

# Fatty liver is associated with impaired activity of PPAR $\gamma$ -coactivator 1 $\alpha$ (PGC1 $\alpha$ ) and mitochondrial biogenesis in mice

Michal Aharoni-Simon, Michal Hann-Obercyger, Svetlana Pen, Zecharia Madar and Oren Tirosh

Accumulating evidence indicates that mitochondria have a key role in non-alcoholic fatty liver disease (NAFLD). C57BL/6J mice were fed a choline-deficient, ethionine-supplemented (CDE) diet. Histological studies demonstrated accumulation of fat vacuoles in up to 90% of hepatocytes in mice fed the CDE diet for 14 days. In addition, a decrease in mitochondrial levels, together with an increase in superoxide radicals' levels were observed, indicating elevation of oxidative stress in hepatocytes. ATP levels were decreased in livers from CDE-fed mice after overnight fasting. This was accompanied by a compensative and significant increase in peroxisome-proliferator-activated receptor- $\gamma$  coactivator 1 $\alpha$  (PGC1 $\alpha$ ) mRNA levels in comparison to control livers. However, there was a reduction in PGC1 $\alpha$  protein levels in CDE-treated mice. Moreover, the expression of mitochondrial biogenesis genes nuclear respiratory factor 1 (NRF-1), mitochondrial transcription factor A (TFAM), mitochondrial transcription factor B1 (TFB1M) and mitochondrial transcription factor B2 (TFB2M), which are all regulated by PGC1 $\alpha$  activity, remained unchanged in fasted CDE-treated mice. These results indicate impaired activity of PGC1 $\alpha$ . The impaired activity was further confirmed by chromatin immunoprecipitation analysis, which demonstrated decreased interaction of PGC1 $\alpha$  with promoters containing NRF-1 and NRF-2 response elements in mice fed the CDE diet. A decrease in PGC1 $\alpha$  ability to activate the expression of the gluconeogenic gene phosphoenol-pyruvate carboxykinase was also observed. This study demonstrates, for the first time, that attenuated mitochondrial biogenesis in steatotic livers is associated with impaired biological activity of PGC1 $\alpha$ .

*Laboratory Investigation* (2011) 91, 1018–1028; doi:10.1038/labinvest.2011.55; published online 4 April 2011

**KEYWORDS:** liver damage; mitochondrial biogenesis; ROS; steatosis

Non-alcoholic fatty liver disease (NAFLD) is becoming more common in Western countries. Non-alcoholic steatohepatitis (NASH) is found in a subset of NAFLD patients who have, in addition to excess fat, evidence of characteristic hepatocellular injury and necro-inflammatory changes.<sup>1</sup> Wanless and Lentz<sup>2</sup> found steatosis in 70% of obese and 35% of lean patients in a consecutive autopsy study. Among obese patients, the prevalence of simple steatosis is about 60%, whereas NASH is found in 20–25% of all subjects.<sup>3</sup>

Fatty liver is found in most men and postmenopausal women when deprived of dietary choline.<sup>4</sup> Choline is a precursor of the membrane phospholipid phosphatidylcholine, which is required for the release of very low-density lipoprotein from the liver. In animal models, choline-deficient (CD) diet leads to an increase in liver triglyceride content and development of NAFLD. If the diet continues,

the liver develops NASH, fibrosis, and cirrhosis, with some animals progressing to hepatocellular carcinoma.<sup>5</sup> It was found that liver injury is more severe when adequate levels of folate and methionine are lacking during a CD diet.<sup>6</sup> Therefore, we have used in the current study a model of choline deficiency and ethionine, a methabolic antagonist of methionine, supplemented diet (CDE diet), which represents steatosis and liver injury.<sup>7–9</sup>

There is accumulating evidence that mitochondria have a key role in NAFLD.<sup>10,11</sup> Mitochondria have many functions in the cell, including  $\beta$ -oxidation, regulation of cell viability and death, and creating ATP in the oxidative phosphorylation machinery.<sup>12</sup> Superoxide radicals ( $O_2^{\bullet-}$ ), generated in the mitochondria as a by-product of the respiratory chain process, can be further transformed into different reactive oxygen species (ROS) such as hydrogen peroxide ( $H_2O_2$ ) and

The School of Nutritional Sciences, Institute of Biochemistry, Food Science and Nutrition, The Robert H Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, Rehovot, Israel

Correspondence: Dr O Tirosh, PhD, The School of Nutritional Sciences, Institute of Biochemistry, Food Science and Nutrition, The Robert H Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, Rehovot 76100, Israel. E-mail: otirosh@agri.huji.ac.il

Received 30 August 2010; revised 7 February 2011; accepted 7 February 2011

hydroxyl radicals.<sup>13</sup> Mitochondrial abnormalities related to an increased generation of ROS have been described in human steatosis of various etiology and in several experimental models of fatty liver, including alcohol administration and drug toxicity.<sup>14</sup>

An important coactivator in liver biology is peroxisome-proliferator-activated receptor- $\gamma$  coactivator 1 $\alpha$  (PGC1 $\alpha$ ).<sup>15</sup> Induction of this coactivator has been documented in experimental models of obesity and diabetes.<sup>16</sup> Activation of PGC1 $\alpha$  results in increased expression of genes important to gluconeogenesis, fatty acid oxidation, lipid transport and mitochondrial biogenesis processes.<sup>17–20</sup> The mitochondrial biogenesis process involves expression of nuclear-encoded proteins that are essential for the replication of mitochondrial DNA (mtDNA) and the expression of mtDNA-encoded genes.<sup>21</sup> PGC1 $\alpha$  also has a role in this metabolic pathway by activating mitochondrial nuclear respiratory factor 1 (NRF-1) and NRF-2 to induce expression of mitochondrial transcription factor A (TFAM). TFAM is important to the stability and the transcription of mtDNA.<sup>22,23</sup> Falkenberg *et al*<sup>24</sup> identified two additional transcription factors, which are necessary for basal transcription of mtDNA: mitochondrial transcription factor B1 (TFB1M) and B2 (TFB2M) (tfb1m and tfb2m in mice). Apparently, the expression of these two proteins (TFBMs) is regulated by the complexes PGC1 $\alpha$ –NRF-1 and PGC1 $\alpha$ –NRF-2.<sup>25</sup>

The goal of the study is to demonstrate impairment in mitochondrial biogenesis during the development of fatty liver disease. We focused on the association between the mitochondrial biogenesis process and the biological activity of PGC1 $\alpha$  in CDE-treated mice as a model of steatosis and liver injury.

## MATERIALS AND METHODS

### Animals

Four- to five-week-old male mice of the C57BL/6J inbred strain (purchased from Harlen Laboratories, Israel) were treated with a CDE diet as previously described.<sup>7–9</sup> Following 14 days on the CDE diet, animals were sacrificed with or without overnight fasting, serum was collected and livers were perfused with PBS to remove blood traces. Liver portions were snap frozen in liquid nitrogen, and kept at  $-80^{\circ}\text{C}$  for further processing. All animals were cared for under the guidelines set forth by the Animal Care and Use Committee of the Hebrew University of Jerusalem.

### Liver Histology

Hepatic steatosis and liver structural changes were assessed by histological evaluation. Removed livers were fixed in 4% formaldehyde (Bio-Lab, Israel) and 5  $\mu\text{m}$  of microtome sections (Leica Microsystems, Wetzlar, Germany) were collected. Hematoxylin–eosin (Sigma-Aldrich, St Louis, MO, USA) staining was used for tissue section visualization. Data were collected from two livers from different mice for each time point. For quantification, cells with fat droplets that are

bigger than the nucleus were counted. The percentage of cells-containing fat was determined in comparison to control.

### Cell Line

Mouse hepatocyte AML-12 cells were grown as described previously.<sup>9</sup> Cells were exposed to 10  $\mu\text{g}/\text{ml}$  oligomycin (O-4786; Sigma-Aldrich) for 6 h.

### Western Blot

Western blots were performed as described previously.<sup>26</sup> Primary antibodies used: mouse monoclonal anti-NDUFA9, Anti-PGC1 $\alpha$  (ab54481), anti-CoxIV (Abcam, Cambridge, UK), mouse anti-manganese superoxide dismutase (anti-MnSOD) (BD Biosciences, Franklin Lakes, NJ, USA). Mouse anti- $\beta$ -actin (BD Transduction Laboratories, Pharmingen, CA, USA) was used as an endogenous control. Secondary antibodies used: horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit (Jackson ImmunoResearch Laboratories, PA, USA). Protein levels were quantified using Gel-Pro Analyzer, version 4.0 (Media Cybernetics, Bethesda, MD, USA).

### Quantitative Real-Time Polymerase Chain Reaction

QRT–PCR technique was performed according to a published procedure.<sup>27</sup> 18S was used as the endogenous control. The list of the primers for all genes can be found in the supplementary data.

### mtDNA Quantification

Total DNA was isolated from control and CDE-fed mice using a kit (Promega, Madison, WI, USA). The mtDNA content relative to nuclear DNA was assessed by QRT–PCR by using 20 ng of total DNA as template and primers for mtCO1 (mitochondrial genome) and RIP140 (nuclear genome).<sup>28,29</sup>

### $\beta$ -Hydroxybutyrate Levels

Generation of  $\beta$ -hydroxybutyrate was evaluated in serum samples of mice fed with a control or a CDE diet for 14 days, followed by 48 h of fasting.  $\beta$ -Hydroxybutyrate concentration was determined using a kit (Cat. No. 700190, Cayman Chemical Company, MI, USA) according to the manufacturer's instructions.

### ROS Measurement

Superoxide levels were detected using dihydroethidium (DHE; Molecular Probes, Eugene, OR, USA). Livers cryosections (20  $\mu\text{m}$ ) from snap frozen liver portions (left lateral lobe) were obtained using a Reichert-Jung 2800 Frigocut cryostat (Cambridge Instruments, Nussloch, Germany). Cryosections were stained with 10  $\mu\text{M}$  DHE at  $37^{\circ}\text{C}$  for 30 min. Superoxide levels are demonstrated by red fluorescent labeling.<sup>30</sup> Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, MP Biomedicals, Solon, OH, USA).

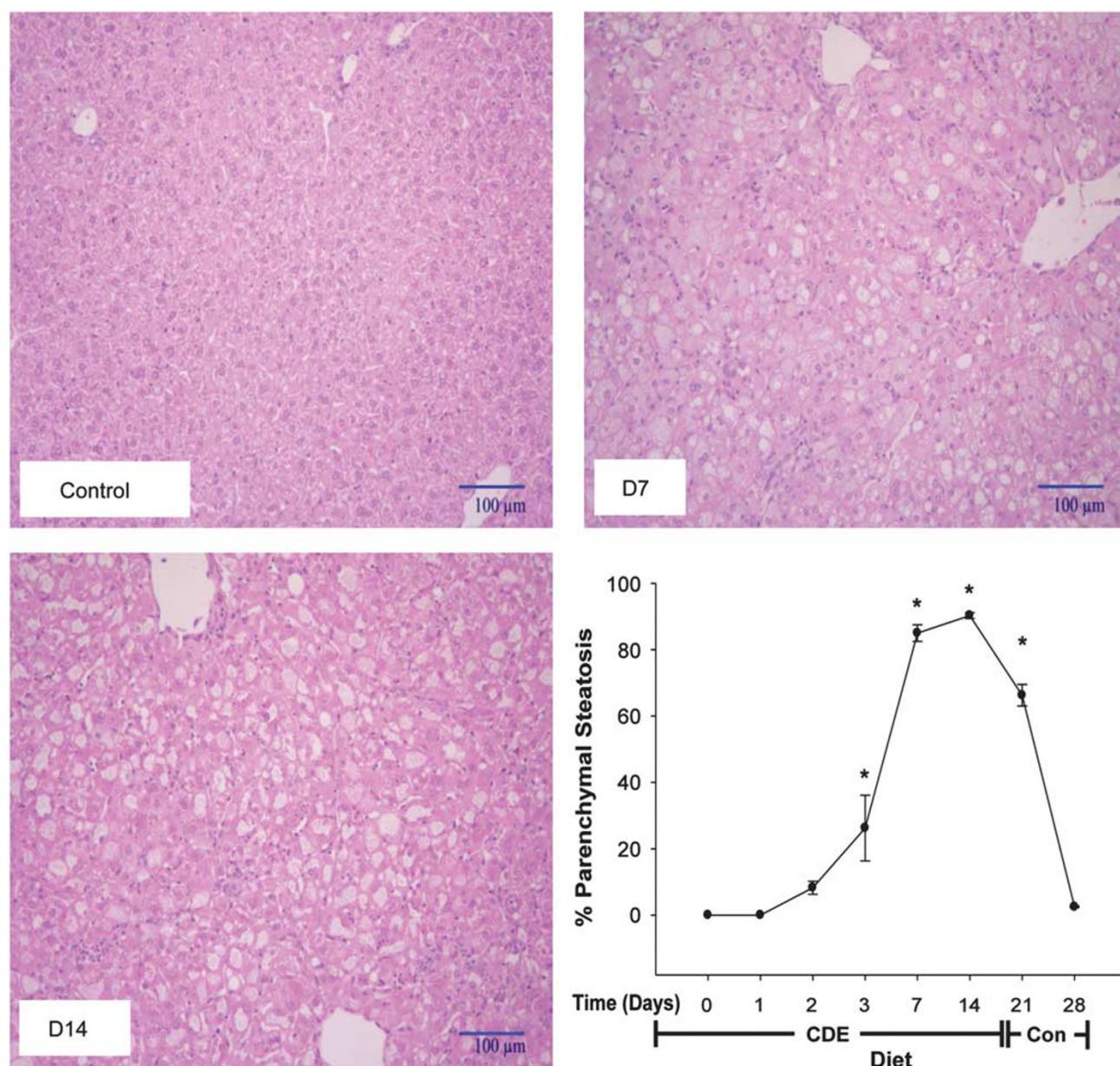
### ATP Measurement

ATP levels in liver or in cell-culture samples were determined using FLE enzyme (F3641; Sigma-Aldrich) in a luminometer according to the manufacturer's instructions.

### Chromatin Immunoprecipitation Analysis

The chromatin immunoprecipitation (ChIP) assay was carried out using a kit (Cat. No. P-2003, Epigentek, NY, USA). Briefly, 50 mg of liver tissue samples was cut into small pieces and cross-linked with 1% formaldehyde for 20 min. After homogenization, the samples were sonicated (Vibra Cell, Sonics, CT, USA) for  $20 \times 10$  s at 20% of maximum power to

produce a soluble chromatin, with average sizes between 300 and 1000 bp. In all, 8 mg of the sonicated chromatin were exposed to anti-PGC1 $\alpha$  antibody (SC-13067, Santa Cruz Biotechnology), anti-RNA Polymerase II (positive control), or to rabbit IgG antibody (negative control). Subsequent incubation and washes were completed according to the manufacturer's instructions. In all, 2  $\mu$ l of DNA was used for each QRT-PCR, which was performed as described before.<sup>27</sup> The data were analyzed using the function  $2^{-\Delta\Delta CT}$  where  $\Delta\Delta CT = (CT, IP-CT, input)_{sample} - (CT, IP-CT, input)_{calibrator}$ . As calibrators, primers were designed to a distal region of the different promoters. Relative promoter



**Figure 1** Development of steatosis in CDE-fed vs control-fed mice. C57BL/6J mice were fed with the CDE diet. At day 21 on CDE diet, the diet was replaced to a control diet for additional 6 days. Liver morphology and fat accumulation were evaluated by H&E staining after the indicated time points (magnification  $\times 200$ ). The graph represents the percentage of parenchymal cells that contain fat vacuoles at each time point. \* $P < 0.05$  compared with control.

enrichment compared with negative control are presented.<sup>31,32</sup>

### Statistical Analysis

Data were collected from 12 mice in each treatment group from at least two independent experiments. All values are expressed as mean values  $\pm$  s.e. Comparisons between two groups were performed with Student's *t*-test. For multiple groups, data were analyzed by analysis of variance. Differences were considered significant at probability levels of  $P < 0.05$  by using the Fisher's protected least significant difference method. Statistical analysis was performed by using the statistical computer program, SPSS version 11 (SPSS, Chicago, IL, USA). In experiments with multiple groups we used common letters in order to indicate significance. The highest average in each experiment was marked with the letter 'a'. The next (lower) significant average was marked with the letter 'b' etc. Means with different letters are significantly different at  $P < 0.05$ .

## RESULTS

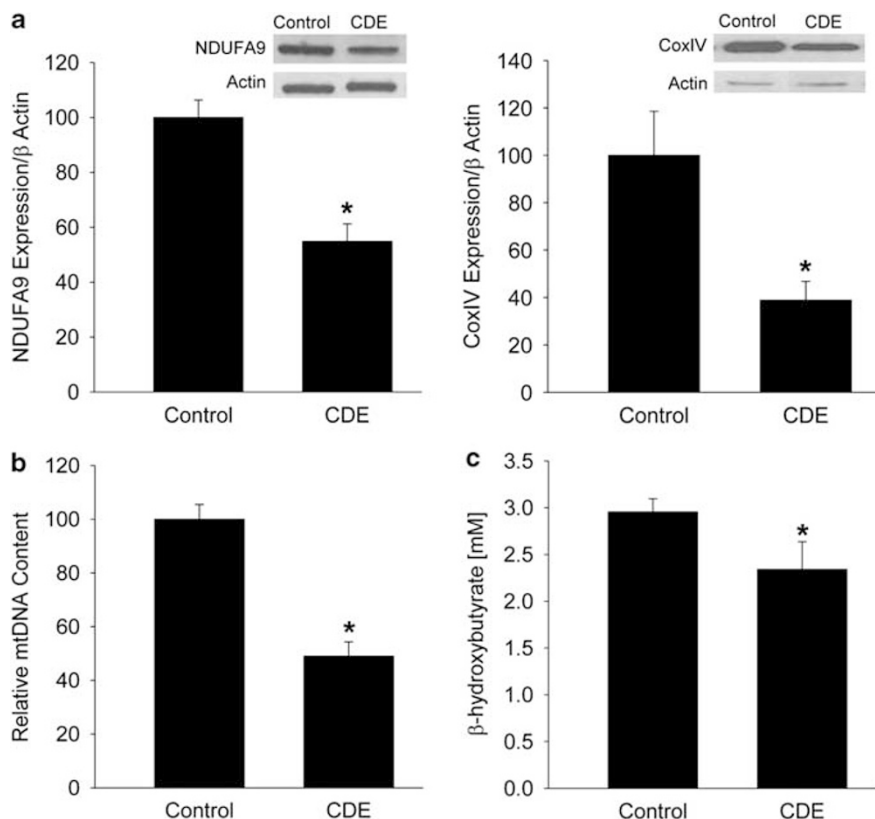
### Development of Steatosis in CDE-Fed Mice

Histological sections demonstrated accumulation of fat vacuoles in up to 90% of hepatocytes in mice fed the CDE

diet for 14 days, as can be seen in the representative pictures and in the quantitative graph that represents the percentage of fat-containing cells at different time points (Figure 1). These results corroborate previously reported data from our laboratory that demonstrated an increase in total liver lipid content and increased liver injury in CDE-treated mice.<sup>9</sup> At day 21, there was a moderate reduction in liver fat content compared with day 14. Replacement of the CDE diet with a control diet after 21 days for additional 6 days decreased fat content in the liver to control levels, with increased mitosis rate, suggesting hepatocellular regeneration (data not shown).

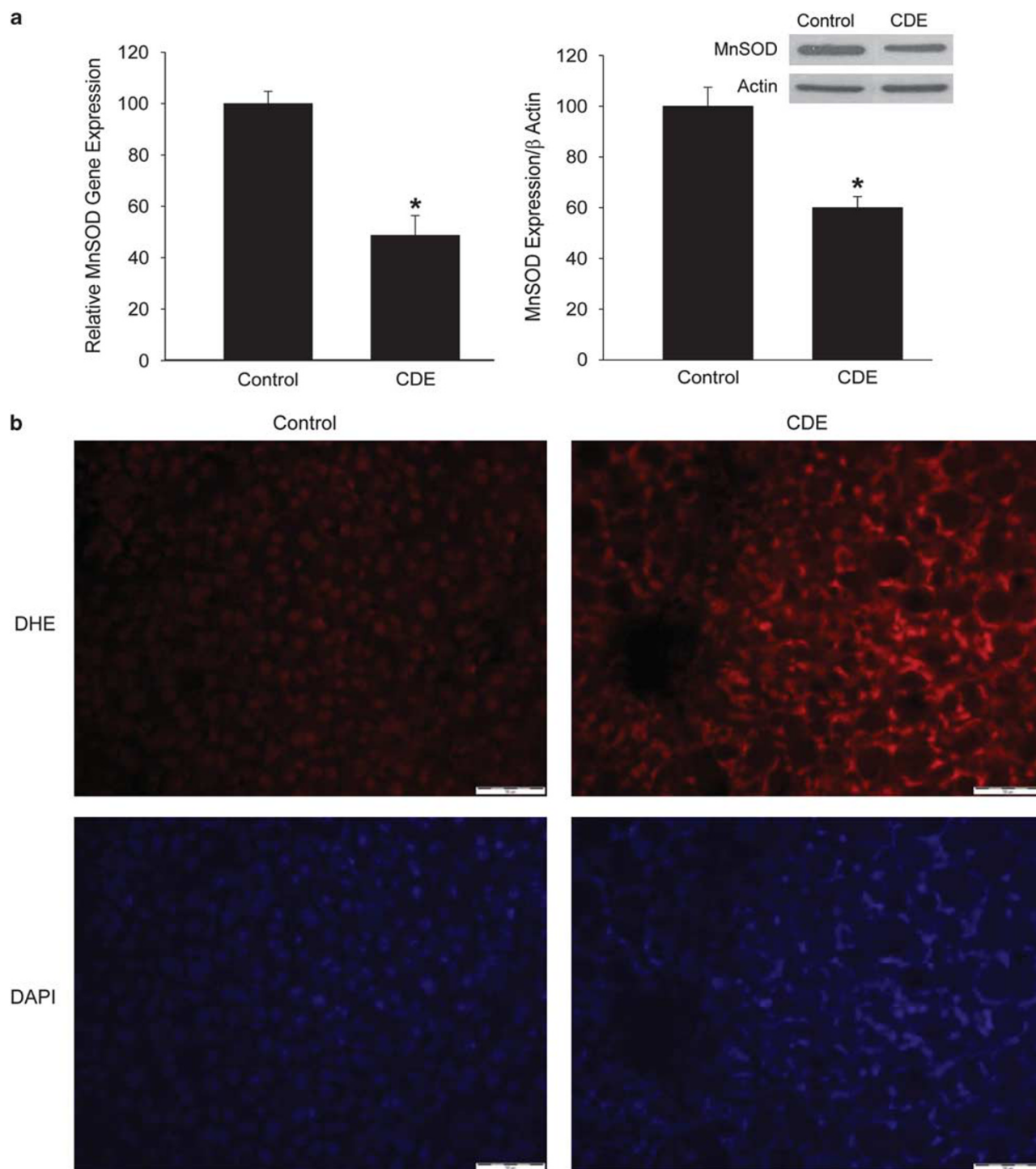
### Mitochondrial Levels in Mice Fed the CDE Diet for 14 Days

We studied changes in different mitochondrial parameters in livers from mice that were fed control or CDE diet for 14 days. A significant reduction in the protein levels of the mitochondrial electron transport chain proteins NADH dehydrogenase (ubiquinone) 1  $\alpha$  subcomplex, 9 (NDUFA9, subunit of complex I) and cytochrome c oxidase IV (Cox IV, subunit of complex IV) was observed (Figure 2a), suggesting a decrease in mitochondrial levels in CDE-fed mice. This was confirmed in quantitative measurements of mitochondrial levels, which indicated a 50% reduction in mtDNA copies in



**Figure 2** CDE diet led to a decrease in mitochondrial levels and the capacity for ketone body formation. Following 14 days on the CDE diet, DNA, RNA and protein were isolated from the perfused livers. (a) Protein levels of NDUFA9 (left graph) and Cox IV (right graph) were examined by western blot. (b) mtDNA content in livers was determined by QRT-PCR. (c)  $\beta$ -Hydroxybutyrate concentration was measured in serum samples following 48 h of fasting as described in Materials and methods. \* $P < 0.05$  compared with control.





**Figure 3** CDE diet led to generation of oxidative stress. **(a)** Following 14 days on the CDE diet, RNA and protein were isolated from the perfused livers. MnSOD mRNA levels were measured by QRT-PCR (left graph), while protein levels were examined by western blot (right graph). \* $P < 0.05$  compared with control. **(b)** Superoxide radical levels were evaluated by DHE staining of liver cryosections as described in Materials and methods. DAPI fluorescence image of the same field is also presented (magnification  $\times 400$ ).

livers from CDE-fed mice, when compared with control-fed mice (Figure 2b). Levels of  $\beta$ -hydroxybutyrate (a ketone body) were measured in serum samples in order to evaluate the efficiency of hepatic fatty oxidation process.<sup>33</sup>  $\beta$ -Hydroxy-

butyrate concentration following 48 h fasting was significantly lower in the serum of CDE-fed mice when compared with control-fed mice, demonstrating decreased capacity for ketone body formation (Figure 2c).

## Oxidative Stress

Staining liver cryosections with DHE revealed increased levels of superoxide radicals after 14 days with the CDE diet (Figure 3b). Measurement of MnSOD levels, an antioxidant enzyme in the mitochondria that is responsible for the dismutation of superoxide radicals into hydrogen peroxide<sup>34</sup> was also carried out. Reduced mRNA and protein levels of MnSOD were found in livers from mice fed with the CDE diet (Figure 3a). This positively correlated with the decrease in NDUFA9 and Cox IV protein levels (Figure 2a). These results demonstrate that changes in redox status in hepatocytes containing excessive amounts of fat lead to increased oxidative stress in the liver.

## Reduction in ATP Production and Activation of PGC1 $\alpha$ by the CDE Diet

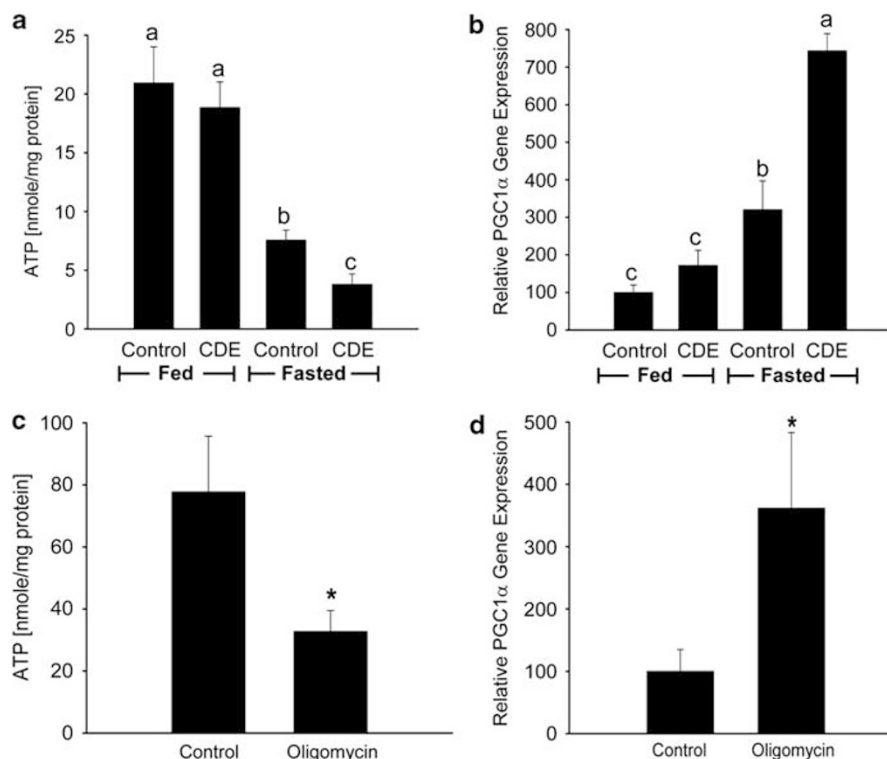
In order to explore whether the decrease in mitochondrial levels and the increase in oxidative stress impairs ATP production, ATP levels were measured in liver extracts following 14 days on the CDE diet. Levels of ATP were determined at the end point of the experiment in both mice that had a free access to food and in mice following an overnight fast. In the fed state, the CDE diet led to a small but non-significant decrease in ATP levels. However, following an overnight fast, ATP levels were significantly lower in CDE-fed mice when compared with control mice (Figure 4a).

The effect of fasting on mitochondrial parameters in CDE-fed mice was evaluated with regard to energy expenditure pathways. mRNA levels of PGC1 $\alpha$  were evaluated. Surprisingly, in the fed state there was no effect of CDE diet on PGC1 $\alpha$  expression when compared with control livers (Figure 4b). After overnight fasting, however, CDE diet led to a significant increase in the mRNA levels of PGC1 $\alpha$  when compared with control mice. It is probable that this increase is an attempt to compensate for the decline in mitochondrial tissue content.

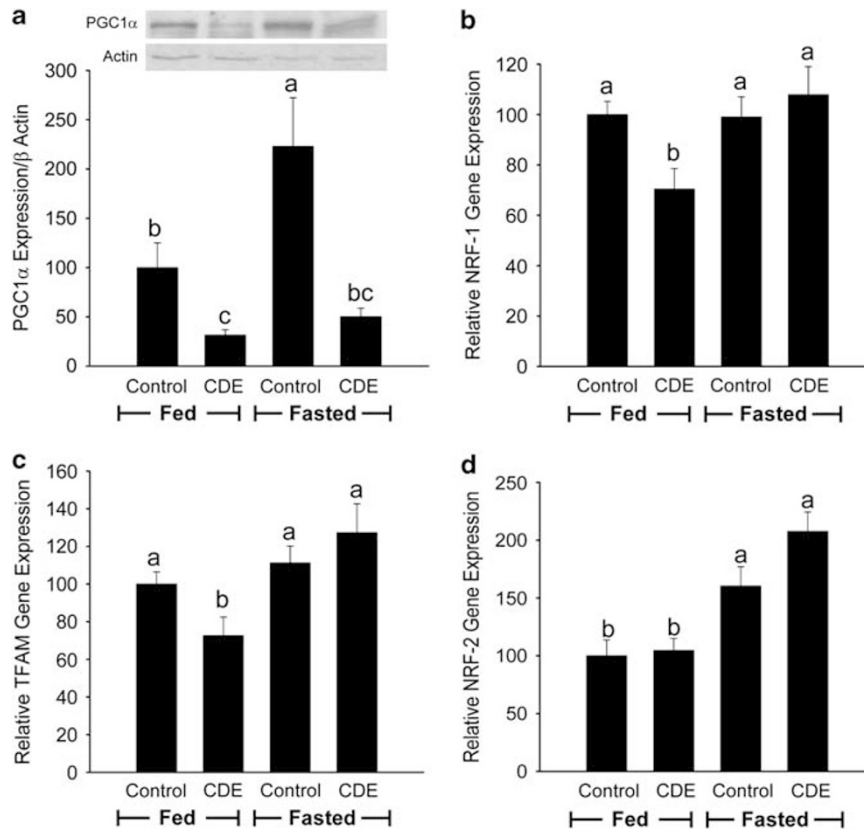
A direct connection between the upregulation of PGC1 $\alpha$  and the downregulation in ATP levels was sought using mouse AML-12 hepatocytes cell line. ATP levels in CDE versus control livers were found to be inversely correlated to the mRNA levels of PGC1 $\alpha$ . AML-12 hepatocytes were treated with oligomycin, an inhibitor of ATP synthase, in order to reduce cellular ATP levels (Figure 4c). As can be seen in Figure 4d, the reduction in ATP levels was accompanied by a significant increase in PGC1 $\alpha$  mRNA levels, indicating a direct link between the capacity of mitochondria to produce ATP and the expression of PGC1 $\alpha$ .

## Effect of CDE Diet on Mitochondrial Biogenesis

Although there was no significant change in PGC1 $\alpha$  mRNA levels in steatotic livers in the fed state (Figure 4b), a 50% reduction in the protein levels of PGC1 $\alpha$  was found



**Figure 4** Impaired ATP production and activation of PGC1 $\alpha$  expression in mice fed with the CDE diet. (a) ATP levels were measured in liver tissue samples as described in Materials and methods. (b) mRNA levels of PGC1 $\alpha$  in the livers were analyzed by QRT-PCR. The depletion in ATP levels is reflected by the expression of PGC1 $\alpha$ . Means with different letter differ at  $P < 0.05$ . AML-12 hepatocytes were exposed to 10  $\mu$ g/ml oligomycin for 6 h. ATP levels (c) and PGC1 $\alpha$  mRNA levels (d) were measured. \* $P < 0.05$  compared with control.



**Figure 5** Expression pattern of PGC1 $\alpha$  and the mitochondrial biogenesis transcription factors NRF-1, NRF-2 and TFAM. Following 14 days on the CDE diet, RNA and protein were isolated from the perfused livers. (a) Protein levels of PGC1 $\alpha$  were examined by western blot. In order to evaluate induction of mitochondrial biogenesis process, the mRNA levels of NRF-1 (b), TFAM (c) and NRF-2 (d) were analyzed by QRT-PCR. Means with different letter differ at  $P < 0.05$ .

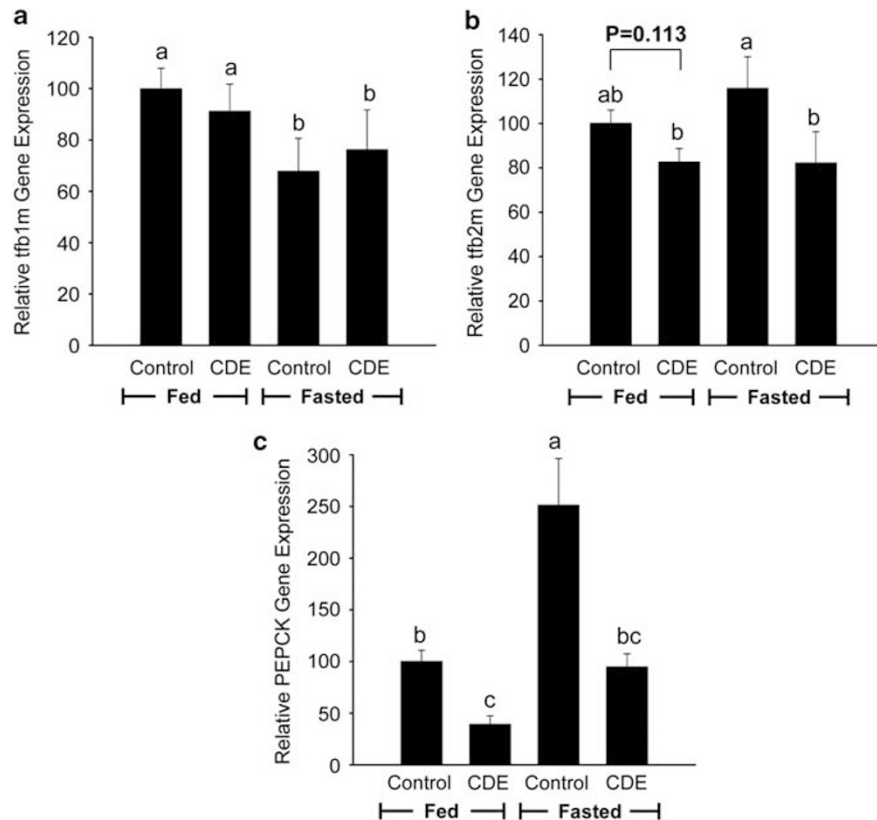
(Figure 5a). This was accompanied by a 30% decrease in the mRNA levels of PGC1 $\alpha$ -target genes NRF-1 and TFAM (Figure 5b and c). Furthermore, the significant increase in the mRNA levels of PGC1 $\alpha$  in steatotic livers after overnight fasting, did not lead to an increase in its protein levels, or to a change in the levels of NRF-1 and TFAM when compared with control livers (Figure 5b and c). NRF-2 is also an important transcription factor in the mitochondrial biogenesis process, yet its expression is not regulated by PGC1 $\alpha$  activity.<sup>23</sup> In contrast to NRF-1 and TFAM expression, the mRNA levels of NRF-2 did not change in steatotic livers when compared with control livers, while overnight fasting, with or without CDE diet, led to an increase in NRF-2 mRNA levels (Figure 5d). This demonstrates that the effect of steatosis is specific to genes whose expression is regulated by the coactivator PGC1 $\alpha$ .

#### Impaired PGC1 $\alpha$ Biological Activity in Livers of CDE-Fed Mice

mRNA levels of the mouse PGC1 $\alpha$ -target genes *tfb1m* and *tfb2m* were measured in liver samples. No change in the expression of *tfb1m* was found in livers of mice fed the CDE diet, while overnight fasting led to a decrease in its expression

compared with the fed state (Figure 6a). The expression of *tfb2m*, however, decreased in steatotic livers when compared with control livers (Figure 6b). The mRNA levels of phosphoenol-pyruvate carboxykinase (PEPCK) were also measured. PEPCK is an important gluconeogenic enzyme in the liver, whose expression is regulated by PGC1 $\alpha$ -induced coactivation of the forkhead box O1 (FOXO1) transcription factor.<sup>35</sup> The pattern of PEPCK expression resembled that of *tfb2m* (Figure 6c). Fasting in control animals led to an increase in both *tfb2m* and PEPCK expression, as expected (Figure 6b and c).

In order to further evaluate PGC1 $\alpha$  activity, a ChIP assay was performed. ChIP analysis using anti-PGC1 $\alpha$  antibody for immunoprecipitation provided information related to the binding of PGC1 $\alpha$ -transcription factors complexes to *tfb1m*, *tfb2m* and PEPCK promoters *in vivo*. As can be seen in Figure 7, there was no change in the binding of the transcriptional complex to the promoter of *tfb1m* (Figure 7a). However, the binding of the complex to *tfb2m* was lower in steatotic livers when compared with control livers in the fed state (Figure 7b), which is consistent with the reduced protein levels of PGC1 $\alpha$  (Figure 5a). Fasting induced a twofold increase in PGC1 $\alpha$  activity, while the binding of the complex



**Figure 6** Impaired PGC1 $\alpha$  activity: expression of its complexes' target genes tfb1m, tfb2m and PEPCK. PGC1 $\alpha$  activity was examined by measuring the expression of its target genes: tfb1m (a), tfb2m (b) and PEPCK (c). mRNA levels of these genes were analyzed by QRT-PCR. Means with different letter differ at  $P < 0.05$ .

to the tfb2m promoter dropped dramatically in CDE-fed mice (Figure 7b). In addition, examining the binding of the complex PGC1 $\alpha$ –FOXO1 to PEPCK promoter illustrated a fivefold increase in the transcriptional complex after overnight fasting (Figure 7c). Nevertheless, the accumulation of fat in the liver significantly decreased the binding of the complex to the promoter both in the fed and the fasted state (Figure 7c).

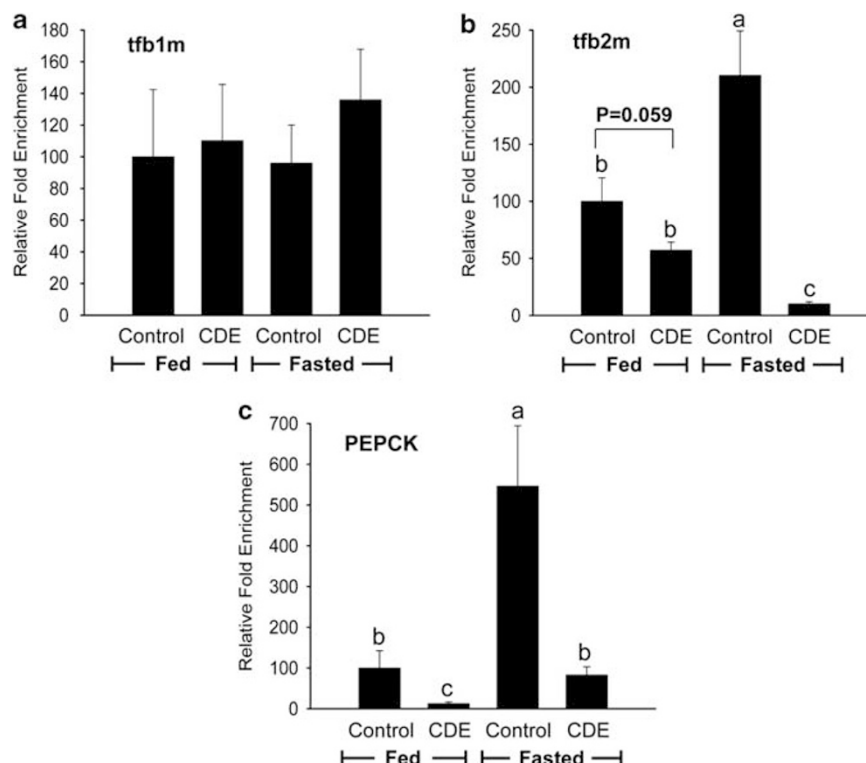
## DISCUSSION

The present study examined the impact of steatosis on hepatocyte metabolism using the CDE-diet model in mice, focusing on mitochondrial biogenesis process. At day 14 from the beginning of the diet, accumulation of fat and generation of oxidative stress are demonstrated (Figures 1 and 3). These results are in accordance to Day and James 'two hit' proposed model for the development of NASH,<sup>14</sup> in which steatosis in the 'first hit' is followed by oxidative stress in the 'second hit'. It could be that the decrease in mitochondrial levels in steatotic livers (Figure 2) mediated the increase in ROS generation.<sup>13,14</sup> However, mitochondria could still effectively generate ATP in the fed state (Figure 4), while a reduction in ATP production was found only in livers of CDE-fed mice after overnight fasting (Figure 4). The

significant decrease in ATP after fasting was accompanied by a reduction in the release of  $\beta$ -hydroxybutyrate from the liver (Figure 2), and also by reduced expression of PEPCK (Figure 6). Taken together, the data suggest that CDE reduced the capability of hepatocytes to utilize nutrients for energy, most probably by affecting cellular pathways that are related to PGC1 $\alpha$  and energy expenditure, such as  $\beta$ -oxidation in the mitochondria, mitochondrial biogenesis, and the gluconeogenesis processes. It would be interesting to further evaluate the direct influence of the diet in isolated mitochondria.

As is shown in Figures 4b and 5a, the liver responses to fasting by increasing expression of PGC1 $\alpha$ , a key transcriptional regulator of energy homeostasis. Similar results were reported previously by others.<sup>36,37</sup> Our both *in vivo* and *in vitro* results suggest that PGC1 $\alpha$  transcription is a sensor for decreased ATP levels. In CDE-fed mice, overnight fasting, which decreased ATP levels, led to a significant increase in PGC1 $\alpha$  expression (Figure 4b). Furthermore, in the mouse hepatocyte cell line AML-12, a direct reduction in cellular ATP levels by oligomycin was followed by induction of PGC1 $\alpha$  expression (Figure 4c and d). The increase in PGC1 $\alpha$  expression could be mediated by the increase in AMP levels and by activation of AMP-activated protein kinase, as reported by Lee *et al.*<sup>20</sup> The induction of PGC1 $\alpha$  expression





**Figure 7** Impaired PGC1 $\alpha$  activity: ChIP analysis. ChIP assay was performed as described in Materials and methods. Fold enrichment in the binding of PGC1 $\alpha$ -transcription factor complex to the promoters of (a) *tfb1m*, (b) *tfb2m* and (c) *PEPCK* is presented in the graphs. Means with different letter differ at  $P < 0.05$ .

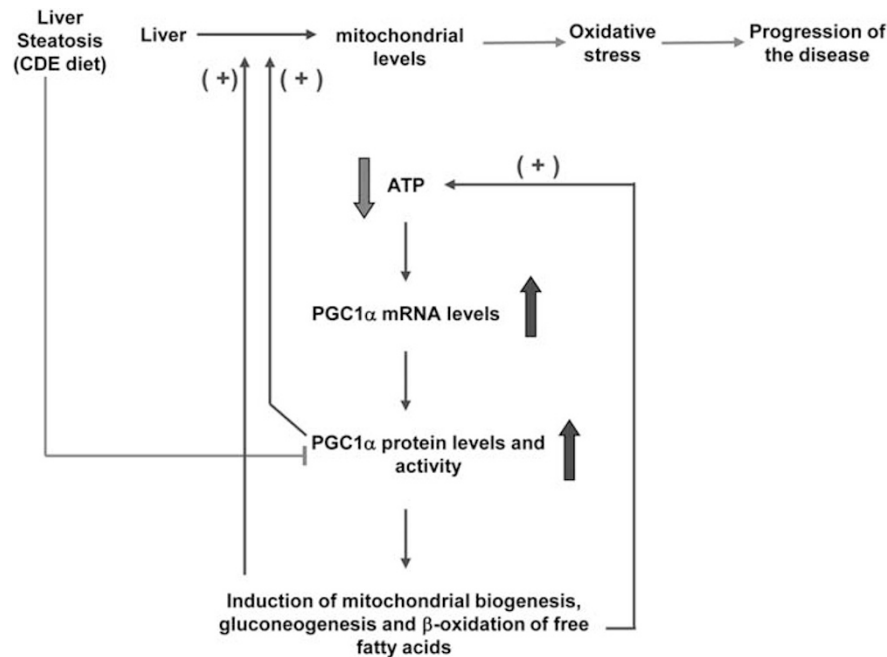
is probably a compensative cellular response, in order to provide the energy demands of the cells. Indeed, it has been shown that transfection of NIH 3T3 fibroblasts with mouse cDNA for PGC1 $\alpha$  leads to an increase in cellular ATP levels.<sup>38</sup> Furthermore, PGC1 $\alpha$  expression mediated the recovery of fibroblast cells from a partial chemical uncoupling of mitochondrial oxidative phosphorylation, with a decrease in ATP levels.<sup>39</sup>

In steatotic hepatocytes, the compensative effect of increased PGC1 $\alpha$  activity is impaired. In contrast to the increase in PGC1 $\alpha$  mRNA levels, its protein levels were significantly lower in CDE-fed mice (Figure 5a). Aoi *et al*<sup>40</sup> found that the microRNA miR-696 is involved in the translational regulation of PGC1 $\alpha$  and skeletal muscle metabolism in mice. It could be that CDE diet affects microRNAs in livers of mice, thus decreasing the translation of PGC1 $\alpha$  mRNA to protein, yet posttranslational modifications may also be involved. In accordance to the reduced protein levels of PGC1 $\alpha$ , reduced mRNA levels of NRF-1, TFAM and *tfb2m* were found in the fed state of CDE-fed mice. Moreover, the synergistic increase in PGC1 $\alpha$  mRNA levels in steatotic livers after fasting did not lead to an increase in NRF-1 and TFAM mRNA levels, with a significant decrease in *tfb2m* expression when compared with control livers (Figures 5 and 6). This effect of CDE feeding on the biological activity of PGC1 $\alpha$  was confirmed by *in vivo* ChIP analysis, which demonstrated a reduction in the binding PGC1 $\alpha$  to the *tfb2m* promoter that

contains NRF-1 and NRF-2 response elements (Figure 7). The decrease in PGC1 $\alpha$  activity is not limited to the mitochondrial biogenesis pathway, as a significant decline in the binding of PGC1 $\alpha$  to PEPCK promoter containing FOXO1 response element in steatotic livers was also observed.

CDE diet decreased the ability of PGC1 $\alpha$  to bind and activate the *tfb2m* promoter, yet it had no effect on its binding and activation of the *tfb1m* promoter (Figure 7). Gleyzer *et al*<sup>25</sup> reported that human TFB1M and TFB2M expression is regulated by the complexes of PGC1 $\alpha$  with NRF-1 or NRF-2. However, it was suggested that the expression of the corresponding mouse genes *tfb1m* and *tfb2m* is mediated only by PGC1 $\alpha$ -NRF-2 complex.<sup>41</sup> Thus, it is possible that the regulation of *tfb1m* expression is mediated by additional transcription factors that compensate for the reduction in PGC1 $\alpha$  biological activity. Nevertheless, although both human TFB1M and TFB2M interact directly with mitochondrial RNA polymerase to induce mtDNA replication and transcription, TFB2M is more active than TFB1M.<sup>24</sup>

In conclusion, impairment of mitochondrial biogenesis process was found in steatotic livers. However, the increase in PGC1 $\alpha$  mRNA expression, which is most likely an attempt of the hepatocytes to compensate for the decrease in mitochondrial number, did not increase mitochondrial mass through the mitochondrial biogenesis pathway or PGC1 $\alpha$  activity. Prolonged dysfunction of the mitochondrial biogenesis process may lead to increased oxidative stress and



**Figure 8** Mechanism by which an impaired mitochondrial biogenesis and NAFLD progression can be developed in liver steatosis induced by the CDE diet. Decrease in mitochondrial levels in fatty liver results in decreased production of ATP after fasting. This is accompanied by a compensative increase in PGC1 $\alpha$  expression and activation, in order to induce mitochondrial biogenesis, gluconeogenesis and  $\beta$ -oxidation of free fatty acids. CDE diet, by reducing PGC1 $\alpha$  protein levels, impairs its biological activity, thus inhibiting the regenerating effect of PGC1 $\alpha$ . As a consequence, there is additional accumulation of fat droplets in the liver, increased damage to the mitochondria, which lead to elevated levels of ROS and may contribute to the progression of the disease.

modify overall cellular function, which may contribute to progression of NAFLD to NASH. Finally, we propose the following scheme (Figure 8) for explaining the consequences of accumulation of fat in the liver and impaired PGC1 $\alpha$  activity on NAFLD progression. To the best of our knowledge, this is the first evidence that directly links impaired biological activity of PGC1 $\alpha$  with mitochondrial tissue content in NAFLD. We also demonstrate that the effect of steatosis on PGC1 $\alpha$  activity influences other metabolic pathways that may also contribute to the progression of the disease.

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

#### ACKNOWLEDGEMENT

This study was supported by a grant no. 377/06 from the Israel Science Foundation to OT and ZM.

#### DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

1. Neuschwander-Tetri BA. Nonalcoholic steatohepatitis and the metabolic syndrome. *Am J Med Sci* 2005;330:326–335.
2. Wanless IR, Lentz JS. Fatty liver hepatitis (steatohepatitis) and obesity: an autopsy study with analysis of risk factors. *Hepatology* 1990;12:1106–1110.
3. Caldwell B, Aldington S, Weatherall M, *et al*. Risk of cardiovascular events and celecoxib: a systematic review and meta-analysis. *J R Soc Med* 2006;99:132–140.
4. Zeisel SH. Choline: critical role during fetal development and dietary requirements in adults. *Annu Rev Nutr* 2006;26:229–250.

5. Mato JM, Corrales FJ, Lu SC, *et al*. S-Adenosylmethionine: a control switch that regulates liver function. *FASEB J* 2002;16:15–26.
6. Kulinski A, Vance DE, Vance JE. A choline-deficient diet in mice inhibits neither the CDP-choline pathway for phosphatidylcholine synthesis in hepatocytes nor apolipoprotein B secretion. *J Biol Chem* 2004;279:23916–23924.
7. Akhurst B, Croager EJ, Farley-Roche CA, *et al*. A modified choline-deficient, ethionine-supplemented diet protocol effectively induces oval cells in mouse liver. *Hepatology* 2001;34:519–522.
8. Knight B, Matthews VB, Akhurst B, *et al*. Liver inflammation and cytokine production, but not acute phase protein synthesis, accompany the adult liver progenitor (oval) cell response to chronic liver injury. *Immunol Cell Biol* 2005;83:364–374.
9. Tirosh O, Artan A, Aharoni-Simon M, *et al*. Impaired liver glucose production in a murine model of steatosis and endotoxemia: protection by inducible nitric oxide synthase. *Antioxid Redox Signal* 2010;13:13–26.
10. Ibdah JA, Perlegas P, Zhao Y, *et al*. Mice heterozygous for a defect in mitochondrial trifunctional protein develop hepatic steatosis and insulin resistance. *Gastroenterology* 2005;128:1381–1390.
11. Zhang D, Liu ZX, Choi CS, *et al*. Mitochondrial dysfunction due to long-chain Acyl-CoA dehydrogenase deficiency causes hepatic steatosis and hepatic insulin resistance. *Proc Natl Acad Sci USA* 2007;104:17075–17080.
12. Bernardi P, Petronilli V, Di Lisa F, *et al*. A mitochondrial perspective on cell death. *Trends Biochem Sci* 2001;26:112–117.
13. Bonawitz ND, Rodeheffer MS, Shadel GS. Defective mitochondrial gene expression results in reactive oxygen species-mediated inhibition of respiration and reduction of yeast life span. *Mol Cell Biol* 2006;26:4818–4829.
14. Serviddio G, Sastre J, Bellanti F, *et al*. Mitochondrial involvement in non-alcoholic steatohepatitis. *Mol Aspects Med* 2008;29:22–35.
15. Staudinger JL, Lichti K. Cell signaling and nuclear receptors: new opportunities for molecular pharmaceuticals in liver disease. *Mol Pharm* 2008;5:17–34.
16. Estall JL, Kahn M, Cooper MP, *et al*. Sensitivity of lipid metabolism and insulin signaling to genetic alterations in hepatic peroxisome

- proliferator-activated receptor-gamma coactivator-1 $\alpha$  expression. *Diabetes* 2009;58:1499–1508.
17. Finck BN, Kelly DP. PGC-1 coactivators: inducible regulators of energy metabolism in health and disease. *J Clin Invest* 2006;116:615–622.
18. Puigserver P. Tissue-specific regulation of metabolic pathways through the transcriptional coactivator PGC1- $\alpha$ . *Int J Obes (Lond)* 2005;29(Suppl 1):S5–S9.
19. St-Pierre J, Drori S, Uldry M, *et al*. Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators. *Cell* 2006;127:397–408.
20. Lee WJ, Kim M, Park HS, *et al*. AMPK activation increases fatty acid oxidation in skeletal muscle by activating PPAR $\alpha$  and PGC-1. *Biochem Biophys Res Commun* 2006;340:291–295.
21. Mantena SK, King AL, Andringa KK, *et al*. Mitochondrial dysfunction and oxidative stress in the pathogenesis of alcohol- and obesity-induced fatty liver diseases. *Free Radic Biol Med* 2008;44:1259–1272.
22. Rantanen A, Jansson M, Oldfors A, *et al*. Downregulation of Tfam and mtDNA copy number during mammalian spermatogenesis. *Mamm Genome* 2001;12:787–792.
23. Wu Z, Puigserver P, Andersson U, *et al*. Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell* 1999;98:115–124.
24. Falkenberg M, Gaspari M, Rantanen A, *et al*. Mitochondrial transcription factors B1 and B2 activate transcription of human mtDNA. *Nat Genet* 2002;31:289–294.
25. Gleyzer N, Vercauteren K, Scarpulla RC. Control of mitochondrial transcription specificity factors (TFB1M and TFB2M) by nuclear respiratory factors (NRF-1 and NRF-2) and PGC-1 family coactivators. *Mol Cell Biol* 2005;25:1354–1366.
26. Aronis A, Aharoni-Simon M, Madar Z, *et al*. Triacylglycerol-induced impairment in mitochondrial biogenesis and function in J774.2 and mouse peritoneal macrophage foam cells. *Arch Biochem Biophys* 2009;492:74–81.
27. Shilo S, Pardo M, Aharoni-Simon M, *et al*. Selenium supplementation increases liver MnSOD expression: molecular mechanism for hepatoprotection. *J Inorg Biochem* 2008;102:110–118.
28. Villena JA, Hock MB, Chang WY, *et al*. Orphan nuclear receptor estrogen-related receptor  $\alpha$  is essential for adaptive thermogenesis. *Proc Natl Acad Sci USA* 2007;104:1418–1423.
29. Bourdon A, Minai L, Serre V, *et al*. Mutation of RRM2B, encoding p53-controlled ribonucleotide reductase (p53R2), causes severe mitochondrial DNA depletion. *Nat Genet* 2007;39:776–780.
30. Zhao ZQ, Corvera JS, Halkos ME, *et al*. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. *Am J Physiol Heart Circ Physiol* 2003;285:H579–H588.
31. Lu YC, Song J, Cho HY, *et al*. Cyclophilin a protects Peg3 from hypermethylation and inactive histone modification. *J Biol Chem* 2006;281:39081–39087.
32. Piantadosi CA, Carraway MS, Babiker A, *et al*. Heme oxygenase-1 regulates cardiac mitochondrial biogenesis via Nrf2-mediated transcriptional control of nuclear respiratory factor-1. *Circ Res* 2008;103:1232–1240.
33. Hashimoto T, Cook WS, Qi C, *et al*. Defect in peroxisome proliferator-activated receptor  $\alpha$ -inducible fatty acid oxidation determines the severity of hepatic steatosis in response to fasting. *J Biol Chem* 2000;275:28918–28928.
34. Fleury C, Mignotte B, Vayssiere JL. Mitochondrial reactive oxygen species in cell death signaling. *Biochimie* 2002;84:131–141.
35. Puigserver P, Rhee J, Donovan J, *et al*. Insulin-regulated hepatic gluconeogenesis through FOXO1-PGC-1 $\alpha$  interaction. *Nature* 2003;423:550–555.
36. Yoon JC, Puigserver P, Chen G, *et al*. Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1. *Nature* 2001;413:131–138.
37. Potthoff MJ, Inagaki T, Satapati S, *et al*. FGF21 induces PGC-1 $\alpha$  and regulates carbohydrate and fatty acid metabolism during the adaptive starvation response. *Proc Natl Acad Sci USA* 2009;106:10853–10858.
38. Liang H, Bai Y, Li Y, *et al*. PGC-1 $\alpha$ -induced mitochondrial alterations in 3T3 fibroblast cells. *Ann N Y Acad Sci* 2007;1100:264–279.
39. Rohas LM, St-Pierre J, Uldry M, *et al*. A fundamental system of cellular energy homeostasis regulated by PGC-1 $\alpha$ . *Proc Natl Acad Sci USA* 2007;104:7933–7938.
40. Aoi W, Naito Y, Mizushima K, *et al*. The microRNA miR-696 regulates PGC-1 $\alpha$  in mouse skeletal muscle in response to physical activity. *Am J Physiol Endocrinol Metab* 2010;298:E799–E806.
41. Rantanen A, Gaspari M, Falkenberg M, *et al*. Characterization of the mouse genes for mitochondrial transcription factors B1 and B2. *Mamm Genome* 2003;14:1–6.