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# Metabolic factors in the development of hepatic steatosis and altered mitochondrial gene expression in vivo

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ABSTRACT

The objective of the study was to understand the role in vivo of elevated plasma free fatty acids (FFA), insulin, and glucose levels in the development of steatosis and altered mitochondrial gene/protein expression. We studied 4 groups of Sprague-Dawley rats: (1) high-fat diet (HFD), (2) high-dose streptozotocin–induced diabetes (T1DM), (3) low-dose streptozotocin–induced diabetic rats on an HFD (T2DM), and (4) controls. Liver histology and expression of genes/proteins related to mitochondrial fatty acid oxidation and biogenesis were analyzed. Despite an attempt to compensate by increasing expression of genes of fatty acid oxidation (carnitine palmitoyl transferase–1/medium chain acyl-CoA dehydrogenase), the HFD and diabetic groups developed marked steatosis and suffered a significant reduction in mitochondrial biogenesis gene expression (nuclear respiratory factor 1/transcriptional factor A, mitochondrial). In T2DM rats, the combination of high glucose and FFA unexpectedly did not lead to greater fat accumulation than HFD alone. Greater steatosis in HFD vs T2DM ($P < .001$) correlated with impairment in the gene expression of PPAR-$\alpha$ (ie, fatty acid oxidation) and PGC1$\alpha$, a major coactivator for mitochondrial biogenesis. Steatosis was not severe in insulin-deficient T1DM rats despite very elevated FFA and glucose levels. Increased carnitine palmitoyl transferase–1/medium chain acyl-CoA dehydrogenase/PPAR-$\alpha$ gene expression suggested inadequate adaptation to high FFA in both T1DM/T2DM rats. Hyperinsulinemia combined with elevated FFA is the key metabolic factor driving hepatic lipogenesis in vivo (HFD rats). Mitochondrial biogenesis (nuclear respiratory factor 1; transcriptional factor A, mitochondrial) is highly susceptible to FFA-induced steatosis. In contrast, hyperglycemia does not have an additive effect (T2DM) and leads to only a modest degree of steatosis in the absence of hyperinsulinemia, even when FFA are extremely elevated as in T1DM rats.



Author contributions: Shaoyun Wang and Amrita Kamat conducted the molecular measurements and assisted in data collection and analysis, data interpretation, and manuscript writing. Pablo Pergola developed the animal groups, followed them, performed all the animal tissue/plasma sample collections, and assisted in data collection and analysis, data interpretation, and manuscript writing. Anita Swamy conducted some of the molecular measurements and assisted in data collection and analysis. Fermin Tio performed the histologic readings. Kenneth Cusi participated directly and supervised all aspects of the study, including the study design, laboratory measurements, data collection and analysis, data interpretation, and manuscript writing.

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## 1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is an increasingly common public health problem [1]. New diagnostic techniques, such as magnetic resonance imaging and spectroscopy, suggest that NAFLD is much more common than previously believed [2,3]. Most patients with NAFLD have muscle/liver insulin resistance (IR), elevated plasma free fatty acids (FFA) from excessive rates of adipose tissue lipolysis, and impaired glucose tolerance or frank type 2 diabetes mellitus (T2DM) [4-7]. The relative contribution of these factors to hepatic steatosis has been challenging to dissect in human studies. Lifestyle modification leading to weight loss [8] and thiazolidinediones [9-12] have proven effective in reverting metabolic and histologic abnormalities in nonalcoholic steatohepatitis (NASH), at least in part by ameliorating hyperinsulinemia, FFA flux to the liver, and hyperglycemia [13,14].

Mitochondrial "dysfunction" (ie, impaired energy generation and fatty acid oxidation) plays an important role in the pathogenesis of NASH. Defects in mitochondrial function and morphology have been described in animal models of NASH and humans, associated with elevated plasma FFA and triglycerides (TG), hyperinsulinemia/IR, and hyperglycemia [15,16]. In humans, adipose tissue IR and increased FFA delivery to the liver are central in the development of lipotoxicity and steatohepatitis in obesity and T2DM [4,5,9,17]. In obesity, chronic hyperinsulinemia is associated with increased hepatic lipogenesis and impaired mitochondrial fatty acid oxidation. Development of NAFLD is common in T2DM, but little is known about hepatic steatosis in type 1 diabetes mellitus (T1DM). Chronic hyperglycemia may directly drive hepatic lipogenesis via activation of carbohydrate response element-binding protein [18]. Hyperinsulinemia, hyperglycemia, and elevated FFA appear to have an additive effect to stimulate hepatic TG accumulation, although previous studies have not attempted to examine these factors directly in vivo. Moreover, whether elevated plasma FFA and glucose may promote the development of a fatty liver in the absence of hyperinsulinemia in vivo is unclear. Without a better understanding of the interrelationship between these factors, it is unlikely that we will be able to develop rationale approaches to patients with NAFLD.

We examined the interplay of these factors on hepatic steatosis and the expression of genes/proteins related to mitochondrial fatty acid oxidation in 3 animal models: (*a*) a rodent model of lipid oversupply (ie, high-fat diet [HFD] group), (*b*) animals in a well-established nongenetic rodent model of T2DM induced by an HFD and low-dose streptozotocin (STZ) with mild to moderate hyperglycemia or T2DM group [19,20], and (*c*) animals given a high-dose of STZ to mimic T1DM (ie, high plasma FFA and glucose concentration but low insulin; T1DM group).

## 2. Research design and methods

### 2.1. Experimental design

All protocols followed the University of Texas Health Science Center at San Antonio Institutional Animal Care and Use Committee. Four groups of Sprague-Dawley rats were studied as validated in previous work [20] based on whether they were fed a regular chow or an HFD and whether they received STZ or not. As shown in Fig. 1, 6-week-old Sprague-Dawley rats weighing 175 to 200 g at study entry (Harlan, Indianapolis, IN) were fed for 5 weeks regular chow (n = 14) or an HFD (60% calories from fat, n = 12). The HFD used (TD 97209) was supplied by Harlan Laboratories (Madison, WI) and composed of 60% kcal from fat, with 73% of fat being from vegetable shortening (partially hydrogenated vegetable oil) and the remainder being hydrogenated coconut oil. Rats were housed in individual cages in a temperature-controlled room with 14/10-hour light-dark cycle (starting at 7:00 AM).

After 5 weeks, the regular chow and HFD rats were each divided into 2 groups. Regular chow rats received either 55 mg/kg of STZ (high-dose STZ, T1DM group, n = 6) or 0.1 mol/L citrate buffer vehicle (control group [CON], n = 8). The rats fed an HFD received either 35 mg/kg of STZ (HFD plus low-dose STZ, T2DM group, n = 6) or 0.1 mol/L citrate buffer vehicle (HFD group, n = 6). In the regular chow group, STZ was given by a single tail vein injection. Animals were followed for another 14 weeks and killed at the end of this observation period.

### 2.2. Collection of blood samples and liver tissue

Animals were fasted for 10 hours before obtaining plasma samples and being killed. Blood was obtained from the tail vein in tubes with 10 $\mu$L ethylenediaminetetraacetic acid, immediately placed in ice, centrifuged at 10 000 rpm (10 minutes), and stored at –80°C. Liver tissue was obtained immediately after the rats were killed and stored at –80°C until analysis.

### 2.3. Measurement of plasma hormones/cytokines and substrates

Plasma glucose was measured by standard glucose oxidase methods (Bayer Elite XL); and $A_{1c}$, by means of a DCA 2000 analyzer (Bayer, Tarrytown, NY). Plasma FFA were measured by standard colorimetric methods (Wako Chemicals, Neuss, Germany). Serum insulin, leptin, adiponectin, transforming growth factor (TGF)–$\beta$, and interleukin (IL)-1$\beta$ were determined by LINCOplex beads (Luminex Instrumentation, Millipore, NJ).

### 2.4. Histology

Liver tissues were stained with hematoxylin and eosin and oil red O. Histopathology was determined by a pathologist (FT)

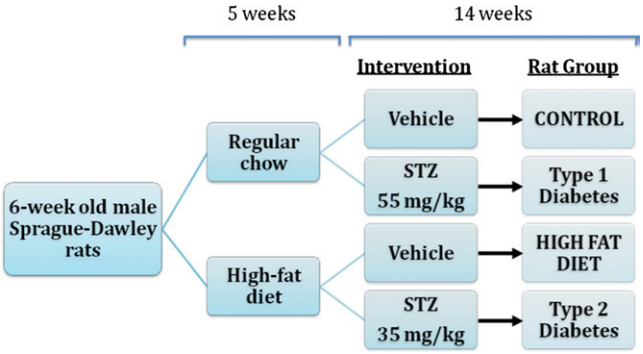



**Fig. 1 – Study design.**

who was blinded as to treatment/dietary group. Quantification of liver fat in the liver slides was made by determining the percentage area of the liver section occupied by the red fat globules isolated by the threshold segmentation method using the Sigma Scan Pro software (SYSTAT, Chicago, IL).

### 2.5. RNA extraction and quantification

RNA from liver tissue was extracted with Trizol (Invitrogen, Carlsbad, CA) and purified with RNeasy columns (QIAGEN, Valencia, CA) according to manufacturers' standard procedure. The quantity and quality of the RNA were determined by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

### 2.6. Determination of gene expression with real-time reverse transcriptase polymerase chain reaction

Gene expression was measured by real-time reverse transcriptase polymerase chain reaction (PCR) performed using a Taqman 100Rxn PCR Core Reagent Kit (Applied Biosystems, Branchburg, NJ). Real-time reverse transcriptase PCR was carried out in an ABI PRISM 7900 sequence detector (Applied Biosystems, Branchburg, NJ) using the following steps: 1 cycle at 48°C for 30 minutes and 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. A standard curve was performed by serial dilution of pooled total RNA, and each gene/sample was compared with this standard curve. Primer probe sets were purchased from Applied Biosystems (Foster City, CA).

### 2.7. Protein extraction and quantification

Proteins were extracted from liver tissue with RIPA buffer from Pierce (Rockford, IL). Protein concentration and quantity were determined with BCA kit from Sigma (Saint Louis, MO).

### 2.8. Determination of protein expression by Western blotting

Equal amounts of protein (10 $\mu$g) were loaded and electrophoresed on the 12% Tris-HCl gel from BioRad (Hercules, CA) and transferred to polyvinylidene difluoride membrane (BioRad). The membrane was then rinsed twice with 20 mmol/L Tris, 150 mmol/L NaCl, and 0.05% Tween 20 (pH 7.5) and blocked for 1 hour at room temperature, followed by 1-hour room-temperature incubation in primary antibody from Santa Cruz Biotechnology (Santa Cruz, CA) with 1:200 dilution. Antimouse or antirabbit (1:20 000 dilution) was used as a secondary antibody as per protocol. Signals were detected by Immun-Star HRP Substrate from BioRad. Immunoblots were quantified by densitometric analysis, and mitochondrial protein values were normalized to loading control ($\beta$-actin) for each sample. Bar graphs represent means ± SE of normalized mitochondrial protein values/$\beta$-actin values expressed as fold change.

**Table 1 – Serum biochemical markers and cytokines of animals at week 14**

| | CON | HFD | T1DM | T2DM |
|---|---|---|---|---|
| Glucose (mg/dL) | 76 ± 2.4 | 84 ± 5 | 395 ± 20[†] | 301 ± 19[*,†,‡] |
| $HbA_{1c}$ (%) | 3.1 ± 0.12 | 3.3 ± 0.09 | 6.3 ± 0.43[*] | 7.0 ± 0.36[*] |
| Insulin (pg/mL) | 0.51 ± 0.03 | 0.65 ± 0.17 | 0.14 ± 0.05[*,†] | 0.67 ± 0.19[#] |
| FFA ($\mu$mol/L) | 310 ± 30 | 620 ± 100[*] | 830 ± 150[*] | 720 ± 80[*] |
| TG (mg/dL) | 60 ± 5 | 80 ± 18 | 295 ± 63[*,†] | 278 ± 71[*,†] |
| Cholesterol (mg/dL) | 86 ± 3.14 | 169 ± 35[*] | 122 ± 10[*] | 103 ± 7 |
| Adiponectin ($\mu$g/mL) | 4.7 ± 0.3 | 4.3 ± 0.2 | 1.9 ± 0.1[*,†,‡] | 4.8 ± 0.3 |
| Leptin (ng/mL) | 3.0 ± 0.1 | 8.0 ± 0.9[*] | 0.2 ± 0.03[*,†,‡] | 3.6 ± 0.4[†,‡] |
| TGF-$\beta$ (ng/mL) | 0.9 ± 0.6 | 77.3 ± 7.1[*,§] | 63.7 ± 10.5[*] | 62.3 ± 7.0[*] |
| IL-1$\beta$ (pg/mL) | ¥ | 69.4 ± 34.6[§] | 21.6 ± 8.0 | 22.7 ± 11.7 |

Plasma biochemical markers and cytokines measured in Sprague-Dawley rats at week 14. In 2 T2DM rats, insulin could not be measured because of hemolysis. $HbA_{1c}$ indicates hemoglobin $A_{1c}$; ¥, undetectable.
[*] $P < .05$-.01 vs CON.
[†] $P < .05$-.01 vs HFD.
[‡] $P < .05$-.01 T1DM vs T2DM.
[§] $P < .05$ HFD vs T1DM and T2DM.
[#] $P < .01$ vs T1DM rats.

### 2.9. Statistical analysis

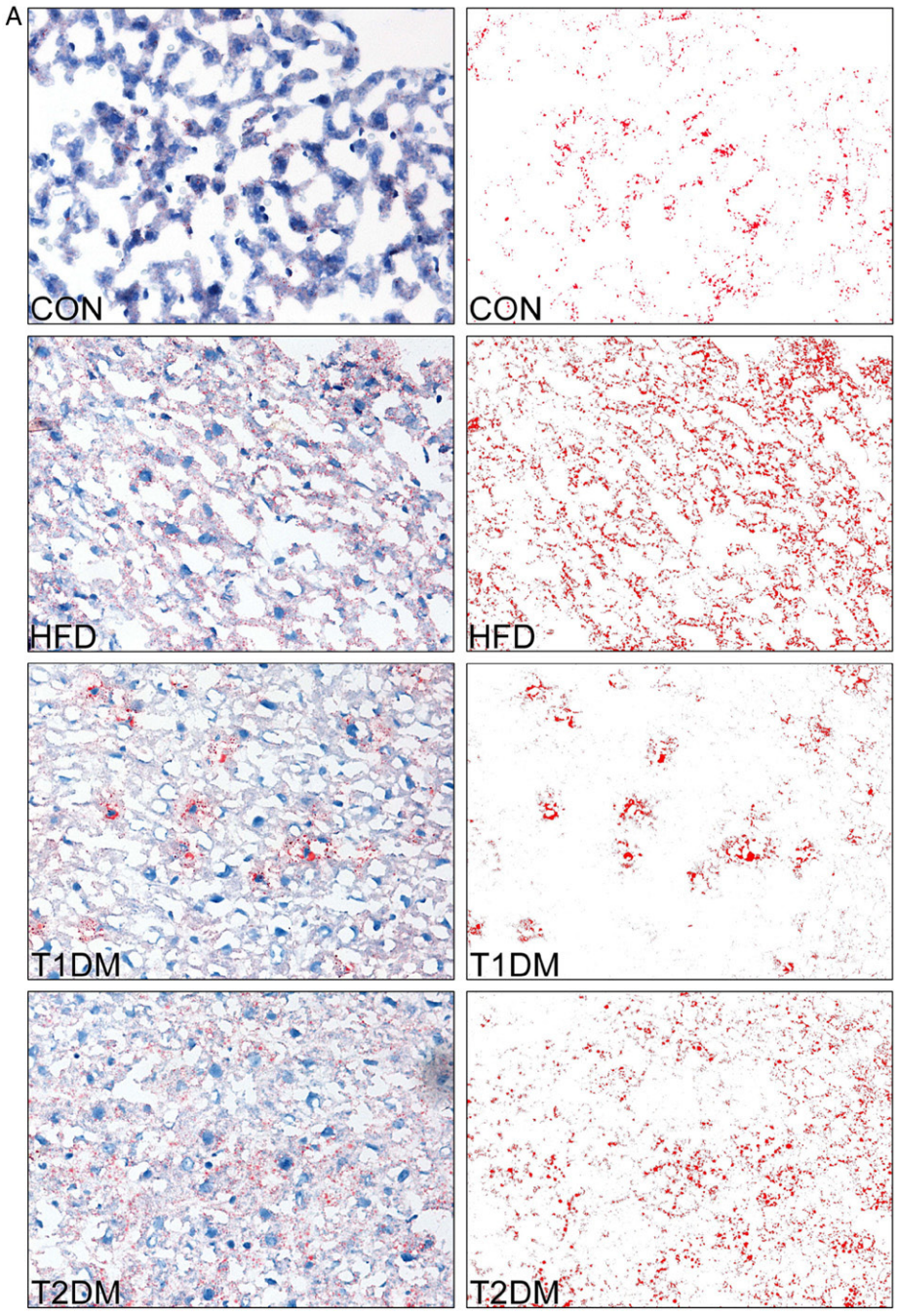
Significant differences were determined by multiple sample comparison methods analysis of variance using JMP software (SAS Institute, Cary, NC). Pearson correlations were performed among related variables. Differences were considered significant at $P < .05$. Data are presented as mean ± SEM.

## 3. Results

### 3.1. Biochemical and hormonal characteristics

Table 1 summarizes the biochemical and hormonal characteristics of the rat groups studied. The HFD and diabetic rats had elevated plasma FFA compared with CON ($P < .01$), with the highest level belonging to T1DM animals. The HFD group had a 2-fold higher plasma FFA but normal plasma glucose

**Fig. 2 – A, Representative fields of rat liver from CON, HFD, T1DM, and T2DM animals stained for neutral fat with oil red O stain and hematoxylin counterstain before (left panel) and after thresholding (right panel) for quantification of the red staining fat content (oil red O, original magnification 28.3×). B, Percentage liver fat from each group as quantified by determining the liver area occupied by the red fat globules isolated by the threshold segmentation method using the Sigma Scan Pro software (SYSTAT). Liver fat was highest in HFD (9.8% ± 0.5%) compared with T2DM (5.5% ± 0.5%), T1DM (2.3% ± 0.8%), and CON (1.2% ± 0.1%). [*]P < .05-.001 vs CON; [#]P < .001 vs HFD; [†]P < .02 T1DM vs T2DM.**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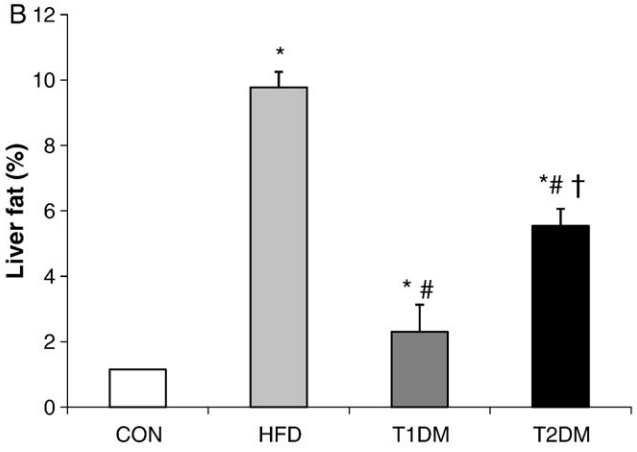
B
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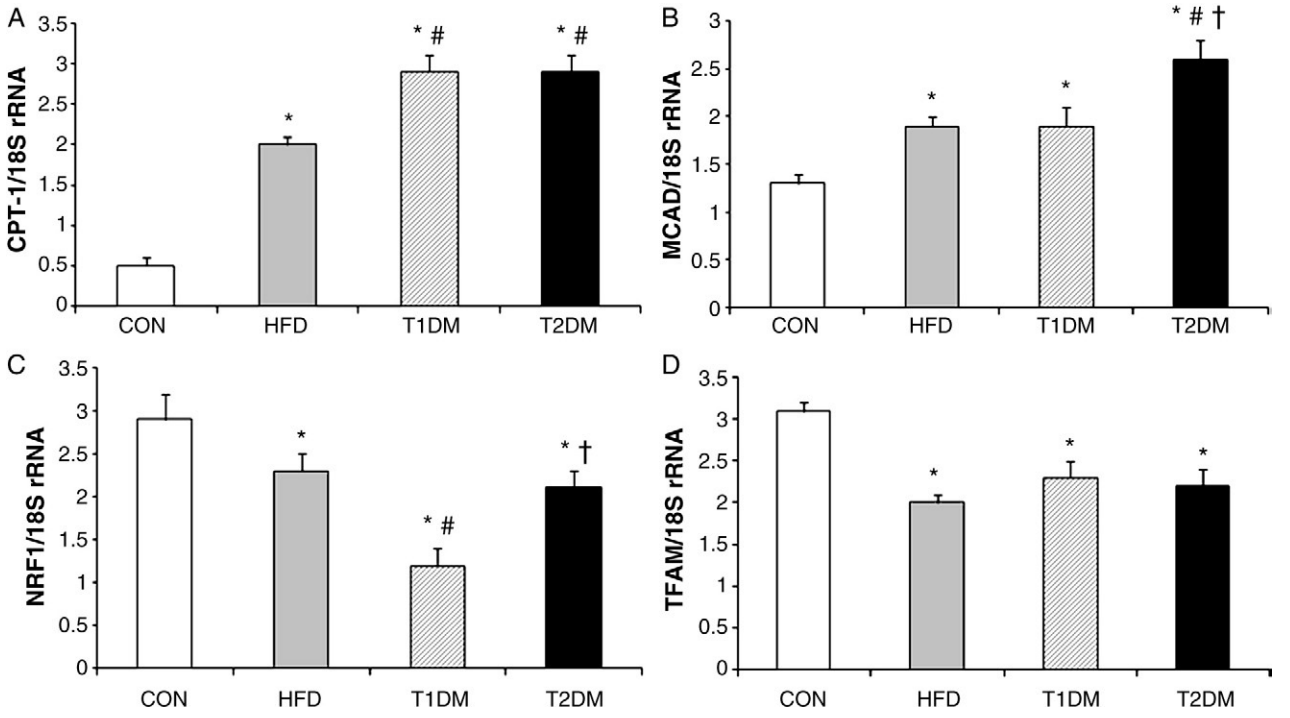


**Fig. 3 – Mitochondrial gene expression of CPT-1 (A), MCAD (B), NRF1 (C), and TFAM (D) in CON, HFD, T1DM, and T2DM rats. The CPT-1 and MCAD gene expressions were markedly increased ($P < .001$), whereas NRF1 and TFAM was decreased ($P < .05$-01), in HFD, T2DM, and T1DM rats compared with CON. In each experiment, mRNA levels were normalized to 18S rRNA. *$P < .05$-.001 vs CON; #$P < .001$ vs HFD; †$P < .02$ T1DM vs T2DM.**

and TG levels compared with CON. The HFD rats were also characterized by significantly higher plasma leptin, TGF-$\beta$, and IL-1$\beta$ vs diabetic rats ($P < .05$-.01). The 2 diabetic groups had very high plasma glucose and TG ($P < .001$ vs HFD/CON), differing as expected when creating the animal models regarding plasma insulin concentrations: low in the T1DM and elevated (but with an inadequate increase for the degree of hyperglycemia) in the T2DM rats.

### 3.2. *Changes in body weight*

Rats fed an HFD for 5 weeks were heavier than those fed a regular rat chow diet at the time of STZ/vehicle injection (370 ± 18 vs 348 ± 12 g, $P < .05$). During the following 14 weeks, HFD rats gained the most weight (HFD: 494 ± 14 g vs nondiabetic CON: 458 ± 13 g) and were heavier than T2DM and T1DM animals throughout the study period and by the end of the study at 14 weeks (378 ± 26 and 324 ± 20 g, respectively; both $P < .01$ vs HFD).

### 3.3. *Hepatic steatosis in HFD, T1DM, and T2DM animals*

Liver fat was highest in HFD, followed by T2DM and T1DM, all significantly higher than the CON (9.77% ± 0.48%, 5.54% ± 0.52%, 2.30% ± 0.83%, and 1.15% ± 0%, respectively; all $P < .001$ vs CON; HFD vs T1DM/T2DM, both $P < .01$; as shown in Fig. 2). There was a direct correlation between the degree of hepatic steatosis and plasma insulin ($r = 0.56$, $P < .05$), TGF-$\beta$ ($r = 0.58$, $P < .05$), and leptin ($r = 0.82$, $P < .01$) concentrations.

### 3.4. *Elevated plasma FFA promote mitochondrial fatty acid oxidation but impair mitochondrial biogenesis gene/protein expression in vivo*

We examined the role of elevated plasma insulin, FFA, and glucose on mitochondrial function by examining gene/protein expression of key enzymes involved in fatty acid transport/oxidation, such as fatty acid carnitine palmitoyl transferase–1 (CPT-1) and medium chain acyl-CoA dehydrogenase (MCAD), and in mitochondrial biogenesis, such as nuclear respiratory factor 1 (NRF1) and transcriptional factor A, mitochondrial (TFAM) (Figs. 3 and 4).

As shown in Fig. 3A, elevated plasma FFA increased CPT-1 messenger RNA (mRNA) (the transporter of fatty acids from cytoplasm to the mitochondria) in HFD, T2DM, and T1DM rats compared with CON (4- to 6-fold increase, all $P < .001$). Medium chain acyl-CoA dehydrogenase (a key enzyme for fatty acid oxidation) was also increased in HFD, T2DM, and T1DM animals compared with CON by 46%, 100%, and 46%, respectively (all $P < .001$; Fig. 3B). Protein expression of MCAD paralleled gene expression within each group (Figs. 3B and 5A), suggesting a functional correlate between gene overexpression and their protein levels stimulated by metabolic factors. A close correlation was observed between plasma FFA and the increase in CPT1 (Fig. 4A) and MCAD (Fig. 4B) gene expression ($P < .05$).

In contrast, mitochondrial biogenesis was greatly affected by FFA as manifested by a decrease in NRF1 in HFD, T2DM, and

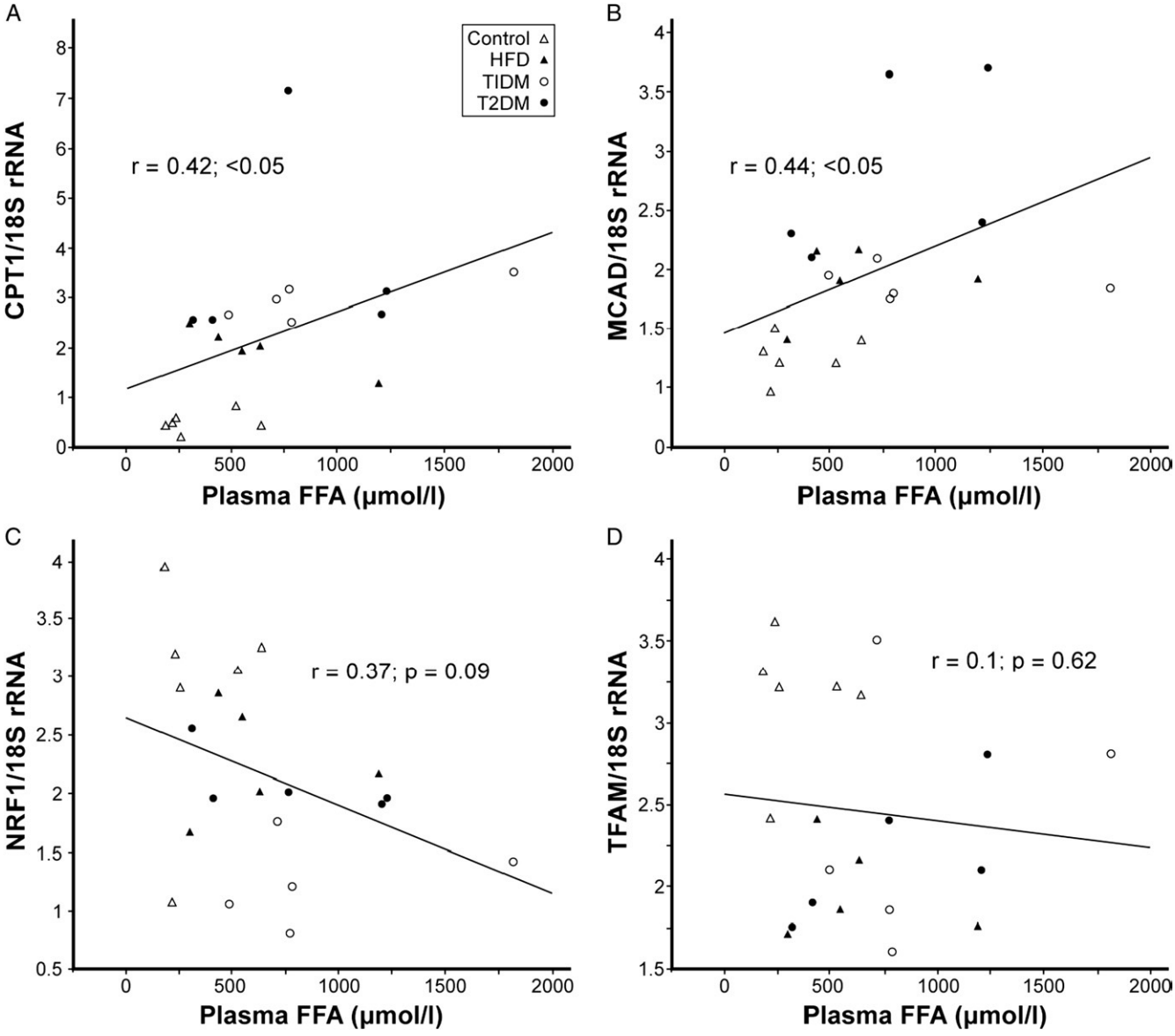


**Fig. 4 – Correlations between plasma FFA concentration and CPT-1 (A), MCAD (B), NRF1 (C), and TFAM (D) in CON (open triangles), HFD (closed triangles), T1DM (open circles), and T2DM (closed circles) rats.**

T1DM animals of –21%, –26% and –60%, respectively (all $P <$ .05-.01 vs CON; Fig. 3C), which had a negative correlation with serum FFA concentration ($P = .09$, Fig. 4C). Plasma FFA also reduced TFAM gene expression in HFD, T2DM, and T1DM by –33%, –27%, and –26%, respectively (all $P < .01$ vs CON, Fig. 4D). As above, Western blot protein quantification of NRF1 paralleled the respective gene expression within each group (Fig. 5B), with a similar trend for TFAM ($P < .01$ for T1DM and T2DM rats vs CON, data not shown).

PGC1$\alpha$ gene expression, a major coactivator for mitochondrial biogenesis, was significantly decreased in HFD and T2DM (–62% and –30%, respectively; both $P < .05$) but not in T1DM rats. The magnitude of this reduction was negatively correlated to liver fat accumulation ($r = –0.57$, $P = .01$). Western blot analysis of PGC1-$\alpha$ protein abundance demonstrated a severe down-regulation of PGC1$\alpha$ in both diabetic and obese rats (Supplemental Fig. 1) that correlated very strongly and inversely with liver fat ($r = –0.84$, $P < .01$).

### 3.5. *PPAR$\alpha$ and PPAR$\gamma$ gene expressions are up-regulated in diabetic rats, but not in HFD rats with elevated plasma insulin and FFA levels*

As shown in Fig. 6A, gene expression of PPAR$\alpha$, modulator of hepatic fatty acid oxidation, was markedly increased in diabetic animals. Compared with CON, it increased by 18% in T1DM ($P < .001$ vs HFD rats) and 54% in T2DM ($P < .001$ vs CON and HFD, $P < .01$ T1DM vs T2DM) animals. A similar trend was observed for PPAR$\gamma$ gene expression, with an increase in diabetic animals compared with CON rats that only reached statistical significance in the T2DM group ($P < .01$, Fig. 6B).

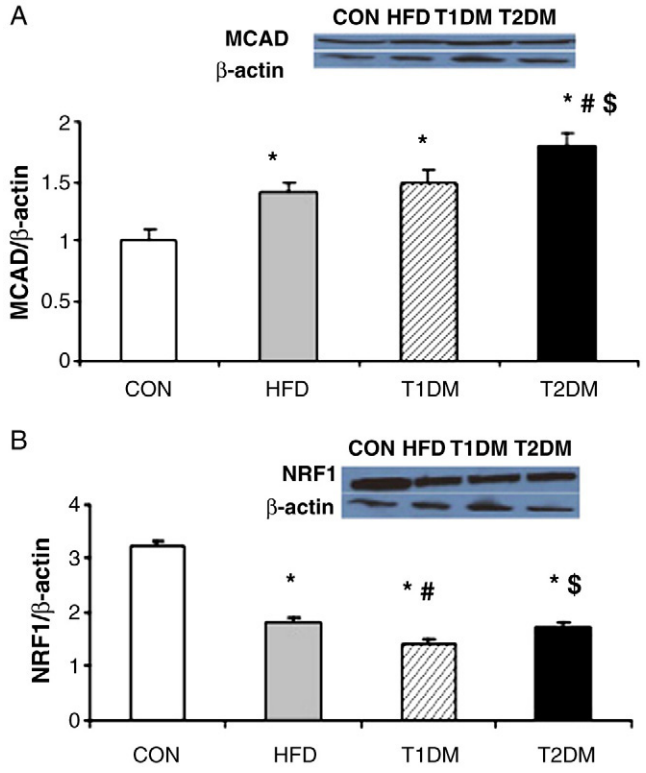


**Fig. 5 – Mitochondrial biogenesis protein expression. Western blots of MCAD (A) and NRF1 (B) in HFD, T1DM, and T2DM rats compared with CON. Protein levels were measured by Western blotting of rat liver tissue lysates, as described in "Research design and methods." The MCAD gene expression was markedly increased ($P < .001$), whereas NRF1 was decreased ($P < .001$), in HFD, T2DM, and T1DM rats compared with CON. $^{*}P < .001$ vs CON; $^{\#}P < .001$ vs HFD; $^{\$}P < .001$ T1DM vs T2DM.**

## 4. Discussion

Elevated plasma FFA and hyperglycemia are main factors for the development of NAFLD [21], but their relative contribution to hepatic fat accumulation is unclear. We took advantage of well-characterized rodent models to study them in vivo in 3 distinct animal models: (*a*) a model evoking clinical obesity with dietary-induced steatosis (normoglycemic but with a high plasma FFA level; HFD group); (*b*) a model of T1DM, with very high plasma FFA and glucose concentrations but with insulinopenia (T1DM group); and (*c*) a model of T2DM fed an HFD, with a more moderate increase in plasma FFA and glucose concentrations (T2DM group). Our results indicate that elevated plasma FFA level (in the presence of adequate insulin levels as in HFD and T2DM rats) is the key determinant of the severity of hepatic steatosis. Compared with the healthy control group, hepatic fat accumulation was highest (8-fold) under the metabolic conditions of the HFD group. In these animals, significant steatosis developed with just an approximately 2-fold elevation in plasma FFA, levels of FFA observed in human obesity. In contrast, minimal steatosis developed in T1DM animals, although plasma FFA (~3-fold) and glucose (~5-fold) concentrations were much higher than in HFD rats. Moreover, hyperglycemia in T2DM animals did not further exacerbate steatosis, an observation of clinical relevance. These findings serve as proof-of-concept of the important role of excess FFA availability (in the presence of permissive plasma insulin levels) to hepatic liver accumulation, whereas hyperglycemia per se plays a minor role. Perhaps even more important to the pathophysiology of NAFLD, we observed that varying degrees of hepatic steatosis (ranging from 1.5- [T1DM] to 8.0-fold [HFD] above CON) led to similar alterations of mitochondrial gene/protein expression, indicating a relatively low threshold for FFA-induced mitochondrial dysfunction. Taken together, these observations have valuable clinical implications to our understanding of the mechanisms involved in the development of NAFLD in vivo.

The strong relationship between the increase in plasma FFA with CPT-1 and MCAD gene expression (Fig. 4A and B, respectively) indicates an adaptive increase in mitochondrial transport and oxidation, respectively, to excess lipid supply. However, in HFD animals, there was evidence of FFA-induced mitochondrial dysfunction. This was based on the inverse relationship between plasma FFA and NRF1/TFAM (Fig. 4C and D) and the reduction in gene expression and protein abundance of NRF1 (Fig. 5B). Nuclear respiratory factor 1 and TFAM are extremely important to stimulate mitochondrial biogenesis [22,23]. In the regulation of hepatic glucose and lipid metabolism, PPAR-$\alpha$ ligands also play a key role to activate peroxisomal and mitochondrial genes encoding for fatty acid $\beta$-oxidation [16,23,24]; and loss of adequate PPAR-$\alpha$ and PGC-1$\alpha$

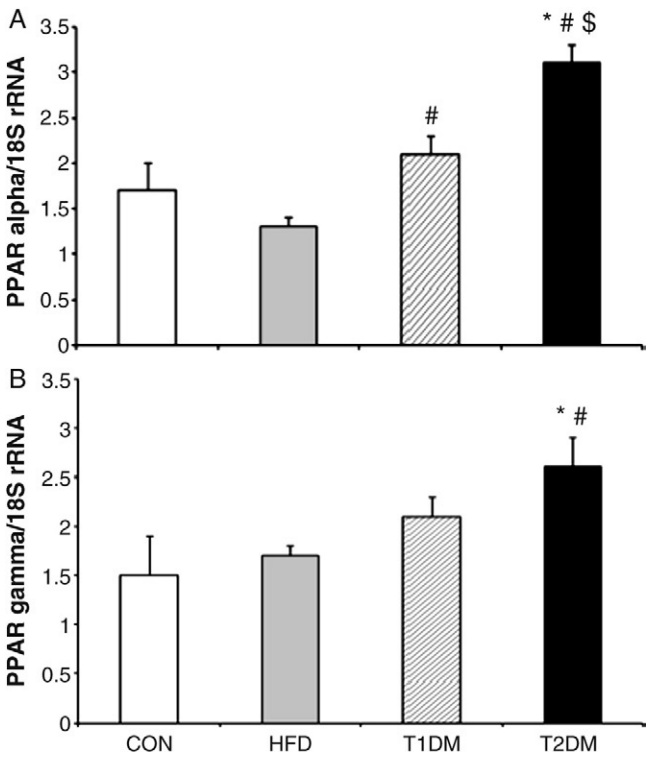


**Fig. 6 – PPAR$\alpha$ (A) and PPAR$\gamma$ (B) gene expression in HFD, T1DM, and T2DM rats compared with CON. $^{*}P < .001$ vs CON; $^{\#}P < .001$ vs HFD; $^{\$}P < .01$ T1DM vs T2DM. In each experiment, mRNA levels were normalized to 18S rRNA.**

gene function leads to hepatic steatosis [25-30]. Although the data in vivo are limited, one may speculate that chronic hyperinsulinemia successfully avoided excessive rates of hepatic glucose production and hyperglycemia in HFD rats by inhibiting gluconeogenesis, at the expense of promoting hepatic lipogenesis and impairing fatty acid oxidation, as recently suggested in humans [31]. The decrease in the transcription of NRF1 and TFAM in HFD animals coupled with a poor PPAR-α compensation likely affected other nuclear-encoded mitochondria subunits of the electron transport chain complex and promoted steatosis [32].

We also found that PGC-1α gene expression and protein abundance were significantly decreased in HFD and T2DM rats and kept an inverse relationship to hepatic fat accumulation. Mice heterozygous for liver-specific PGC-1α (having only one functional PGC-1α allele) develop hepatic IR as a consequence of a mild but chronic reduction in fatty acid oxidation [33]. This is consistent with studies in rodents [34] and humans [35] in which an experimental increase in plasma FFA decreases skeletal muscle PGC-1α gene expression. Obese [36] and nonobese [35] insulin-resistant subjects have decreased muscle and adipose tissue PGC-1α gene expression that may improve with physical training [36], although this response appears to be blunted when compared with that of insulin-sensitive individuals [37]. The role of PGC-1α and other nuclear-encoded mitochondrial genes in human disease remains incompletely understood. However, it is believed to play an important role in the adipose tissue inflammation and resistance to the action of insulin frequently observed in obesity [16,17,38]; and the resulting elevation of plasma FFA concentration plays an important role in the pathogenesis of NAFLD [39] and NASH [9,40].

Other factors could have also contributed to steatosis in HFD animals, such as leptin deficiency and/or resistance [41,42]. An HFD may cause leptin resistance in rodents [43], being consistent with the close correlation between plasma leptin levels and liver fat accumulation in this study. Reduced leptin receptor gene expression has been reported in obese individuals with NASH [44], although the role of leptin in NAFLD in humans remains controversial [45]. There are also leptin receptors on hepatic stellate cells, and the hormone plays a critical role in hepatic fibrogenesis [46]. Leptin resistance may explain the elevated levels of TGF-β in HFD rats, a cytokine closely involved in the activation of hepatic stellate cells and liver fibrosis. Adiponectin resistance can also be invoked for the observed hepatic steatosis in HFD rats, as plasma levels were similar to CON but failed to activate PPAR-α gene expression and prevent a fatty liver. This is consistent with a recent report of hepatic steatosis with reduced AMPK and PPAR-α activation due to adiponectin resistance in a mice model of NASH fed a methionine- and choline-deficient diet [47]. Impaired PPAR-α activation/fatty acid oxidation and hepatic steatosis due to leptin and adiponectin resistance have been also recently reported in fructose-fed rats [48]. Other factors, such as aging and in obesity induced by HFD, may also reduce plasma adiponectin concentration and alter adiponectin receptor expression (AdipoR1 and AdipoR2) and the response to the hormone in muscle and liver [49,50]. In obese [51] and nonobese [52,53] insulin-resistant subjects, plasma adiponectin levels and/or adiponectin receptors in target tissues are reduced, a finding also reported in liver [54] and adipose tissue [55] of patients with NASH. The important metabolic role of adiponectin in NASH has been recently highlighted by the clinical observation of a close correlation between the increase in plasma adiponectin concentration with thiazolidinedione treatment and improvement in insulin sensitivity and liver histology [56].

An unexpected finding in our T1DM and T2DM rat models with severe hyperglycemia was that the increase in liver fat was much less than in the HFD group. In the setting of insulin deficiency (ie, T1DM model), elevated FFA and glucose alone had a minimal ability to promote steatosis (Fig. 2). This is consistent with clinical reports of minimal steatosis in patients with poorly controlled T1DM [57], although this has not been systematically examined in humans. PPAR-α resistance has been recently reported in hyperglycemic (ZDF) rats [58]. Absolute (ie, T1DM) or relative (ie, T2DM) insulin deficiency also promotes very low-density lipoprotein oversecretion. Both observations (ie, PPAR-α resistance with FFA shifting from oxidation to lipogenesis and hepatic very low-density lipoprotein TG oversecretion) could explain the dramatic increase in plasma TG concentration observed in T1DM and T2DM groups. Of note, a lesser degree of steatosis in diabetic rats compared with HFD rats may be explained by the observation that FFA derived from plasma carbohydrates Of note, a lesser degree of steatosis in diabetic rats compared to HFD rats may be explained by the observation that FFA derived from plasma carbohydrates (so-called "new fat" [59]) in hyperglycemic T1DM/T2DM rats would be a powerful stimulus for fatty acid oxidation through PPAR-α (Fig. 6) and CPT1/MCAD (Fig. 3) activation.

Given the multiple effects of PPAR-γ on lipid/glucose metabolism, energy balance, and inflammation [13-18], we were interested in observing the effect of FFA, insulin, and glucose on hepatic PPAR-γ gene expression. In mice, PPAR-γ activation in the liver is adipogenic [47]; and liver-specific PPAR-γ–deficient animals are protected from steatosis [24,60]. Consistent with this, we observed that PPAR-γ was markedly increased in T2DM rats. However, our group has established that thiazolidinediones reduce steatosis and have anti-inflammatory effects in patients with NASH and T2DM [9]. Others have confirmed these findings in nondiabetic patients with NASH [10-12]. One may speculate that rosiglitazone and pioglitazone function as partial PPAR-γ agonists [60] that may exert their effects by partially preventing overt activation of hepatic PPARγ receptors, a hypothesis that awaits confirmation in patients with NASH. However, caution must be taken before extrapolating results in rodents to humans. One should be aware that both PPAR-α and PPAR-γ receptors are much more abundant in rodent liver than in humans and that there are significant differences between both species regarding the metabolic effects of stimulating PPAR pathways in rodent models of diabetes and/or NAFLD. For example, fenofibrate markedly reduces hepatic steatosis and improves hepatic and muscle insulin sensitivity in mice models of HFD-induced IR and steatosis [47]. In contrast, fenofibrate does not improve liver transaminases or hepatic/muscle insulin sensitivity in obese subjects with the metabolic syndrome [51,61]. A similar discordance between species occurs with rosiglitazone, as it has been reported to improve elevated liver transaminases and steatosis in patients with NASH [10]; but in *ob/ob* mice,

rosiglitazone increases hepatic transaminases and worsens necroinflammation and steatosis [62].

In summary, this study provides a comprehensive look at how FFA and hyperglycemia may promote hepatic steatosis and mitochondrial dysfunction in vivo. Although the cross talk between these factors is complex, elevated plasma FFA concentrations associated with adequate or increased plasma insulin levels emerge as the major determinants for the development of hepatic steatosis in vivo. Our work also suggests a low threshold for alterations in mitochondrial biogenesis gene expression, as observed even with a modest liver fat accumulation (ie, T1DM). From a clinical perspective, our results render support for targeting dysfunctional adipose tissue and increased FFA availability in NAFLD, either by lifestyle interventions or pharmacologic therapy with thiazolidinediones. Future work should focus on the underlying mechanism(s) of FFA-induced IR and lipotoxicity in human disease.

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