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

Running Title: Fatty acids and OXPHOS

Inmaculada García-Ruiz¹, Pablo Solís-Muñoz², Daniel Fernández-Moreira³, Teresa Muñoz-Yagüe¹, José A. Solís-Herruzo¹

¹ Research Center. Laboratory of Gastroenterology and Hepatology. University Hospital “12 de Octubre”. Complutense University. 28041-Madrid. Spain

² Anglo-American Medical Unit. 28001-Madrid, Spain.

³ Department of Bromatology and Food Hygiene. Military Center of Veterinary of Defense. 28024-Madrid. Spain

Corresponding author:

Inmaculada García-Ruiz
Centro de Investigación Hospital Universitario “12 de Octubre”.
Avenida de Córdoba S/N, 28041-Madrid, Spain.
E-mail address: inmagr86@hotmail.com
Telephone: +34 913908764; Fax: +34 913908544

Keywords: mitochondrial respiratory chain; nonalcoholic steatohepatitis; NADPH oxidase; oxidative phosphorylation; proteomic; nitro-oxidative stress.
**List of Abbreviations:**

CYP2E1, cytochrome P450-2E1; iNOS, nitric oxide synthase; MnTBAP, manganese [III] tetrakis (5,10,15,20 benzoic acid) porphyrin; mtDNA, mitochondrial DNA; NADPHox, nicotinamide adenine dinucleotide phosphate-oxidase; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; nDNA, nuclear DNA; 3NT, 3-nitrotyrosine; 8-OHdG, 8-hydroxy-2’-deoxyguanosine; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species; TBARS, thiobarbituric acid-reacting substances. XDH, xanthine oxidase.
Abstract

Activity of the oxidative phosphorylation (OXPHOS) is decreased in patients and mice with non-alcoholic 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 the OXPHOS activity, OXPHOS complexes and their subunits, gene expression and half-life of OXPHOS complexes, nitro-oxidative stress, 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 OXPHOS inhibition. Exposure of cultured HepG2 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 fully assembled OXPHOS complexes and the amount 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 human and animal with non-alcoholic steatohepatitis.
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 patients who do not consume 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). While 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 the oxidative phosphorylation (OXPHOS) is defective in patients with NASH (Pérez-Carreras M et al., 2003), in ob/ob mice with NAFLD (Garcia-Ruiz et al., 2001) and in mice on a high fat diet (García-Ruiz et al., 2014, in press). We also demonstrated that this mitochondrial dysfunction can be prevented by treating mice with antioxidants and antiperoxinitrites, like melatonin or uric acid, indicating that oxidative and nitrosative stress may play a critical role in the pathogenesis of this defect. However, the cause of this stress remains unclear. Potential sources of nitrooxidative stress are multiple, including cytochrome P450-2E1 (CYP2E1) (Weltman et al., 1998), nicotinamide adenine dinucleotide phosphate-oxidase or NADPH oxidase (NADPHox) (de Minicis et al., 2006), mitochondrial electron transport chain (Fridovich, 2004), and xanthine oxidase (XDH) (Spiekermann, 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 anion, a very potent reactive oxygen species (ROS). Activity and expression of this enzyme is increased in the liver of human 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 type 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 I et al., 2014, in press) and other authors have found the same changes in mice fed 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., in press), 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 NASH patients and in obese mice. OXPHOS dysfunction, in turn, may 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 the NADPHox in 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 enzyme activity of these complexes to about 66.8±4.3% of control activity (Fig. 1A). These effects were abrogated by pretreating cells with 4mM manganese [III] tetrakis (5,10,15,20 benzoic acid) porphyrin (MnTBAP), a mimic of manganese superoxide dismutase. As the final product of the OXPHOS is ATP, we measured the ATP content in HepG2 cells treated with 200 µM palmitic, stearic or oleic acid for 24 hours. As figure 1B 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 (Fig. 1C). Treatment of cells with 200 µM oleic acid did not affect cellular ATP. Likewise, the ATP content and the ATP/ADP ratio were 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.

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.
As a decrease in these subunits may 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). On the contrary, 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). Measurement of 8-hydroxy-2’-deoxyguanosine (8-OHdG) content in nDNA was identical in cells treated with fatty acids than in untreated cells (Fig. 2C). On the contrary, this marker for oxidative DNA damage was significantly increased in mtDNA from cells treated with palmitic or stearic acids but not in those treated with the monounsaturated oleic acid.

**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 figure 3 shows, palmitic acid decreased 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 and 4C). 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, 4F).

**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 figure 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, an index of oxidative stress. On the contrary, 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-benzoxazol-2-yl-3-benzyl-3H-[1,2,3]triazolo[4,5,d]pyrimidin-7-yl-sulfide-7-(1,3-benzoxazol-2-yl-sulfanyl)-3-benzyl-3H-[1,2,3]triazolo[4,5-d]pyrimidine), or 40 µM chlormethiazole, inhibitors of XDH, NADPHox, or CYP2E1, respectively. As shown in figure 5B, the oxidative effect caused by palmitic acid was totally avoided by inhibiting NADPHox and partially by blocking CYP2E1. As expected, this effect was also prevented by treating cells with MnTBAP. On the contrary, inhibiting XDH did not avoid the effects of palmitic acid on TBARS concentration. In order to ensure these results, we silenced XDH, CYP2E1, or NADPHox with appropriated interfering RNAs (siRNA). These cells were then treated with palmitic acid. As figure 5C shows, the effects of palmitic acid persisted in cells with silenced XDH, but not in cells with silenced RAC1, a component of the NADPHox. Silencing CYP2E1 reduced but did not abolish entirely the effect of palmitic acid on cellular TBARS.

The effects of palmitic acid on OXPHOS activity were totally prevented by inhibiting or silencing NADPHox.

As NADPHox seemed to be involved in causing the oxidative stress induced by palmitic acid, we wanted to know whether NADPHox also mediated the inhibitory effects of palmitic acid on OXPHOS complexes. Thus, we treated HepG2 cells with 200 µM palmitic acid for 24 hours in the absence or presence of 0.3 mM allopurinol, 10 µM VAS2870 or 40 µM chlormethiazole. As figure 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, while 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. As figure 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 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 increase NADPHox activity and gene expression in cultured HepG2 cells.**

Because NADPHox seemed to play a critical 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 \( \mu \)M palmitic, stearic or oleic acids. While 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 \( \mu \)M of either palmitic or stearic acids elevated significantly \( p22^{\text{phox}} \), \( p47^{\text{phox}} \), \( RAC1 \), \( NOX2 \), and \( NOX4 \) gene expression (Fig. 7C). On the contrary, 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 \( NADPHox \) gene expression but also induced phosphorylation of \( p47^{\text{phox}} \), 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 contents in muscle cells (Lambertucci et al., 2012; Hirabara et al., 2010). Our findings allow us to suggest that fatty acids, which concentrations are increased in plasma and liver of NASH patients (Allard et al., 2008) and obese mice (Garcia-Ruiz et al., 2014, in press), may be responsible for the low OXPHOS enzyme activity we found in these patients and animals (Garcia-Ruiz et al., 2006; García-Ruiz I 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 may 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, since complex subunits and the amount of fully assembled complexes were markedly reduced in mitochondria of treated cells (Fig. 1D and 1E). This low amount of complex subunits may be caused by a reduced synthesis of these subunits, by their increased degradation or by a combination of both defects.

Our study shows that palmitic acid decreased significantly gene expression of mtDNA-encoded polypeptides, while expression of nDNA-encoded subunits was not affected by this acid (Fig. 2A and 2B). 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 may be attributed to the oxidative damage of the mtDNA, as accumulation of mtDNA lesions may 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) since it is not covered by protective histones and other DNA-associated proteins, allowing direct exposure to ROS. Moreover, mitochondrial DNA repair systems appear to be less efficient (Druzhyna, et al. 2008; Gao D, et al, 2004). Finally, mtDNA is located near to the damaged OXPHOS chain, another major source of reactive oxygen substances (ROS). Therefore, mitochondrial generated ROS may 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 accelerated degradation of OXPHOS complexes, reducing half-lives of fully assembled complexes by about six-fold (Fig. 3). This effect seems to be dependent on the nitrosative stress, since pretreating cells with uric acid, a natural scavenger of peroxynitrite anion (Whiteman, 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 and 4F). 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, fully assembled of OXPHOS complexes, and 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 (O$_2^-$). A number of studies have shown that NO and superoxide anion formation is increased in the liver of NASH patients (Laurent et al., 2004; Sanyal et al., 2001) and obese mice (Garcia-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, which concentrations are significantly increased in the liver of obese mice (Wang et al., 2011), are able to elevate markedly NADPHox activity in HepG2 cells (Fig. 7A and 7B). Therefore, the oxidative stress found in HepG2 cells exposed to saturated fatty acids may 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 became 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 increased in NADPHox activity was due to an up-regulation of NADPHox components 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 CYP2E1 or XDH, were less marked or even absent (Fig. 7D). There is no information about the effect of fatty acids on the NADPHox activity in HepG2 cells. Nevertheless, a number of authors have shown that palmitate generated ROS via NADPHox in a several cell lines (Han et al., 2012; Lambertucci et al., 2008).

The 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 with 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 the OXPHOS and to decrease concentration of complex I subunits (Kozieł 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, in press), 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 patients 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, 5C) and prevented partially the effect of palmitic acid on OXPHOS complexes (Fig. 5D and
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 NADPHox activity was decreased in cells with silenced CYP2E1 (Fig. 6). Other authors have found that inhibition of CYP2E1 with anti-CYP2E1 IgG antibody resulted in the complete inhibition of NADPHox-dependent generation of TBARS (Ekström and Ingelman-Sundberg, 1989). Likewise, induction of CYP2E1 resulted in an increased activity of NADPHox and oxidative stress, all of which could be prevented with chlormethiazole (Seitz and Mueller 2012). Therefore, the improvement of OXPHOS function after inhibition of CYP2E1 may be attributed to effects of CYP2E1 on 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, as inhibition or silencing this oxidase did not prevented the effects of palmitic acid on oxidative stress or OXPHOS complex activity.

In conclusion, our study demonstrates that saturated fatty acids decrease OXPHOS enzyme activity by reducing OXPHOS complexes and their subunits. These effects are mediated by the nitrooxidative stress caused by these acids, which results in reduction of mtDNA-encoded subunits gene expression and in accelerated degradation of OXPHOS complexes. NADPH oxidase and, to a lesser extent, CYP2E1 mediate these effects of fatty acids. Antioxidants and antiperoxinitrites prevent all these effects of fatty acids and might be useful in the treatment and prevention of NASH in human.
Material 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 5x10⁶/80-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 manufacture’s protocol (Sigma-Aldrich, Alcobendas, Spain).

Nitration of cellular proteins by peroxinitrite [3-nitrotyrosine (3NT)] was assessed as described elsewhere (García-Ruiz et al., 2006).

MRC activity assays. HepG2 cells (approximately 5x10⁶ 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 5,000 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 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 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 DNase 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; 42ºC, 60 min; 95ºC, 5 min, and 4ºC, 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 et al. (Livak and Schmittgen, 2001). Sequence of primers used in these experiments are shown in supplementary table 1. Expression of protein genes was normalized to that corresponding β-actin or GAPDH mRNA. The amplification conditions were 45 cycles of denaturation at 95ºC for 10 s, annealing at 59ºC for 5 s, and extension at 72ºC for 20 s. (Bustin, 2000). The correct size and purity of the amplified products was verified by agarose gel electrophoresis.

**RNA interference.** XDH, CYP2E1, and Rac1-specific siRNA and nonspecific 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 (Solís-Herruzo et al., 1999). After electrotransfer, the filters were incubated with appropriated polyclonal antibody against 3-nitrotyrosine (Upstate Biotechnology. Lake Placid. NY), inducible nitric oxide synthase (iNOS), p47phox, TOM 20, 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 Ibérica (Madrid, Spain)).

**Immunoprecipitation.** The immunoprecipitation assays were performed as previously described (Lang et al., 2000). Proteins were precipitated with appropriated 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-assembly of MRC complexes.** Mitochondria were isolated from HepG2 cells according 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 NDUFA9, complex II subunit SDHA, complex III subunit UQCRC2, complex IV subunit MTCO1, complex V subunit ATP5A1 (Molecular Probes Inc. Eugene. OR.), and TOM complex subunit TOM 20 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 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 manufactured 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, 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, NDUFA6, NDUFB6, NDUFA9, NDUFS3, NDUFB8, MTND1, MTND4L, and MTND6, complex II subunit SDHA, complex III subunits UQCRC1 (Core1), UQCRC2 (Core2), UQCRFS, and MTCYB, complex IV subunits COX4 and MTCO1, and complex V subunits ATP5A1 and MTATP8 (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, USA) according to the manufacturer’s protocol. The ADP/ATP ratio was measured by luminometry using the commercial assay kit ApoSENSORTM ADP/ATP Ratio Assay Kit (BioVision Research Products, Mountain View, CA).
Measurement of 8-hydroxy-2’-deoxyguanosine (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 manufacture’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).

Lipid peroxidation was determined by measuring thiobarbituric acid-reacting substances (TBARS) in cells as described by Ohkawa et al. (Ohkawa et al., 1979).

NADPH oxidase 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, USA). 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.

Conflict of Interest: The authors of this study declare that they have nothing to disclose and that they have not received any support from the pharmaceutical industry in the form of a grant, equipment or drug.

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. M.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.
**Funding.** This study was supported in part by Grants from the “Fundación Mutua Madrileña” (AP7257-2010; AP8540-2011; AP11223-2013) and from the “Fondo de Investigación Sanitaria” (PI10/0312), Spain, two non-profit institutions to support medical research.
References.


Pivotal role of superoxide anion and beneficial effect of antioxidant molecules in murine steatohepatitis. *Hepatology* 39: 1277–1285


**Figure 1.** Saturated fatty acids decreased enzyme activity of the OXPHOS complexes, ATP content, and the amount of fully assembled complexes and their subunits. (A) Enzyme activity of the OXPHOS complexes and citrate synthase was measured in HepG2 cells treated for 24 hours with 200 µM oleic acid, 200 µM palmitic acid, 200 µM stearic acid or 4 mM MnTBAP, normalized to the level of the specific activity of citrate synthase, and expressed as fold the activity in untreated cells. Values are expressed as mean ± SD. ***, p<0.001 compared with untreated cells.** (B) ATP content in control HepG2 cells (Ctr) and cells exposed 200 µM palmitic acid (Pal), 200 µM stearic acid (Ste) or 200 µM oleic acid (Ol) for 24 hours. (C) ATP/ADP ratio in HepG2 cells treated with 200 µM palmitic acid (Pal), 200 µM stearic acid (Ste) or 200 µM oleic acid (Ol) for 24 hours. (D) Mitochondrial complexes isolated from HepG2 cells and treated with increasing doses of palmitic acid were separated on a BN-PAGE system. Western blot analysis of mitochondrial proteins was performed using antibody against complex I subunit NDUFA9, complex II subunit SDHA, complex III subunit core 2 protein, complex IV subunit MTCO1, complex V subunit ATP5A1, and TOM complex subunit TOM20. Expression of TOM complex (TOM COMP.) was used as loading control. (E) Mitochondrial complexes extracted from HepG2 cells treated with 200 µM palmitic acid for 24 hours were separated in the first dimension using BN-PAGE and in the second dimension using SDS-PAGE. Presence of individual subunits of these complexes was identified by immunoblotting using appropriated antibodies. Expression of VDAC1 was used as loading control. -fold, amount of subunit in palmitic acid-treated cells divided by the amount of the same subunit in control cells.

**Figure 2.** Palmitic acid decreased gene expression of OXPHOS subunits. (A and B) Gene expression of representative subunits encoded by genomic- and mitochondrial-DNA was measured in HepG2 cells treated with 200 µM palmitic acid (Palm.) for 24 hours in the absence or presence of 4 mM MnTBAP (TBAP). Messenger RNA of the subunits was analyzed by RT-PCR following the procedure described in “Material and methods”. The subunit mRNA/β-Actin mRNA ratio was calculated. *, p<0.05; **, p<0.01; vs. control untreated cells (Ctr.); b, p<0.01; c, p<0.001 vs. cells treated with palmitic acid. A9, NDUFA9; B6, NDUFB6; S3, NDUFS3; SDH, SDHA; Co1, UQCRCl;
Co2, UQCR2; FeS, UQCRFS1; ATP5, ATP5A1; ND1, MTND1; ND2, MTND2; ND4, MTND4; ND4L, MTND4L; ND6, MTND6; CYB, MTCYB; CO1, MTCO1. (C) HepG2 cells were cultured in absence (C) or presence of 200 μM palmitic (P), stearic (S) or oleic (O) acids for 24 hours. 8-Hydroxy-2′-deoxyguanosine (8-OHdG) content was measured in nDNA) and mtDNA of the same cells. ***, p<0.001 as compared with untreated cells.

**Figure 3.- Palmitic acid induced OXPHOS complex degradation.** Confluent HepG2 cells were exposed to control medium or to 200 μM palmitic acid for 24 h prior to inhibition of gene transcription with 5 μM actinomycin D. At 3, 6, 12 and 24 h after the addition of actinomycin D, fully assembled OXPHOS complexes were analyzed by BN-PAGE. The same experiments were repeated in the absence or presence of 200 μM palmitic acid and 1 mM uric acid. These experiments were repeated twice with similar results.

**Figure 4.- Palmitic acid induced 3-tyrosine nitration of OXPHOS subunits.** (A) Confluent HepG2 cells were exposed to control medium or to 200 μM palmitic acid in the absence or presence of 1 mM uric acid for 24 hours. Cells were then fixed for 1 hour in 1% paraformaldehyde and permeabilized with Triton X before labelling with anti-3-nitrotyrosine (3NT) specific antibody and goat anti-rabbit IgG FITC-conjugated secondary antibody. Nuclear counterstain of these cells was done with Fluoroshield (Sigma-Aldrich, Alcobendas, Spain) with DAPI (Bars, 30µm). Membranes containing mitochondrial proteins from control, untreated HepG2 cells (B) or from cells treated with 200 μM palmitic acid (C) or with palmitic and 1 mM uric acid (D) were probed with specific antibody against 3-nitrotyrosine. (E) Gene expression of iNOS was measured in HepG2 cells treated with 200 μM palmitic acid (Palm.) for 24 hours in the absence or presence of 4 mM MnTBAP (TBAP). Messenger RNA of iNOS was analyzed by RT-PCR following the procedure described in “Material and methods”. The iNOS mRNA/GAPDH mRNA ratio was calculated. ***, p<0.001 vs. control untreated cells. (F) Western blots showing the effect of increasing doses palmitic acid on iNOS protein expression.
Figure 5. Effects of fatty acid on TBARS contents in HepG2 cells. (A) Cells were pretreated with 200 μM palmitic acid (P), 200 μM stearic acid (S), or 200 μM oleic acid (O) for 24 hours. Cellular content in TBARS was measured as described under Methods. (B) Cellular TBARS were measured in cells treated with 200 μM palmitic acid (Palm. Ac.) in the presence or absence of 4 mM MnTBAP, 0.3 mM allopurinol (Allopur.), 10 μM VAS2870 (VAS), or 40 μM chlormethiazole (CMZ). (C) NADPH oxidase (RAC1), CYP2E1 (CYP) or xanthine oxidase (XDH) were silenced with appropriated small interfering RNAs (siRNA) in HepG2 cells in the presence (+) or absence (—) of 200 μM palmitic acid. TBARS content was measured in these cells. Western blots showing expression of XDH, CYP, RAC1, and β-actin (ACTB) after knocking down XDH, CYP, RAC1, respectively. (D) Enzyme activity of the OXPHOS complexes and citrate synthase was measured in HepG2 cells treated for 24 hours with 200 μM palmitic acid in the absence or presence of 0.3 mM allopurinol, 10μM VAS2870, or 40 μM chlormethiazole. This activity was normalized to the level of the specific activity of citrate synthase, and expressed as fold the activity in untreated cells. Values are expressed as mean ± SD. *, p<0.05; **, p<0.01; ***, p<0.001 compared with untreated cells.

Figure 6. The inhibitory effect of the palmitic acid on the 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 appropriated 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 vs. 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 (panel A), CYP (panel B), and XDH (panel C) after knocking down expression of these genes, respectively.

Figure 7. Saturated fatty acids increase NADPHox activity and gene expression in cultured HepG2 cells. (A) Cells were cultured in the presence of increasing doses of palmitic, stearic, or oleic acids for 6 hours. NADPH oxidase activity was measured as described under Methods. (B) NADPHox enzyme activity was measured in cultured HepG2 cells treated with 200 μM palmitic acid for 0.5 to 24 hours.
(C) The effect of 200 µM palmitic, stearic acid or oleic acid on gene expression of p22, $p47^{\text{phox}}, RAC1, NOX2$ and $NOX4$ was analyzed by RT-PCR as described in 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 ± SD of one representative experiment performed in quadruplicate. (D) The effect of 200 µM palmitic acid on $RAC1$ (NADPH oxidase), $XDH$, 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 ± SD 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-$p47^{\text{phox}}$ ($p47^{\text{phox}}$) and subsequently immunoblotted (IB) with either anti-phosphoserine (p-serine) or anti-$p47^{\text{phox}}$ ($p47^{\text{phox}}$) to evaluate equal loading.
**Translational Impact:**

**Clinical Issue:** Nonalcoholic fatty liver disease is a worldwide problem that represents the most frequent histological finding in patients with abnormal liver tests in the Western countries. Its pathogenesis remains undefined but we have found that oxidative phosphorylation (OXPHOS) is decreased in patients with this disease. The OXPHOS system, comprised of five multimeric enzyme complexes, is the metabolic pathway by which mitochondria use energy released by oxidation of nutrients to produce ATP. The latter supplies energy to cell metabolism. Mitochondria are involved in the oxidation of fatty acids and are important sources of ROS. Therefore, defective OXPHOS might contribute to the accumulation of fat in the liver and to cause oxidative stress resulting in inflammation and progression of the disease to steatohepatitis and cirrhosis. In mice fed a high fat diet, we also found a marked decrease in OXPHOS activity due to a reduced amount of OXPHOS complex subunits. This defect seemed to be related with the nitro-oxidative stress. The use of antioxidants or antiperoxynitrite prevented all these OXPHOS defects. The aims of the present study were to determine whether fatty acids are implicated in the pathogenesis of this mitochondrial defect and to know the role played by the NADPH oxidase in the generation of the oxidative stress and the mitochondrial dysfunction.

**Results.** In HepG2, saturated fatty acids cause a decrease in the OXPHOS activity that is due to a decrease in the amount of OXPHOS complex subunits. These fatty acids provoke nitro-oxidative stress, 3-tyrosine nitration of mitochondrial proteins and accelerate their degradation. Likewise, oxidative damage of mitochondrial DNA reduces the synthesis of mitochondrial OXPHOS subunits. NADPH oxidase seems to play a critical role in the pathogenesis of the nitro-oxidative stress and OXPHOS dysfunction, as the effects of saturated fatty acids are prevented in the absence of NADPH oxidase activity. Moreover, these fatty acids activate NADPH oxidase gene expression and increase NADPH oxidase activity.

**Implications and future directions:** We conclude that saturated fatty acids decrease OXPHOS enzymatic activity, which is due to a decreased amount of OXPHOS complex subunits. The use of antioxidants or antiperoxynitrites prevents all these changes. Therefore, treatment with these agents or with inhibitors of the NADPH oxidase as well as measures for reducing hepatic free fatty acid concentration might be useful in preventing the progression of NAFLD in humans.
<table>
<thead>
<tr>
<th>OXPHOS Complexes</th>
<th>Control</th>
<th>Palmitic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Half-life 29.2</td>
<td>2.8 hours + Uric Acid</td>
</tr>
<tr>
<td></td>
<td>Half-life 27.2</td>
<td>15.2 hours</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Half-life 27.3</td>
<td>5.1 hours + Uric Acid</td>
</tr>
<tr>
<td></td>
<td>Half-life 20.3</td>
<td>21.2 hours</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Half-life 23.3</td>
<td>4.2 hours + Uric Acid</td>
</tr>
<tr>
<td></td>
<td>Half-life 31.5</td>
<td>29.8 hours</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Half-life 24.1</td>
<td>6.8 hours + Uric Acid</td>
</tr>
<tr>
<td></td>
<td>Half-life 34.0</td>
<td>32.4 hours</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Half-life 15.8</td>
<td>3.1 hours + Uric Acid</td>
</tr>
<tr>
<td></td>
<td>Half-life 22.7</td>
<td>24.3 hours</td>
</tr>
</tbody>
</table>

hours: 0 3 6 12 24