

Central energy metabolism remains robust in acute steatotic hepatocytes challenged by a high free fatty acid load

Jens Niklas^{1,*}, Anne Bonin¹, Stefanie Mangin¹, Joachim Bucher¹, Stephanie Kopacz², Madlen Matz-Soja³, Carlo Thiel³, Rolf Gebhardt³, Ute Hofmann² & Klaus Mauch¹

¹Insilico Biotechnology AG, Meitnerstrasse 8, D-70563 Stuttgart, ²Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology and University of Tübingen, Auerbachstrasse 112, D-70376 Stuttgart, ³Institute of Biochemistry, Faculty of Medicine, University of Leipzig, Johannisallee 30, D-04103 Leipzig, Germany

Overnutrition is one of the major causes of non-alcoholic fatty liver disease (NAFLD). NAFLD is characterized by an accumulation of lipids (triglycerides) in hepatocytes and is often accompanied by high plasma levels of free fatty acids (FFA). In this study, we compared the energy metabolism in acute steatotic and non-steatotic primary mouse hepatocytes. Acute steatosis was induced by pre-incubation with high concentrations of oleate and palmitate. Labeling experiments were conducted using [U-¹³C₅,U-¹⁵N₂] glutamine. Metabolite concentrations and mass isotopomer distributions of intracellular metabolites were measured and applied for metabolic flux estimation using transient ¹³C metabolic flux analysis. FFAs were efficiently taken up and almost completely incorporated into triglycerides (TAGs). In spite of high FFA uptake rates and the high synthesis rate of TAGs, central energy metabolism was not significantly changed in acute steatotic cells. Fatty acid β -oxidation does not significantly contribute to the detoxification of FFAs under the applied conditions. [BMB Reports 2012; 45(7): 396-401]

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a multifactorial disorder mainly caused by overnutrition in the Western population with an estimated incidence of 15-20% (1, 2). NAFLD refers to hepatic steatosis (fatty liver), which is the accumulation of lipid droplets in hepatocytes accompanied by an increase in liver size, followed by progressive stages resulting eventually in fibrosis and cirrhosis (2). NAFLD is significantly related to obesity, type II diabetes, and metabolic syndrome.

*Corresponding author. Tel: +49-711-460594-24; Fax: +49-711-460594-10; E-mail: jens.niklas@insilico-biotechnology.com
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One important characteristic shared by these disorders is elevated levels of free fatty acids (FFAs) (3). FFAs can be taken up efficiently by hepatocytes and converted to triglycerides (TAGs) resulting in steatosis. It was reported that plasma FFA levels are significantly increased in NAFLD patients and that these are the primary source for TAGs in hepatocytes (4).

Different effects of high free fatty acid load on metabolism have been reported. As regards respiratory chain activity, for example, different groups (5-7) have reported increased, unchanged, and decreased activity under a high fatty acid load. Similarly, both increase and decrease in mitochondrial β -oxidation have been reported (8, 9). Recently, Vial *et al.* showed that a high-fat diet resulted in an inhibition of fatty acid oxidation and oxidative phosphorylation (10).

High concentrations of free fatty acids are toxic by mediating lipoapoptosis (11). Under normal physiologic conditions, intracellular fatty acid pools are low, and fatty acids are oxidized in β -oxidation or released after conversion to triglycerides via lipoprotein secretion. High fatty acid load results in a pathophysiologic lipid metabolism characterized by increased intracellular triglyceride pools, increased lipid peroxidation products, as well as reactive oxygen species formation (2).

A better understanding of the mechanisms and physiologic adaptations of the liver to FFAs and, in particular, steatosis would be highly desirable for developing new preventative strategies and therapies. A quantitative understanding of cellular metabolic response to FFAs under steatotic conditions is an important step on the roadmap to a more comprehensive model of NAFLD.

The best method for an in-depth analysis of alterations in cellular metabolism is probably ¹³C metabolic flux analysis (¹³C MFA), which was applied in the past to shed light on a number of scientific questions (12, 13). Transient ¹³C MFA is an additional improvement compared to traditional stationary ¹³C MFA (14, 15).

In this study, we used comprehensive metabolite analyses in combination with transient ¹³C MFA to study cellular alterations in steatotic hepatocytes under FFA excess conditions. The goals were to find out (i) in which way FFAs are metabolized in steatotic cells, (ii) how the central metabolism adapts

to steatotic conditions in combination with elevated FFA levels, and (iii) is this a significant burden for cellular metabolism? The answers to these questions are important for an improved understanding of NAFLD.

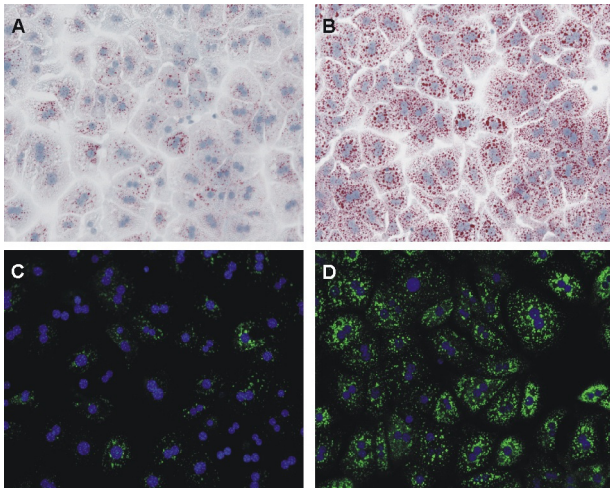


Fig. 1. Staining of neutral lipids in cultured normal and acute steatotic hepatocytes. Hepatocytes were cultured in normal culture medium (A, C) or in medium supplemented with 200 μ M sodium-oleate, 100 μ M sodium-palmitate and insulin at a concentration of 0.01 g/L (B, D) for 24 h. Fixed cells were stained with Oil Red O (A, B) and Bodipy (C, D). Cell nuclei were counterstained with hematoxylin (A, B) or with DAPI (C, D). Magnification: 250 \times .

RESULTS

Addition of oleate and palmitate results in a strong increase in intracellular triglyceride pools

The addition of the fatty acids oleate and palmitate into the medium resulted in a strongly increased intracellular TAG content and the visible accumulation of lipid droplets after 24 h (Fig. 1 and 2A). After 24 h, when the main experiment was started, oleate and palmitate were taken up in the steatotic cells at high rates (Fig. 2B), also resulting in high synthesis rates for TAGs (Fig. 2C), which were much higher than those in the control. The total TAG synthesis rates were 24 fmol/cell/h in steatotic and 6 fmol/cell/h in control cells. It is interesting to note that the additional amount of TAGs produced in steatotic cells viz. 18 fmol/cell/h matches the measured total fatty acid uptake rate of \sim 54 fmol/cell/h perfectly since three fatty acid molecules are required for the synthesis of one TAG molecule. This is a first indication that the detoxification of free fatty acids in steatotic hepatocytes might be mainly accomplished through TAG generation and storage. However, cellular metabolism is extremely complex and must be analyzed in much more detail to allow definite conclusions. High levels of fatty acids and intracellular TAG synthesis and accumulation might also lead to specific changes in intracellular physiology. Increased triglyceride synthesis, even from external FFAs, might burden energy metabolism to a certain extent. Fatty acids might also be used to generate energy via β -oxidation simultaneously with the observed TAG synthesis. In order to answer these questions properly, tracer experiments were carried out followed by a comprehensive metabo-

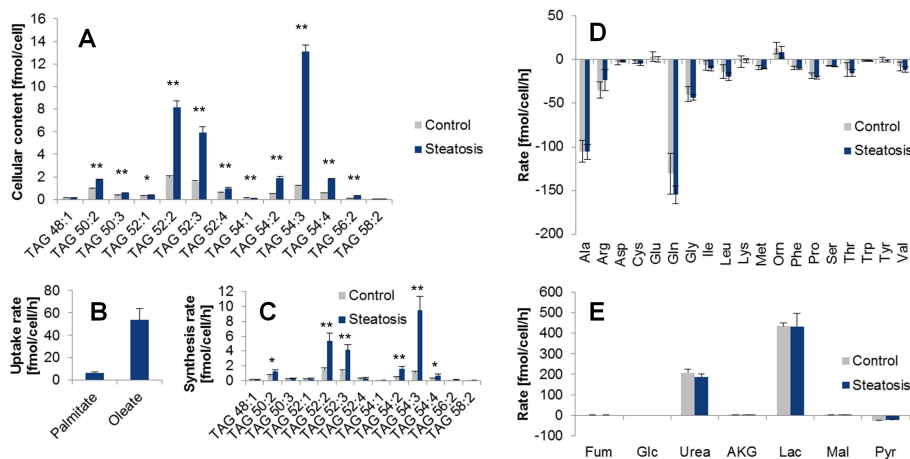


Fig. 2. Metabolic profile of control and steatotic hepatocytes cultured with high free fatty acid concentrations. Mean values and standard deviations of three biological replicates. * $P < 0.05$; ** $P < 0.01$ (unpaired, two-tailed Students T-test). (A) Triglyceride (TAG) content. Different TAGs are indicated by the respective total fatty acid residues in the format carbon atoms: double bonds. (B) Uptake rates of palmitate and oleate. (C) Synthesis rates of different TAG species. (D) Uptake and secretion rates of amino acids. Negative values indicate uptake of the respective amino acid, positive values production. Standard abbreviations are used. (E) Uptake and secretion rates of additional extracellular metabolites. Fum, fumarate; Glc, glucose; AKG, α -ketoglutarate; Lac, lactate; Mal, malate; Pyr, pyruvate. Negative values indicate uptake, positive values production.

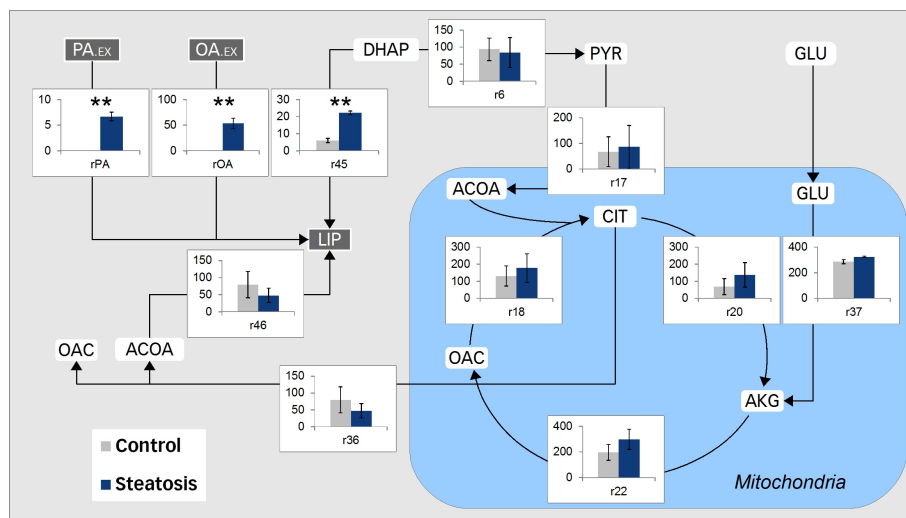


Fig. 3. Metabolic fluxes in control and steatotic hepatocytes cultured with high free fatty acid concentrations. Selected, most important fluxes are shown. Detailed data are provided in Table S2 of the supplementary material. Mean values and standard deviations (three biological replicates). ** $P < 0.01$ (unpaired, two-tailed Student's T-test). Fluxes are given in fmol/cell/h. ACOA, acetyl-CoA; AKG, α -ketoglutarate; CIT, citrate; DHAP, dihydroxyacetone phosphate; GLU, glutamate; LIP, lipids; PA, palmitate; PYR, pyruvate; OA, oleate; OAC, oxaloacetate; flux numbers are given in Fig. S2 (metabolic network).

lite analysis and, finally, transient ^{13}C MFA.

Extracellular fluxes are not significantly altered in steatotic cells

We calculated the uptake and secretion rates of extracellular metabolites from measured time courses. Interestingly, we did not observe remarkable differences in the uptake and secretion rates of most amino acids (Fig. 2D). The same holds true for organic acids, glucose, urea, and lactate (Fig. 2E).

Adaptation of intracellular fluxes and metabolite pools to steatotic conditions

Metabolic fluxes were calculated using transient ^{13}C metabolic flux analysis. The most important fluxes are depicted in Fig. 3. Total metabolic flux distributions as well as measured and estimated intracellular metabolite pools are provided in the supplementary material (Tables S2 and S3). Central energy metabolism was not significantly affected by the strongly increased influx of fatty acids and generation of TAGs. Though not significant, TCA cycle activity seemed slightly increased in steatotic cells. The flux of dihydroxyacetone phosphate (DHAP) to lipids was significantly higher in the steatotic cells but only approximately 10% of the estimated glycolytic flux from DHAP to phosphoenolpyruvate (PEP). Measured and estimated metabolite pools (Table S3) were similar in control and steatotic hepatocytes except for the α -ketoglutarate (AKG) pools which were higher in the control.

DISCUSSION

In order to be able to set-up a comprehensive, predictive model of the human liver, it is necessary to analyze metabolic activities quantitatively under different nutritional states. The following is a detailed analysis of physiological changes induced by high fatty acid load in acute steatotic cells. Generally speaking, knowing that high levels of FFAs can result in increased intra-

cellular TAG synthesis and formation of lipid droplets (3, 4), one might speculate that this enormous change in cell homeostasis might result in large adaptations in the central energy metabolism. In addition, the higher precursor demand for the synthesis of the TAGs might be an additional burden for cellular metabolism. However, if this is quantitatively relevant in the context of the total fluxes in the central energy metabolism cannot be answered immediately and requires detailed quantitative analyses, as presented in this study.

Our findings indicate that the central energy metabolism is not significantly affected under high FFA load in acute steatotic hepatocytes. The specific and significant adaptations observed in those parts of the metabolism involved in the synthesis of TAGs, i.e. increased flux from DHAP to lipids, are a direct consequence of the increased TAG synthesis. In general, palmitate and oleate were reported to induce changes in the expression of selected genes involved in β -oxidation and glucose metabolism, but within a longer period of time (16, 17). Our results do not indicate significant changes in either of the two pathways under the applied experimental conditions. Small differences in the glycolytic pathway might exist since increased amounts of DHAP are required as precursor for TAGs in the case of a high load of FFAs, as mentioned before. Overall, this flux is, however, not quantitatively important since it is only $\sim 10\%$ of the estimated glycolytic flux even under conditions of high TAG synthesis. From an evolutionary point of view, uptake of FFAs and conversion to TAG without losing energy by up-regulating central energy metabolism make sense. The fact that the cellular energy metabolism must fit to certain energetic requirements may prevent the cells from modifying central metabolic pathways. In another study, it was reported that the addition of different fatty acids did not increase glycolytic activity in rat hepatocytes, and it was proposed that the presence of fatty acids might have little effect on glucose metabolism (18). Another explanation for the observed low influence of steatosis and free fatty acids on central metabo-

lism and, in particular, for the low activity of β -oxidation under applied conditions might be related to the control of metabolism, e.g. through insulin (19), which was present in the culture, or intracellular mediators (20). Insulin is known to influence fatty acid oxidation and might antagonize possible oxidation of fatty acids. This issue could be tackled in additional quantitative studies by performing experiments with combinations of different levels of insulin and fatty acids.

The ease with which the synthesis and storage of TAGs is accomplished without deranging the central metabolism of the hepatocytes also emphasizes the danger possibly associated with constantly high plasma FFA levels *in vivo* caused by overnutrition. Without significant fasting periods, a prolonged low carbohydrate and fat diet, or increased energy demand in peripheral organs due to exercise, the hepatic metabolism will not be able to reduce TAG content in the hepatocytes. The problem is well-known (3, 21) and brings hormonal regulation into play. It is important to keep in mind that the metabolic conditions chosen for this study ($\sim 2 \mu\text{M}$ insulin) are standard conditions for an *in vitro* hepatocyte cell culture and correspond to an absorptive nutritional state in the liver. It might be interesting to study the response to FFAs in steatotic cells further under different insulin levels as this could change cell physiology (18, 19). Linking hormonal signal transduction, kinetic metabolic network models, and spatiotemporal models of the liver to one large predictive liver model could be a big help for understanding NAFLD better as well as liver-associated physiological processes in general. Additionally, this might open up new avenues for improved therapies and possibilities for cost-effective *in silico* testing of medication in the future. However, the predictive strength of these models and their validity will rely inherently on the data basis on which they are constructed and verified. Therefore, the data presented in this study is essential for validating the outcome of predictive models of liver physiology.

In summary, the metabolic response of acute steatotic hepatocytes cultured at a high FFA supplementation was studied. The hepatic energy metabolism is able to cope with this challenge without significant adaptations. Even high rates of TAG synthesis resulted only in moderate alterations in the respective precursor demand and related fluxes. FFAs were not significantly metabolized via the β -oxidation under the applied conditions, but were just converted to TAGs leading to visible intracellular accumulation of lipid droplets.

MATERIALS AND METHODS

Animals, hepatocyte isolation, cell culture, and experimental procedure

Primary hepatocytes from C57BL/6-N mice were isolated by collagenase perfusion of the liver as described by (22). The cells were seeded in 12-well plates at a cell density of 3.5×10^5 cells/well. The cells were cultured in William's Medium E (WME, Invitrogen #A12176-01) supplemented with 1% pen-

icillin/streptomycin (10,000 U/ml and 10 g/L, respectively), 2 mM glutamine, 0.1 μM dexamethasone, and 1% ITS-G (Invitrogen #41400-045, corresponding to a final insulin concentration of 0.01 g/L), and incubated at 37°C in a 5% CO₂ atmosphere. The experiments were carried out in accordance with the EU directive 2010/63/EU for animal experiments.

A scheme showing the experimental workflow is given in the supplementary material (Fig. S1). The cells were incubated for 24 h in normal WME (1 ml per well) with 0.01% fatty acid free bovine serum albumine (control) or in WME which was additionally supplemented with 200 μM sodium oleate and 100 μM sodium palmitate to induce steatosis in the respective hepatocytes. The following experimental setup for the determination of metabolite and mass isotopomer time courses as well as metabolic fluxes was as described in detail in (23). In a first experimental part, extracellular rates and intracellular concentrations were determined as follows. The medium was replaced by 0.5 ml of new medium using the two different media as described before (control and steatosis). Extracellular samples were taken over time for 4 h and cells were prepared for determination of intracellular metabolite concentrations as described earlier (23). The second experimental part was started immediately after the first and focused on the determination of the dynamics of intracellular mass isotopomer distributions as well as on the time courses of triglycerides in steatotic hepatocytes. As tracer we applied [U -¹³C₅,¹⁵N₂] glutamine (Eurisotop, Saarbrücken, Germany). The media for the labeling experiment were prepared in the same way as described before with the same molar composition except that labeled glutamine was used. The experiment was started by adding 0.6 ml of the respective tracer medium to the remaining wells which were not used in the first part of the experiment. The total cultivation volume was 1.1 ml. Cell samples for determination of mass isotopomer distributions of intracellular metabolites as well as triglyceride concentrations were taken over time (23). Each experiment was performed with three replicates.

Metabolite, fatty acid, and triglyceride analyses

Extracellular and intracellular metabolites as well as mass isotopomer distributions of intracellular metabolites were determined according to Hofmann *et al.* (23) and Maier *et al.* (24).

For determination of palmitate and oleate, 2 μl of medium samples were spiked with an internal standard mixture consisting of [¹³C₁₆] palmitate and [¹³C₁₈] oleate. After evaporation to dryness, samples were derivatized to the pentafluorobenzyl (PFB) esters with 25 μl of 1% pentafluorobenzyl bromide in acetonitrile and 25 μl of 1% *N,N*-diisopropylethylamine for 20 min at room temperature, evaporated again and redissolved in 40 μl of isoctane. Two μl of the samples were used for GC-MS analysis in Negative Ion Chemical Ionization (NCI) mode. The PFB esters were detected as [M-PFB]⁻ ions in SIM mode (*m/z* 255 and 271 for palmitic acid and its internal standard, and *m/z* 281 and 299 for oleic acid and its internal standard,

respectively). The GC oven program started at 150°C and was held for 1.5 min. Temperature was increased by 15°C/min to 300°C for 18.5 min, with a total run time of 30 min.

Triglycerides were analyzed by LC-MS on an Esquire HCT plus ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) coupled to an Agilent 1100 HPLC System (Agilent, Waldbronn, Germany). Ionization mode was electrospray (ESI), polarity positive, nitrogen was used as a drying and nebulizer gas. The mass spectrometer conditions were as follows: nebulizer pressure, 45 psi; dry gas flow, 9.5 l/min; dry gas temperature, 300°C; capillary -4,500 V; end plate offset, -500 V; scan range, 100 -1,100 m/z; ultra-scan mode. Cell pellets were extracted according to Wu et al. (25). Chromatographic separation was carried out at 80°C on a Poroshell 120 RP18 column (2.1 × 100 mm, 2.7 µm particle size, Agilent Technologies, Waldbronn, Germany) at a flow rate of 0.4 ml/min. Mobile phase A consisted of acetonitrile:water 95 : 5 (v/v) with 20 mM ammonium acetate and mobile phase B was isopropanol with 20 mM ammonium acetate. Gradient runs were programmed as follows: 5% B, increased to 15% B within 2 min, remaining at 15% B to min 4, increased to 50% B by min 8, 60% B by min 11, 70% B by min 23, and 90% B by min 24. After this step, the system was reconditioned to initial conditions (5% B) and held for 5 min. TAGs were analyzed as ammonium adducts [M+NH₄]⁺ using triheptadecanoin as internal standard.

Lipid staining

Medium was removed from cultured hepatocytes and cells were fixed with 4% paraformaldehyde in PBS for 10 min. For Oil red O staining of neutral lipids, fixed cells were washed 3 times with distilled water followed by 60% isopropanol for 10 min and then dried. Thereafter, cells were stained with Oil Red O for 10 min, shortly treated with 60% isopropanol and washed 3 times with distilled water. Cell nuclei were counterstained with hematoxylin-solution (Holborn & Söhne, Leipzig, Germany). Fluorescent staining of neutral lipids in fixed hepatocytes was performed using the fluorophore Bodipy 493/503 (Invitrogen). Bodipy stock (1 mg/ml in ethanol) was diluted 1 : 1,000 in PBS, filtered through 0.2 µm filters and added directly to the fixed cells. Cells were stained at room temperature for 20 min. Cell nuclei were counterstained with DAPI (Sigma). Fluorescent images were digitally captured using a DFC 350FX fluorescence camera.

Carbon atom transition model for ¹³C metabolic flux analysis

A carbon atom transition network of the central energy metabolism in hepatocytes was set up based on the stoichiometry of a large scale stoichiometric hepatocyte model published recently by Maier et al. (14). The carbon atom transitions are given in the supplementary material section (Table S1). A scheme showing the applied metabolic network model is additionally provided in Fig. S2.

Transient ¹³C metabolic flux analysis

Transient ¹³C metabolic flux analysis was carried out as described in detail in previous publications (14, 26). Theory of metabolic flux analysis (MFA) and, in particular, transient ¹³C MFA can be found in several reviews (12, 15, 27). The optimizations were performed on a high-performance computing cluster using an evolution strategy with covariance matrix adaptation (pCMAlib, (28)).

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