with control cells. Results represent mean values ± s.d. of one representative experiment performed in quadruplicate. (E) HepG2 cells were treated with 200 μM palmitic, stearic or oleic acids for 30 and 60 minutes. Cellular proteins were immunoprecipitated (IP) with anti-p47phox (p47phox) and subsequently immunoblotted (IB) with either anti-phosphoserine (p-serine) or anti-p47phox (p47phox) to evaluate equal loading.

In conclusion, our study demonstrates that saturated fatty acids decrease OXPHOS function after inhibition of CYP2E1 might be attributable to the effects of CYP2E1 on the NADPHox system. In HepG2 cells, the role played by XDH in the fatty-acid-induced oxidative stress and inhibition of OXPHOS activity seems to be minor, because inhibition or silencing of this oxidase did not prevent the effects of palmitic acid on oxidative stress or OXPHOS-complex activity.

In our study, we demonstrated that saturated fatty acids decrease OXPHOS enzyme activity by reducing the amount of OXPHOS complexes and their subunits. These effects are mediated by the nitro-oxidative stress caused by these acids, which results in reduced gene expression of mtDNA-encoded subunits, and in accelerated degradation of OXPHOS complexes. NADPHox and, to a lesser extent, CYP2E1 mediate these effects of fatty acids. Antioxidants and antiperoxinotrites prevent all these effects of fatty acids and might be useful in the treatment and prevention of NASH in humans.

**MATERIALS AND METHODS**

**Cell culture**

The HepG2 cell line obtained from American Type Culture Collection (Manassas, VA) was grown at 37°C in an atmosphere of 5% CO2, 95% air in cell culture flask using 10 ml of Dulbecco’s Modified Eagle’s Medium (Lonza Iberica SA, Barcelona, Spain) containing 10% fetal calf serum, 1% L-glutamine, 1% penicillin, 1% streptomycin, 1% fungizone. Cells were plated at a density of 5×10⁶/80-cm² flask. The effect of fatty acid was examined by addition of these agents to the cell cultured in medium with 2% fetal calf serum. Palmitic and stearic fatty acids were dissolved as described by Joshi-Barve et al. (Joshi-Barve et al., 2007). Oleic acid was prepared according to the manufacturer’s protocol (Sigma-Aldrich, Alcobendas, Spain).
Nitrification of cellular proteins by peroxinitrite [3-nitrotyrosine (3NT)] was assessed as described elsewhere (Garcia-Ruiz et al., 2006).

Oxidative phosphorylation (OXPHOS) activity assays

HepG2 cells (approximately 5 × 10⁵ cells) were collected by trypsinization, washed twice with phosphate-buffered saline (PBS), and resuspended in 2 ml of ice-cold solution containing 20 mM MOPS, 0.25 M sucrose, and 200 μg of digitonin. After centrifugation at 5000 g for 3 minutes at 4°C, the pellet was resuspended in 0.5 ml of 10 mM K-phosphate buffer, pH 7.4, and frozen-thawed twice. These digitonin-permeabilized homogenates were used to measure the activities of OXPHOS enzymes and citrate synthase (CS) using a DU-650 spectrophotometer (Beckman Instruments, Palo Alto, CA). Incubation temperatures were 30°C for complexes I, II, III, V and CS, and 38°C for complex IV. Enzyme activities were performed in supernatants as described elsewhere (Pérez-Carreras et al., 2003), expressed as nanomoles of substrate used per minute per milligram of protein and, to correct for the hepatic content of mitochondria, referred to as a percentage of the specific activity of CS. Enzyme assays were performed in triplicate.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from cultured HepG2 cells using the TRI-Reagent (Sigma-Aldrich, Steinheim, Germany) according to the manufacturer’s instructions. RNA was treated with DiNAse I to remove DNA contamination (Sigma-Aldrich, Steinheim, Germany). cDNA was generated from 1 μg 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 cDNA, 0.5 μM primers and 2 μl FastStart DNA Master SYBR Green I (Roche Applied Science, Mannheim, Germany). Data from the real-time, quantitative PCR were analyzed following the 2-ΔΔCT method as described by Livak and Schmittgen (Livak and Schmittgen, 2001). The sequences of primers used in these experiments are shown in supplementary material Table S1. Expression of protein genes was normalized to that corresponding to GAPDH activity of CS. Enzyme assays were performed in triplicate.

RNA interference

XDH-, CYP2E1-, and Rac1-specific siRNA and non-specific control RNA used as a negative control were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). For the transfection experiments, we followed the procedure described elsewhere (Díaz-Sanjuán et al., 2009).

Western blot

Mitochondria were isolated from cultured cells by differential centrifugation as described by Turko et al. (Turko et al., 2001). Proteins were separated and transferred to an Immobilon membrane (Millipore, Bedford, MA) as previously described (Solis-Herruzo et al., 1999). After electrotransfer, the filters were incubated with appropriate polyclonal antibody against 3-nitrotyrosine (Upstate Biotechnology, Lake Placid, NY), inducible nitric oxide synthase (iNOS), p47phox, TOM20, XDH, CYP2E1, Rac1, VDAC1 (Santa Cruz Biotechnology, Santa Cruz, CA) and phosphorylated serine (Sigma-Aldrich, Alcobendas, Spain). Signals were detected using the ECL Western Blotting Detection Reagent (Amersham Iberica, Madrid, Spain).

Immunoprecipitation

The immunoprecipitation assays were performed as previously described (Lang et al., 2000). Proteins were precipitated with appropriate polyclonal antibodies (anti-p47phox). Immune complexes were recognized using specific antibodies (p47phox, anti-phosphoserine). Signals were detected using the ECL detection kit.

Assessment of fully-assembled OXPHOS complexes

Mitochondria were isolated from HepG2 cells according to the procedure described by Nijtmans et al. (Nijtmans et al., 2002). Mitochondrial complexes were separated on a 3-12% acrylamide blue native–polyacrylamide gel (BN-PAGE) as described elsewhere (Garcia-Ruiz et al., 2010). Western blotting of these proteins was performed using primary antibodies against complex I subunit NDUFV1, complex II subunit SDHA, complex III subunit UQCR2C, complex IV subunit MTCO1, complex V subunit ATP5A1 (Molecular Probes Inc., Eugene, OR) and TOM complex subunit TOM20 on blocking buffer for 2 hours. After washing, blots were incubated for 1 hour with peroxidase-conjugated anti-mouse antibody as a secondary antibody, prepared at 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. Blots were finally exposed to Hyperfilm MP (Amersham, GE Healthcare, Madrid, Spain). ECL signals were quantified using the ImageJ image analysis software (Rasband, 2007).

Second-dimension electrophoresis for assessing complex subunits

For second-dimension BN/SDS-PAGE, a lane containing mitochondrial complexes was excised from the one-dimension gel, as previously described (Garcia-Ruiz et al., 2010). Western blotting was performed using primary antibodies against: VDAC1 (Santa Cruz Biotechnology, Santa Cruz, CA); complex I subunits NDUFV1, NDUFV2, NDUF6, NDUF8, NDUF9, NDUF53, NDUF8B, MTND1, MTND4L and MTND6; complex II subunit SDHA; complex III subunits UQCR1 (Core1), UQCR2C (Core2), UQCRFS and MTCYB; complex IV subunits COX4 and MTCO1; and complex V subunits ATP5A1 and MITATP8 (Molecular Probes Inc., Eugene, OR) on blocking buffer for 2 hours. After washing, blots were treated as indicated above.

Measurement of total ATP content and ATP:ADP ratio in mouse liver

Cells were homogenized in perchloric acid and centrifuged at 15,000 g for 2 minutes. Supernatants were collected and 30 μl was added to a 96-well plate and then brought up to 50 μl with ATP assay buffer. ATP reaction mix and ATP measurement was performed using the ATP Colorimetric/Fluorometric Assay Kit (BioVision Research Products, Milpitas, CA) according to the manufacturer’s protocol. The ATP:ADP ratio was measured by luminometry using the commercial assay kit ApoSENSOR™ ADP/ATP Ratio Assay Kit (BioVision Research Products, Mountain View, CA).

Measurement of 8-OHdG in nuclear and mitochondrial DNA

Nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) were isolated from HepG2 cells 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-deoxyguanosine EIA Kit, Cayman Chemical Co., Ann Arbor, MI).

Lipid peroxidation

Lipid peroxidation was determined by measuring TBARS in cells as described by Okawa et al. (Okawa et al., 1979).

NADPH oxidase activity

NADPHox activity was measured following the procedure described by Jalil et al. (Jalil et al., 2005).

Statistical analysis

These analyses were carried out using 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 ± s.d. P-values <0.05 were considered significant.

Competing interests

The authors declare no competing or financial interests.
Author contributions I.G.-R. performed many of the experiments and participated in the design, analysis and interpretation of data. P.S.-M. was involved in the acquisition and interpretation of data and in the critical revision of the manuscript for important intellectual content. D.F.-M. performed many experiments and analyzed and interpreted data. T.M.-Y. contributed by designing the study, and in the acquisition, analysis and interpretation of data. J.A.S.-H. conceived the study, participated in its design and coordination, in the analysis and interpretation of data, and in writing the manuscript. All authors read and approved the final manuscript.

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Supplementary material

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