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Mitochondrial dysfunction during in vitro hepatocyte steatosis is reversed by omega-3 fatty acid–induced up-regulation of mitofusin 2

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Abstract

We examined the effects and mechanisms of omega-3 polyunsaturated fatty acid (PUFA) administration on mitochondrial morphology and function in an in vitro steatotic hepatocyte model created using HepG2 cells. Reverse transcriptase polymerase chain reaction and Western blot analyses were performed to determine the expression levels of mitofusin 2 (Mfn2), and immunofluorescent MitoTracker Mitochondrion-Selective Probes were used to detect changes in mitochondrial morphology. Adenosine triphosphate (ATP) synthesis and reactive oxygen species (ROS) production were measured to assess mitochondrial function. Mitofusin 2 expression was significantly suppressed ($P < .05$), ATP levels were decreased ($P < .05$), ROS production was increased ($P < .05$), and the normal tubular network of mitochondria was fragmented into short rods or spheres. Model cells were incubated with eicosapentaenoic acid or docosahexaenoic acid at a final concentration of 50 μmol/L for 1 hour. Both eicosapentaenoic acid and docosahexaenoic acid increased the expression of Mfn2 ($P < .01$) and caused an increase in the length of mitochondrial tubules. The omega-3 PUFAs also increased the levels of ATP ($P < .05$) and decreased the ROS production ($P < .05$). However, these changes were not seen in Mfn2-depleted steatotic HepG2 cells, created by RNA interference before incubation with the omega-3 PUFAs. This study demonstrated that, in steatotic hepatocytes, omega-3 PUFAs may change mitochondrial morphology and have beneficial effects on recovery of mitochondrial function by increasing the expression of Mfn2.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver injury, with a prevalence estimated to be at 20% to 30% among the general populations of Western countries. The illness includes a spectrum of liver injuries characterized histologically by hepatic steatosis in individuals who do not consume significant amounts of alcohol and who have no viral, congenital, or autoimmune liver disease markers [1]. Studies indicate that NAFLD is strongly associated with features of the metabolic syndrome, especially obesity. The prevalence of NAFLD is almost universal among people with diabetes and obesity [2]. Steatosis may be associated with steatohepatitis or nonalcoholic steatohepatitis (NASH), which can progress to fibrosis, cirrhosis, liver failure, or hepatocellular carcinoma [3]. Nonalcoholic fatty liver disease is increasingly recognized as a major cause of liver-related morbidity and mortality [4].

The omega-3 polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) cannot be synthesized by the human body and so must be derived from exogenous sources, which are almost exclusively marine in origin. There is a general view that omega-3 PUFAs are beneficial in human disease states [5]. A recent study in a rat model of parenteral nutrition–induced steatosis demonstrated that supplementation with omega-3 PUFAs may protect the liver [6]. However, the mechanisms responsible for this therapeutic benefit are not fully understood.

The factors that lead to progressive hepatocellular damage in the setting of hepatic steatosis are not well elucidated; recent evidence suggests the involvement of mitochondrial dysfunction during the several steps from obesity to the development of NASH [7-10]. Nonalcoholic steatohepatitis mitochondria exhibit ultrastructural lesions and markedly

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decreased activities of the respiratory chain complexes. Mitochondria are very important eukaryotic organelles, playing a crucial role in many cellular functions such as respiration, substrate oxidation, adenosine triphosphate (ATP) production, and apoptosis. Mitochondria form dynamic organized networks of filaments that undergo constant fission and fusion [11-13]. A balance between this fusion and fission is crucial for the maintenance of correct mitochondrial morphology and proper functional dynamics. In many adherent cell types, mitochondria form elongated tubules that continually divide and fuse to form a dynamic interconnecting network [14-16]. Interruption of this fusion process results in a loss of inner mitochondrial membrane potential, which is critical for maintenance of function [17]. These dynamic mitochondrial networks could be involved in the repartition throughout the cell of lipids, proteins, or even mitochondrial DNA molecules. Maintaining these networks could increase transport rates to allow for efficient delivery of these molecules to different areas of the cell. Excessive mitochondrial fission appears to be a requisite step in intrinsic apoptosis pathways, at least for the normal rate of cytochrome *c* release and caspase activation [18].

Alterations in some of the proteins that participate in mitochondrial dynamics are linked to human pathology. Recent observations indicate that mitochondrial metabolism is regulated by manipulation of the proteins involved in mitochondrial dynamics [19,20], particularly the mitofusin 2 (Mfn2) protein. Mitofusin 2 is a multifunctional protein that participates in cell proliferation and metabolism and is also required for normal endoplasmic reticulum morphology. Alterations in Mfn2 activity have been reported to modify cell respiration, substrate oxidation, and oxidative phosphorylation subunit expression in cultured nonmuscle and muscle cells. Mitofusin 2 expression in skeletal muscle is subject to regulation; and conditions characterized by reduced mitochondrial activity, such as type 2 diabetes mellitus, are associated with suppressed Mfn2. Previous studies have shown that mutations in Mfn2 genes cause the neurodegenerative diseases Charcot-Marie-Tooth type 2A and Kjer disease/autosomal dominant optic atrophy [21-23]. Reductions of Mfn2 protein levels are observed in muscle tissues in obese Zucker rats and in obese humans [22,24]. It was shown that Mfn2 expression may be associated with obesity [24].

In this study, an in vitro steatotic hepatocyte model was established; and then these cells were incubated with omega-3 PUFAs. Our goal was to investigate in steatotic hepatocytes the relationships between and among mitochondrial function, Mfn2 messenger RNA (mRNA), and protein expression, and the therapeutic protection afforded by omega-3 PUFAs.

2. Materials and methods

2.1. HepG2 cell cultures

HepG2 cells were purchased from the Chinese Type Culture Collections (Shanghai, China) and were cultured in Dulbecco modified Eagle medium supplemented with 7% fetal calf serum, penicillin (50 U/mL), and streptomycin (50 μg/mL). For subculturing purposes, cells were detached by treatment with 0.25% trypsin/0.02% EDTA at 37°C. Cultures were used at 80% confluence.

2.2. Steatosis induction and oil red O staining of HepG2 cells

Fat overloading of cells was performed according to previously established methods [25]. The HepG2 cells at 80% confluence were incubated for 48 hours in a high concentration of long-chain free fatty acids (HFFA)—a mixture of oleate and palmitate—in the final ratio of 2:1 and final concentration of 1 mmol/L. Following incubation, cells were fixed and stained with oil red O/Mayer hematoxylin using the method described by Mori et al [26].

2.3. Fluorimetric determination of quantities of fat content in cells

The lipid content in cultured cells was determined fluorimetrically using Nile red [27,28]. Cell monolayers were washed twice with phosphate-buffered saline (PBS) and incubated for 15 minutes with Nile red solution at a final concentration of 1 mg/mL in PBS at 37°C. Monolayers were washed thereafter with PBS, and lipid droplet fluorescence staining intensity was measured by microplate fluorometer (excitation, 488 nm; emission, 550 nm) [29]. Cell protein content was determined by the Lowry method adapted to 96-well plates [30].

2.4. In vitro transfection with Mfn2

Human Mfn2 short hairpin RNA (shRNA) and scrambled negative control shRNA were designed by and purchased from Santa Cruz Biotechnology (Santa Cruz, CA; sc-43928). Steatotic HepG2 cells were transfected with shRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, while being maintained in the HFFA medium. Cells were routinely harvested at 24 hours posttransfection for polymerase chain reaction (PCR) and Western blot analyses.

2.5. Preparation of PUFA and treatment conditions

Stock solutions of DHA and EPA sodium salts were prepared as previously described [31], reconstituted in endotoxin-free water to a final concentration of 100 μmol/L, and then stored in the dark at −80°C to prevent peroxidation. HepG2 cells were incubated for 1 hour in DHA or EPA at a final concentration of 50 μmol/L in the following experimental groups: DHA or EPA; DHA or EPA + Mfn2-shRNA; and DHA or EPA + control-shRNA. Control (steatotic HepG2 cells) and experimental groups were maintained in HFFA during the treatment phase.

2.6. Reverse transcriptase PCR

RNA was extracted using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. DNase I (Invitrogen) was added to the RNA sample to remove any genomic DNA contamination. First-strand complementary DNA was synthesized using the SuperScript III First-Strand Synthesis kit (Invitrogen). Reactions were carried out in the following conditions: 94°C for 5 minutes, 94°C for 15 seconds, 60°C for 25 seconds, and 72°C for 15 seconds, through 30 amplification cycles. Mitofusin 2 primer sequences were as follows: forward primer, 5′-ATGCATCCCCACTTAAGCAC-3′; reverse primer, 5′-CCAGAGGGCAGAACTTTGTC-3′. G3PDH primer sequences were follows: forward primer, 5′-ACCACAGTCCATGCCATCAC-3′; reverse primer, 5′-TCCACCACCCTGTTGCTGTA-3′. Quantitation was performed by densitometry, with G3PDH as the internal control. Products were analyzed on a 1.5% agarose gel system. A computerized image analysis system (Kontron IBAS2.0, Munich, Germany) was used to quantify band intensity. Results were evaluated as a relative unit determined by normalization of the optical density of the Mfn2 band to that of the G3PDH band (giving the ratio Mfn2:G3PDH).

2.7. Western blot analysis

A rabbit antibody against the Mfn2-specific peptide was purchased from Research Genetics (Huntsville, AL). Sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blots were performed according to standard protocols, using G3PDH as the loading control. Signals were detected by secondary antibodies labeled with Alex Fluor 680 (Invitrogen), and a computerized image analysis system (Kontron IBAS2.0) was used to quantify band intensity. Results were evaluated as a relative unit determined by normalization of the optical density of the Mfn2 band to that of the G3PDH band (giving the ratio Mfn2: G3PDH).

2.8. Fluorescence microscopy and image analyses

Prewarmed (37°C) staining solution containing 1 mmol/L MitoTracker Green Probes (Invitrogen) was added to cells growing on coverslips inside a Petri dish followed by incubation for 40 minutes in experimental growth conditions. The staining solution was replaced with fresh prewarmed media, and cells were identified using fluorescence microscopy. Mitochondrial morphology in individual cells was evaluated. Fragmented mitochondria were shortened, punctate, and sometimes rounded, whereas filamentous mitochondria consisted of thread-like tubular structures. In rare cases of mixed morphologies, we classified the cells based on the majority (>70%) of mitochondria. For each sample, several random fields of cells (>100 cells per dish) were evaluated for mitochondrial morphology.

2.9. Determination of ATP and reactive oxygen species production

Mitochondrial ATP synthesis was carried out on digitonin-permeabilized cells as described by Ouhabi et al [32]. Steady-state ATP synthesis was initiated by adding 2 mmol/L adenosine diphosphate and was recorded for 2 minutes as follows: after adenosine diphosphate addition, 10-μL aliquots were taken every 30 seconds, quenched in 100 μL dimethyl sulfoxide, and then diluted in 5 mL of ice-cold distilled water. Adenosine triphosphate was measured using bioluminescence in a Luminoskan using the ATP Bioluminescence Assay Kit HS II (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions. The rate of ATP synthesis was calculated using linear regression. Rates were expressed in nanomoles ATP per minute per 10^6 cells. Changes in mitochondrial reactive oxygen species (ROS) levels were monitored using the MitoSox red probe (Invitrogen). The probes were added to the cell suspension and incubated for 30 minutes at 37°C according to the manufacturer's protocol. Cells were washed in PBS, and fluorescence was measured at steady state on a spectrofluorometer (SAFAS, NJ).

2.10. Statistical analysis

Each experiment was performed in at least triplicate, and data were expressed as mean ± SE. Unpaired Student *t* tests for normally distributed numerical variance and nonparametric Student *t* tests (Mann-Whitney) for skewed data were conducted using SPSS (Chicago, IL) 13.0. Linear correlation was used to explore association between 2 variables. Differences were considered statistically significant at $P < .05$.

3. Results

3.1. Effects of fatty acids on lipid droplet formation and mitochondrial morphology in HepG2 cells

Twenty-four hours of culturing HepG2 cells with HFFA markedly induced lipid droplet formation as detected by oil red O staining (Fig. 1A, B). The lipid content, estimated fluorimetrically using the Nile red assay, was 5-fold higher in HFFA-overloaded HepG2 cells than control cells (Fig. 1C). Mitochondrion-Selective Probes were used to demonstrate the morphology of the mitochondrial networks (Fig. 1D, E), which were found to be significantly more fragmented in the HFFA-treated group than in the control group (Fig. 1F).

3.2. Significant inhibition of Mfn2 mRNA and protein expression in steatotic hepatocytes

Reverse-transcriptase (RT) PCR revealed that Mfn2 expression in the HFFA group was significantly lower than that in the HepG2 control cells, with values of 0.307 ± 0.066 and 0.773 ± 0.029, respectively (Fig. 2A, n = 21, $P < .01$).

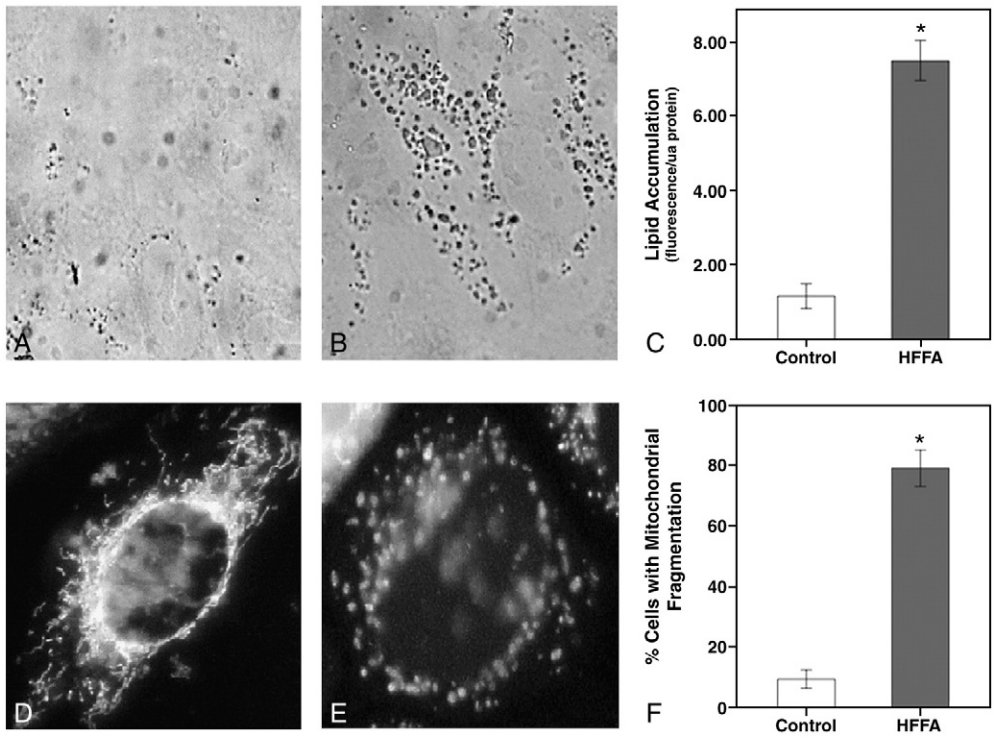


Fig. 1. Effects of HFFA on lipid droplet formation and mitochondrial fragmentation in HepG2 cells. HepG2 cells were cultured for 48 hours in 10% fetal bovine serum with 1 mmol/L HFFA, fixed with formalin, stained with oil red (A and B). A, Lipid droplet formation in HFFA-treated cells. B, Control HepG2 cells. C, Lipid content in HFFA-overloaded HepG2 cells vs control cells. The lipid content was estimated by Nile red staining. Results are expressed as fluorescence arbitrary units per microgram of protein. *$P < .05$ in relation to the control cells. Mitochondrial morphology was stained with mitochondrial immunofluorescent probes (MitoTracker) and analyzed by fluorescence microscopy and (D and E). D, Control HepG2 cells. E, HFFA-treated HepG2 cells. F, Percentages of mitochondrial fragmentation were quantified by cell counting. Data are means ± SD of 3 separate experiments. *Significantly different from the control group.

Western blot analysis revealed that the Mfn2 protein levels in experimental and control groups were 0.395 ± 0.042 and 1.0146 ± 0.062, respectively (Fig. 2B, n = 21, $P < .01$).

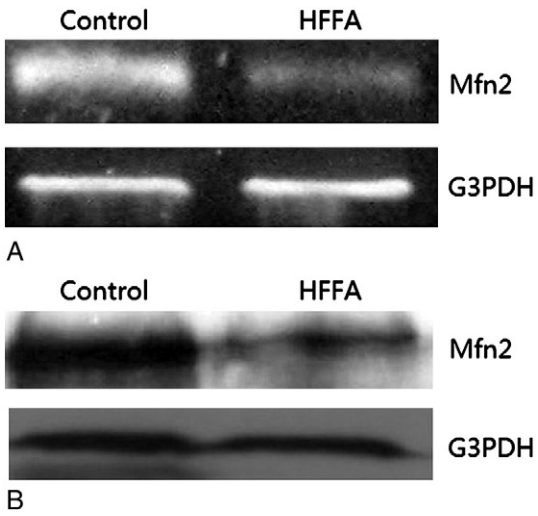


Fig. 2. High concentration of long-chain free fatty acids decreases expression of Mfn2 in HepG2 cells. HepG2 cells were incubated with HFFA for 48 hours. A, Reverse transcriptase PCR (n = 21, $P < .01$). B, Western blot with anti-Mfn2 monoclonal antibody (n = 21, $P < .01$). Anti-G3PDH as loading control.

3.3. Decreased ATP levels and increased ROS levels in steatotic hepatocytes

Compared with control cells, ATP levels in steatotic HepG2 cells were significantly decreased (6.15 ± 0.21 vs 3.17 ± 0.19 μmol/g, respectively) (n = 21, $P < .05$). Reactive oxygen species production was significantly increased in HepG2 cells cultured with HFFA compared with control cells (76.48 ± 5.31 vs 48.79 ± 4.36, respectively) (n = 21, $P < .05$) (Table 1). A partial correlation analysis was done on the levels of Mfn-2, ATP, and ROS. The data show that Mfn2 levels correlated positively with ATP levels ($R = 0.809$, $P = .003$), but negatively with ROS levels ($R = -0.715$, $P = .001$). These results indicate that there are physiologically relevant interrelationships between and among Mfn2, ATP, and ROS (Tables 2 and 3).

Table 1
Levels of ATP and ROS in HFFA-treated and control cells ($x \pm s$, n = 21)

Groups	ATP (μmol/g)	ROS (FI)
HFFA treated	3.17 ± 0.19*	76.48 ± 5.31*
Control	6.15 ± 0.21	48.79 ± 4.36

FI indicates fluorescence intensity.
* $P < .05$ vs control group.

Table 2
Partial correlation analysis among the 3 variables: Mfn2, ATP, and ROS

Control variables	2 Random variables	Correlation coefficient (R)	Significance (P)
ATP	Mfn2/ROS	−0.715	.001
ROS	Mfn2/ATP	0.809	.003

3.4. Omega-3 PUFAs induced Mfn2 transcription and increased protein levels in steatotic hepatocytes

Our data show that Mfn2 mRNA levels were significantly higher in DHA- or EPA-treated steatotic HepG2 cells compared with controls (Fig. 3A), as were Mfn2 protein levels (Fig. 3B).

3.5. Mfn2 is required for omega-3 PUFA-induced changes in mitochondrial morphology

Both EPA and DHA caused an increase in the length of mitochondrial tubules in most steatotic HepG2 cells (Fig. 4C-E). To study whether the change in mitochondrial morphology induced by omega-3 PUFAs was mediated mainly through Mfn2 activity, knockdown of Mfn2 was performed by RNA interference. Mitofusin 2 shRNA (h), which was designed to inhibit Mfn2 expression in human cells, and a scrambled control, shRNA, were separately transfected into steatotic HepG2 cells. Target-specific shRNA significantly decreased the expression of Mfn2 compared with control cells (Fig. 4A, B). Neither EPA nor DHA was able to promote mitochondrial elongation in Mfn2-depleted steatotic HepG2 cells (Fig. 4C-E). These data demonstrate the requirement for Mfn2 expression for omega-3 PUFA-mediated changes in mitochondrial morphology.

3.6. Omega-3 PUFAs increase the ATP level and decrease ROS production by up-regulation of Mfn2 expression in steatotic hepatocytes

Adenosine triphosphate levels were higher and ROS levels were lower than in control cells in steatotic HepG2 cells after incubation with DHA or EPA, but these levels in Mfn2-depleted cells were not influenced by the same treatment (Table 1). Therefore, the results presented in these experiments showed that up-regulation of Mfn2 is sufficient to effect the omega-3 PUFA-induced increase in ATP levels and decrease in ROS production in this steatotic hepatocyte model.

Table 3
Effect of omega-3 PUFAs on ATP levels and ROS production in Mfn2-shRNA–transfected and control steatotic HepG2 cells (mean ± SD, n = 21)

Groups	ATP (μmol/g)	ROS (FI)
EPA treated		
EPA + Mfn2-shRNA	3.87 ± 0.36	68.38 ± 4.33
EPA + control-shRNA	5.98 ± 025*	43.68 ± 5.76*
EPA + untransfected	6.11 ± 0.31*	45.62 ± 3.99*
Untransfected (control)	3.79 ± 0.29	69.07 ± 5.84
DHA treated		
DHA + Mfn2-shRNA	4.14 ± 0.21	67.69 ± 4.77
DHA + control-shRNA	6.33 ± 0.20†	42.71 ± 5.02†
DHA + untransfected	6.51 ± 0.33†	53.47 ± 3.81†
Untransfected (control)	4.18 ± 0.19	69.88 ± 4.45

* $P < .05$ vs control groups.
† $P < .05$ vs control groups.

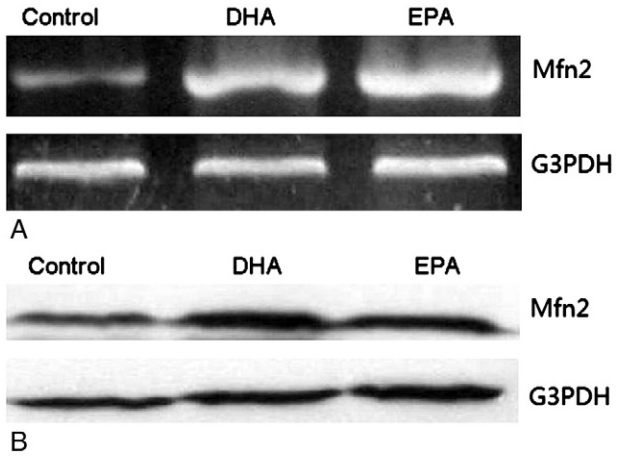
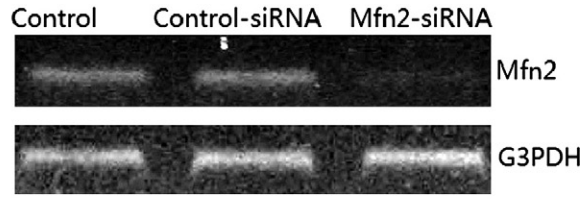


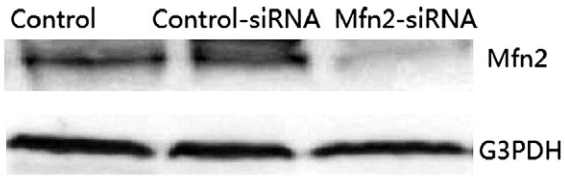
Fig. 3. Omega-3 polyunsaturated fatty acids up-regulate the expression of Mfn2 in steatotic hepatocytes. Steatotic HepG2 cells were incubated with DHA (50 μmol/L), EPA (50 μmol/L), or 0.05% dimethyl sulfoxide (control) for 1 hour. A, Reverse transcriptase PCR: Mfn2 mRNA levels in EPA- or DHA-treated cells were significantly higher (1.023 ± 0.031 and 1.109 ± 0.033, respectively) than in control cells (0.317 ± 0.033, n = 21, $P < .01$). B, Western blot: Mfn2 protein levels were significantly higher in EPA- or DHA-treated cells (0.975 ± 0.040 and 1.079 ± 0.034, respectively, n = 21, $P < .01$) than in control cells (0.514 ± 0.041). G3PDH was referenced to express results.

4. Discussion

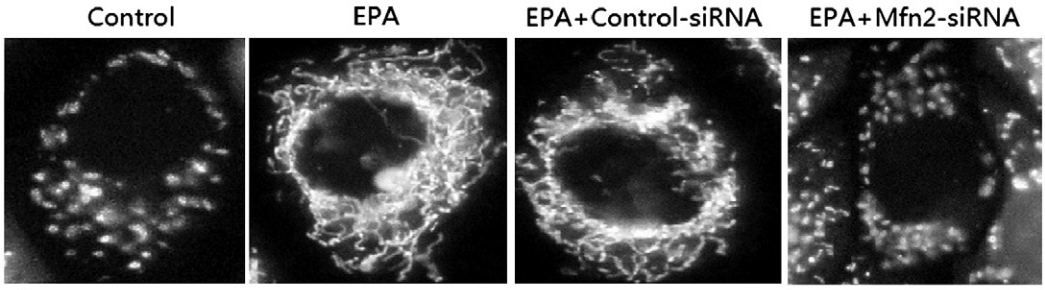
The omega-3 PUFAs are precursors for the long-chain products DHA and EPA. Several studies in rats have shown that an omega-3 PUFA-rich diet increases insulin sensitivity, reduces intrahepatic triglyceride content, and ameliorates steatohepatitis [33,34]. Some studies in NAFLD patients have shown that omega-3 PUFA supplementation decreases blood triacylglycerol concentrations, liver enzymes, fasting glucose, steatosis, and liver damage [35,36]. However, studies on the possible molecular mechanisms involved are still lacking. In this study, a steatotic hepatocyte in vitro model was created by treating HepG2 cells with HFFA. Abnormal mitochondrial morphologic changes resulted. We found a very extensive mitochondrial network in HepG2 cells; but in steatotic hepatocytes, fragmented mitochondria were observed. These results led us to conclude that overloading with FFAs results in an increase in mitochondrial fission. Although the mechanisms leading to obesity, insulin resistance, diabetes, hepatic steatosis, and NASH are not fully understood, recent evidence suggests the involvement of mitochondrial dysfunction [7,37-40]. Mitochondrial dysfunction, characterized by decreased activity of respiratory chain


Control
Control-siRNA
Mfn2-siRNA
Mfn2
G3PDH

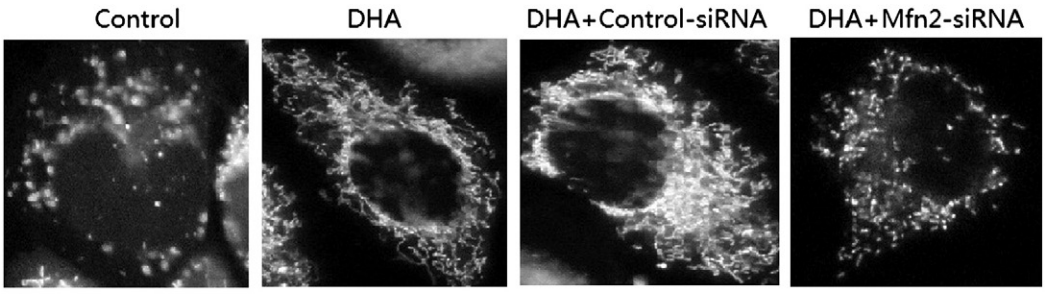
A


Control
Control-siRNA
Mfn2-siRNA
Mfn2
G3PDH

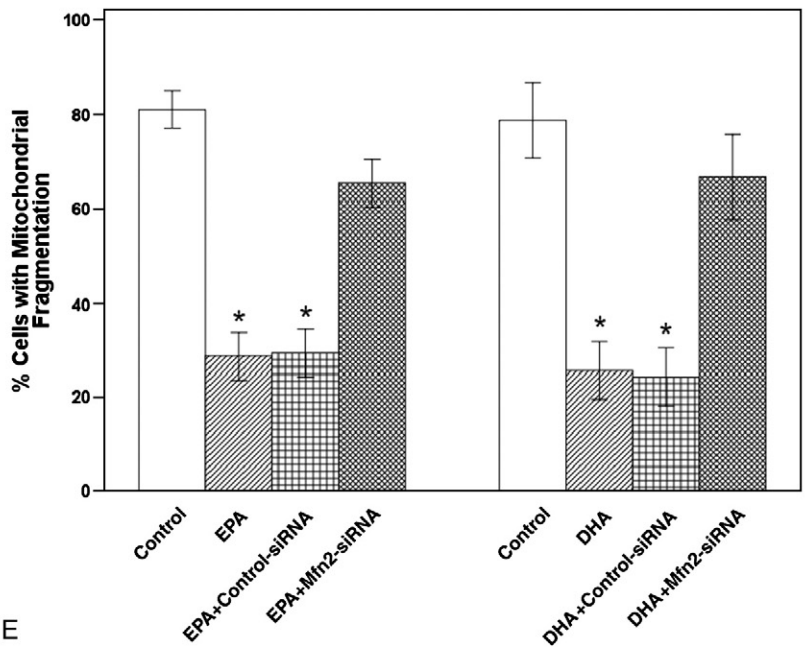
B


Control
EPA
EPA+Control-siRNA
EPA+Mfn2-siRNA

C


Control
DHA
DHA+Control-siRNA
DHA+Mfn2-siRNA

D


% Cells with Mitochondrial Fragmentation
100
80
60
40
20
0
*
*
*
*
Control
EPA
EPA+Control-siRNA
EPA+Mfn2-siRNA
Control
DHA
DHA+Control-siRNA
DHA+Mfn2-siRNA

E

complexes and impaired mitochondrial β-oxidation, makes the liver susceptible to the "second hit" phenomenon, including oxidative stress and decreased hepatic ATP production [41]. Mitochondrial dysfunction is crucial to the pathogenesis of NAFLD. Polyunsaturated fatty acids are involved in various mitochondrial processes including mitochondrial calcium homeostasis, gene expression, respiratory function, ROS production, and mitochondrial apoptosis [42]. Therefore, mitochondria play a central role in the mechanisms underlying the protective effects of PUFAs.

Tissues with high and variable rates of aerobic metabolism, such as skeletal or heart muscle, show a more prominent development of the mitochondrial network, although this remains unexplained. These data suggest that the mitochondrial network, or proteins that participate in its generation, may be involved in the control of mitochondrial energy metabolism. A recently identified mitochondrial membrane protein, Mfn2, a GTPase mitofusin protein, participates in the control of morphology and electrochemical operation of the mitochondrial network and in mitochondrial metabolism [11]. Although there is a link between Mfn2 and NAFLD, its cellular basis is poorly elucidated. We speculated that Mfn2 could also be a factor in the development of steatosis. Our data demonstrated that when the expression of Mfn2 was significantly inhibited, the network of mitochondria fragmented into short rods or spheres. Previous studies have proven that cellular repression of Mfn2 decreases the mitochondrial membrane potential and the cellular rate of glucose oxidation and represses mitochondrial proton leak, all occurring in the absence of changes in coupled respiration [18]. Overloading with HFFA can suppress the expression of Mfn2, demonstrating that Mfn2 may play a role in the progression of NAFLD.

The most important observation of our study was that both EPA and DHA exhibit an up-regulatory effect on expression of Mfn2 in steatotic HepG2 cells. We wondered whether it was linked to changes in mitochondrial morphology because mitochondrial elongation was simultaneously observed in these cells. To confirm the involvement of Mnf2 in omega-3 PUFA–induced changes in mitochondrial morphology, RNA interference was performed before incubation of the steatotic hepatocytes with omega-3 PUFAs. The levels of Mfn2 were reduced in cells transfected with the shRNA vector compared with cells transfected with the vector control. Treatment of steatotic cells with either EPA or DHA caused a change in mitochondrial morphology characterized by the recovery of mitochondrial network architecture, but no obvious changes in mitochondrial morphology were detected in Mfn2-depleted steatotic HepG2 cells. These results led us to conclude that omega-3 PUFAs regulate the expression of Mfn2 involved in mitochondrial dynamics in steatotic hepatocytes.

Several reports provide evidence that defective Mfn2 may contribute to impaired mitochondrial function [18,24]. On the basis of our observations, we propose that increased mitochondrial fusion caused by omega-3 PUFA-induced Mfn2 contributes to optimal mitochondrial activity in steatotic hepatocytes.

In addition, the production of extracellular ROS was assessed because mitochondria are the major cellular source of ROS and mitochondrial dysfunction can directly lead to ROS production. Our findings indicate that HFFA significantly increased ROS levels in steatotic HepG2 cells. Reactive oxygen species activate c-Jun N-terminal kinase, which regulates hepatocellular injury and insulin resistance; and it has been reported that patients with NASH have increased ROS production [43,44]. We found that EPA and DHA may decrease the production of ROS in steatotic HepG2, but omega-3 PUFAs were almost ineffective in cells treated with Mfn2 shRNA. Our in vitro data confirmed a significant decrease in ROS production in HepG2 cells treated with HFFA through up-regulation of Mfn2 expression.

Higher oxidative activity is associated with a decrease in the number of ATPs synthesized, and decreased ATP production is often found in patients with NAFLD [45,46]. Reactive oxygen species causes hepatocyte injury through up-regulating inflammatory molecules such as tumor necrosis factor–α and C-reactive protein [47]. Chronically elevated tumor necrosis factor–α levels may promote hepatocellular death through alterations in ATP synthesis. Loss of ATP results in an impairment of mitochondrial lipid metabolism. Our data showed that ATP levels were sharply reduced in HepG2 cells treated with HFFA, accompanied by suppression of Mfn2, whereas the ATP levels were significantly higher in steatotic HepG2 cells incubated with EPA and DHA. Oxygen electrode studies demonstrate that both endogenous and uncoupled respiration rates are reduced in Mfn2-null cells [24,48]. Attenuation of electron transport rates in respiration complexes I, III, and IV contributes to this decrease [49]. Uncoupled mitochondrial respiration leads to dissipation of the proton gradient generated by the respiratory chain, producing heat instead of ATP [50]. This

Fig. 4. Knockdown of Mfn2 using RNA interference and omega-3 PUFAs blocks mitochondrial fragmentation through increased expression of Mfn2 in steatotic HepG2 cells. Steatotic HepG2 cells were transfected with vector alone (scrambled sequence; control) or vector encoding small interfering RNAs (siRNAs) specific for Mfn2 knockdown. G3PDH was referenced to express results. A, Reverse transcriptase PCR: 24 hours after transfection, Mfn2 mRNA levels in Mfn2-siRNA–transfected cells (0.070 ± 0.019) were significantly lower than in control steatotic cells (0.385 ± 0.041, n = 21, $P < .01$) or control siRNA-transfected groups (0.312 ± 0.019, n = 21, $P < .01$). B, Western blot: Mfn2 protein levels in Mfn2-siRNA–transfected cells (0.069 ± 0.022) were significantly lower than in control steatotic cells (0.404 ± 0.036, n = 21, $P < .01$) or control shRNA-transfected groups (0.388 ± 0.022 n = 21, $P < .01$). Immunofluorescence microscopy reveals EPA-induced (C) and DHA-induced (D) morphologic changes in mitochondria of steatotic cells transfected with control and Mfn2 shRNA. E, Percentages of mitochondrial fragmentation were quantified by cell counting. Data are means ± SD of 3 separate experiments. *Significantly different from the control group.

is supported by the fact that the viability of ATP does not change when Mfn2 RNA interference cells are coincubated with omega-3 PUFAs. Our results demonstrated that Mfn2 is necessary for omega-3 PUFA-induced ATP production in steatotic hepatocytes.

In conclusion, the results of this study proved that omega-3 PUFAs may significantly increase mitochondrial fusion by up-regulating Mfn2 expression in steatotic hepatocytes. Moreover, omega-3 PUFAs may also increase ATP levels and reduce ROS production through Mfn2 activity. Although our study provides further support for the role of Mfn2 in the pathogenesis of NAFLD, the precise mechanisms by which omega-3 PUFAs regulate Mfn2 remain poorly understood. Still needing further research, in vitro and clinical setting then can establish the connection among omega-3 PUFAs, Mfn2, and NAFLD. Experimental studies exploring the effects of these compounds during NASH progression should be encouraged. In the future, increasing the expression of Mfn2 by omega-3 PUFA administration may become a novel therapeutic intervention for the treatment of NAFLD.

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