Hydroxymethylglutaryl CoA synthase 2 (HMGCS2), the gene that regulates ketone-body production, is barely expressed in cultured cell lines. In this study, we restored HMGCS2 expression and activity in HepG2 cells, thus showing that the wild-type enzyme can induce fatty acid β-oxidation and ketogenesis, while a catalytically inactive mutant C166A did not generate either process. PPARα expression also induces fatty acid β-oxidation and endogenous HMGCS2 expression. Interestingly, PPARα-mediated induction was abolished when HMGCS2 expression was down-regulated by RNAi. These results indicate that HMGCS2 expression is both sufficient and necessary to the control of fatty acid oxidation in these cells. Next, we examined the expression pattern of several PPARα target genes in this now “ketogenic” HepG2 cell line. Fibroblast growth factor 21 (FGF21) expression was specifically induced by HMGCS2 activity or by the inclusion of the oxidized form of ketone bodies (acetoacetate) in the culture medium. This effect was blunted by sirtuin 1 (SirT1) RNAi, so we propose a SirT1-dependent mechanism for FGF21 induction by acetoacetate. These data suggest a novel feed-forward mechanism by which HMGCS2 could regulate adaptive metabolic responses during fasting. This mechanism could be physiologically relevant, since fasting-mediated induction of liver FGF21 was dependent on SirT1 activity in vivo.

The liver plays a central role in the adaptive response to fasting. The plasma hormone profile in this situation, low insulin and high glucagon, induces the release of large amounts of fatty acids from the adipose tissue to be used by peripheral tissues in order to spare glucose consumption. Therefore, the liver of a starved animal actively oxidizes fatty acid, which provides the energy necessary to sustain gluconeogenesis. It also supplies the acetyl-CoA needed for active ketone-body synthesis, which replaces glucose as energy substrate for the brain and other tissues (1).

Two enzymes determine the metabolic fate of fatty acids in the liver of starved animals: carnitine palmitoyltransferase-1 (CPT1) A (EC: 2.3.1.21) and hydroxymethylglutaryl CoA synthase 2 (HMGCS2) (EC: 4.1.3.5). CPT1A encodes a malonyl-CoA sensitive protein that regulates mitochondrial long-chain fatty acid oxidation (1), while HMGCS2 encodes a mitochondrial protein that controls the HMG-CoA cycle, by which acetoacetate, β-hydroxybutyrate, and NAD⁺ are generated (2). The expression of both genes is regulated by peroxisome proliferator activated receptor alpha (PPARα) (3-6), a fatty acid-activated nuclear receptor that regulates metabolic changes in the liver associated with starvation (7). Another gene directly regulated by PPARα in liver is fibroblast growth factor 21 (FGF21), a signaling molecule induced in the ketogenic state (8, 9).

Consistently, during starvation, PPARα null mice show severe hypoglycemia and hypoketonemia (7). The hypoglycemia is due to a reduced capacity for hepatic gluconeogenesis secondary to a 70% lower rate of fatty-acid oxidation (10). However, the decrease in fatty acid oxidation is not due to inappropriate expression of hepatic CPT1A, which is similar in both genotypes, but to impaired HMGCS2 expression in the PPARα null mouse liver (10).

It has been proposed that HMGCS2 interacts with PPARα and acts as a co-activator to up-regulate transcription from the PPRE of its own gene (11, 12). The HMGCS2-PPARα interaction is enhanced by HMGCS2 palmitoylation (12), underlying a putative mechanism by which PPARα is activated by one of its target gene products when fatty acid are available. However, this is a specific mechanism for HMGCS2, since other PPARα target genes are not co-activated by HMGCS2 expression (11).
In addition to this network of genes regulated by PPARα activation, another enzyme may contribute to the metabolic adaptation to fasting: sirtuin 1 (SirT1). This NAD+ dependent protein deacetylase is a general regulator of energy homeostasis in response to nutrient availability (13). Hepatic deletion of SirT1 alters PPARα signaling, especially the induction of FGF21 mRNA by PPARα ligands (14).

A common feature in hepatoma cell lines is the low capacity for long-chain fatty acid oxidation and ketone body production, which correlates with low expression of HMGCS2 (15). Over expression of PPARα in HepG2 cell can restore the expression of this and other genes and it induces fatty acid β-oxidation (6). Therefore, we examined whether PPARα-mediated expression of HMGCS2 acts as co-activator of this process.

In this paper we show that, in HepG2 cells, wild-type human HMGCS2 expression induces both fatty acid oxidation and ketogenesis. Using shRNAs, we also show that HMGCS2 expression is necessary for PPARα-mediated induction of fatty acid oxidation. In addition, we show that HMGCS2 expression stimulates FGF21 expression. We also report that FGF21 is induced by starvation by a mechanism involving SirT1 activity. Finally, we show that these events are dependent on HMGCS2 activity, since a catalytic dead mutant (C166A) failed to induce either fatty acid β-oxidation or FGF21 expression, while acetoacetate (an oxidized form of ketone bodies) can stimulate FGF21 mRNA induction by a SirT1-dependent mechanism. We propose a feed-forward model in which ketogenesis activates a SirT1-mediated response and long-chain fatty-acid oxidation.

**Experimental Procedures**

**Plasmids**

pcDNA3-HMGCS2-wt was cloned by EcoRI digestion of human HMGCS2 cDNA (16) and ligated into pcDNA3. pcDNA3-HMGCS2-C166A mutant plasmid was generated from pcDNA3-HMGCS2-wt by site-directed mutagenesis using Qiaquick Mutagenesis Kit (Qiagen) with the following oligonucleotides: Forward: 5'-gataccacagtgecgctacggttgtagtctcc-3' and Reverse: 5'-ggagccagctaccaagcaggctttgtagttctcc-3', following the manufacturer’s instructions. For GST pulldowns, pGEX-4T-human PPARα (17) was used.

**Animal experiments**

SirT1 liver-specific knockout (SirT1-LKO) mice were a gift from Dr. L. Guarente (18). SirT1-LKO mice were generated by crossing a mice with a SirT1 allele containing a floxed exon 4 (19) with Cre-expressing mice driven by the liver-specific albumin promoter on the C57BL/6 background. All mice were housed in cages on a 12:12-h light:dark cycle at controlled temperature (25 ± 1°C).

Four-month-old SirT1 LKO mice and their age-matched littermate Lox controls (Cre+/− , SirT1 flox/flox) were either fed ad libitum a standard laboratory chow diet or subjected to a 15h overnight fast. All animals were sacrificed at zeitgeber time (ZT) 3 (i.e., 3 h after the onset of the 12 h light span). Livers were extracted and immediately snap frozen in liquid nitrogen and stored at −80°C until analysis. Blood was collected by cardiac puncture and kept on ice until centrifugation (1500x g, 15 min at 4°C). The serum obtained was either used immediately for assays or stored at −80°C until analysis. All experimental protocols with mice were performed with approval of the animal ethics committee of Universitat de Barcelona, Barcelona, Spain.

**Cell Culture**

The human hepatocellular carcinoma cells HepG2 were cultured at 37°C in a humidified atmosphere containing 5% CO2 in Eagle’s Minimum Essential Medium (MEM) supplemented with 100 U/ml penicillin G, 100 µg/ml streptomycin and heat-inactivated 10% foetal bovine serum. HEK293 cell line was cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with antibiotics and 10% foetal bovine serum. All cell culture products were obtained from Invitrogen. Acetoacetate and 3-hydroxybutyrate were obtained from Sigma Aldrich (St. Louis, MO).

**Adenoviruses generation**

Recombinant adenoviruses were constructed as described previously (20). Briefly, cDNAs from wt and mutant forms of HMGCS2 were obtained from pcDNA3-HMGCS2 wt and pcDNA3-HMGCS2-C166A, respectively, by KpnI and Xhol digestion and cloned into pAdTrack-CMV shuttle vector. The resultant plasmid was linearized by digesting with Pmel and co-transformed into E. coli BJ5183 cells with pAdEasy-1 plasmid which contains the adenoviral backbone. This E. coli strains allows homologous recombination between both plasmids, giving rise to recombinant adenoviruses. When their identity had been confirmed by restriction endonuclease analyses, these viruses were transfected into the adenovirus packaging cell line HEK293. Recombinant adenoviruses were amplified in these cells, purified by CsCl gradient and titrated using the Adeno-X Rapid Titer Kit (BD Biosciences). As a negative control, we generated adenoviruses expressing only green fluorescent
protein (GFP). The parent plasmids were a gift from B. Vogelstein (The John Hopkins Oncology Center).

Adenoviral infection

In general, HepG2 cells were seeded 24h before infection. Cells were infected with MEM supplemented with 10% FBS at a multiplicity of infection of 40 for 48h.

HepG2 shRNA stable cell lines

Several retroviral-based plasmids containing a short hairpin RNA (shRNA) to human HMGCS2 were purchased from Origene (Rockville, MD). Scrambled shRNA was used as a negative control. The sequence of the human HMGCS2-specific 29-mer shRNA with highest efficacy reported here was CGTCTGTTGACTCCAGTGAAGCGCATTCT. Stable clones were generated using Phoenix™ Retroviral Expression System (Orbigen, San Diego, CA). Briefly, Phoenix cells were seeded at 3x10^6 cells per 100mm plate and transfected with 10 µg of shRNA plasmids using Lipofectamine LTX (Invitrogen) according to the manufacturer’s recommendations 18h later. 48h after transfection, retrovirus in the supernatant were harvested and filtered with 0.45 µm low binding protein filter and added to HepG2 cells with 4 µg/ml Polybrene (Sigma Aldrich). HepG2 cells were placed under selection with 1.0 µg/mL puromycin (Sigma Aldrich) until clones were formed. Clones were transferred with glass cylinders (Sigma Aldrich) and split when they reached confluence. Successful knockdown of specific gene products was confirmed by Western blot.

siRNA transfection

HepG2 cells were seeded 24h before transfection at a density of 4x10^4 cells/well in 6-well plates. Specific siGENOME SMARTpool against human HMGCS2 (M-010179-01) and SIRT1 (T2004-01) were purchased from Thermoscientific Dharmacon. siGENOME Non-Targeting siRNA #1 (D-001210-01-05) was used as a control. A concentration of 10 nM was transfected with Dharmafect 4 (Thermo Scientific Dharmacon) according to the manufacturer’s instructions. Cells were harvested 72h post-transfection and successful knock down was assessed Western Blot analysis.

Mitochondria isolation

To assay HMGCS2 activity, mitochondria were obtained from 48h infected HepG2 cells with indicated adenoviruses. Cells were harvested in cold PBS and centrifuged (500x g for 10 min). The pellet obtained was resuspended in 0.4 ml of homogenization buffer (150 mM KCl, 5 mMTris-HCl pH 7.2) and lysed by 10 cycles with each of the pestles of a mechanical Douncer homogenizer. The supernatant of a first centrifugation (250x g for 10 min) was submitted to a second centrifugation (16,000x g for 30 min). The resulting pellet was resuspended in three volumes of Resuspension buffer (0.4 mM DTT, 1.5% Triton X-100, 100 mM Tris-HCl pH 8) and desalted through a Bio-Spin chromatography columns (BioRad). Mitochondrial protein was quantified following Bradford and stored at -80ºC.

Enzymatic activity assay

HMGCS activity determination was carried out as described previously (21). HMGCS activity was measured as the incorporation of [1-14C]acetyl-CoA into HMG-CoA at 30ºC in 10 min. The reaction was initiated by adding mitochondrial protein preparation to a reaction mixture [100 mM Tris-HCl, 1 mM EDTA, 20 µM acetoacetyl-CoA, 200 µM and 12000 cpm/nmol [14C]acetyl-CoA (ITISA Biomedica)] in a final volume of 200 µl. After 10 minutes, the enzymatic reaction was stopped by adding 300 µl of 6 N hydrochloric acid and was incubated for 2 h at 100ºC. Radiolabelled HMG-CoA was recovered from the vials, diluted in Ecolite Scintillation Liquid (ICN) and counted in an automatic analyzer. HMGCS activity is expressed as nmols of produced HMG-CoA per minute.

Palmitate oxidation

Palmitate oxidation was performed in MW24 plates as previously described (22). Briefly, cells were incubated with 0.5mL/well of forces media (glucose and pyruvate-deprived DMEM containing 0.5mM palmitate, 0.1mM fatty acid free-BSA, 3mM glucose, 0.2mM carnitine) for 16h before the assay. Then, cells were incubated for 2h in forced media with radiolabelled palmitate at a final concentration of 0.5 mM (2.8 µCi/µmol [1-14C]palmitate (Amersham Biosciences). The reaction was stopped with 0.7mM perchloric acid, and radiolabelled released 14CO2 was recovered for 1h in Whatman paper soaked with 25 µl of β-phenylethylamine (Sigma Aldrich). Then, the trapped 14CO2 in the Whatman paper was quantified in a scintillation analyzer. To measure the acid soluble products (ASP), we followed the assay as previously described (23). Briefly, cells were scrapped, neutralized with 0.5N KOH and incubated at 60ºC for 30 min. The media was then acidified by addition of 150mM sodium acetate and 0.3N H2SO4. Cells were centrifuged at 1,000x g for 7 min and extracted with 1:1 chloroform/methanol. The aqueous phase was counted in a scintillation analyzer (ASP). Total palmitate oxidation was calculated as the sum of CO2 trapped plus ASPs recovered.

Ketone body determination

The concentration of total ketone bodies in the media of infected HepG2 cells was determined using
Autokit Total Ketone Bodies (Wako, Germany), according to the manufacturer’s instructions. When the sample (media from HepG2) is mixed with R1 (20mM phosphate buffer, pH 7.4, 2.7mM thio-NAD\textsuperscript{+}) and R2 (0.2M Good’s buffer, pH 9.0, 3200 IU/mL 3-Hydroxybutyrate dehydrogenase -3-HBDH- and 2.65 mM NADH), AcAc and 3-HB present in the media are converted to 3-HB and AcAc, respectively, in the presence of 3-HBDH, NADH, and Thio-NAD. Then, the 3-HB and AcAc produced in the enzymatic reactions are reverted to AcAc and 3-HB, again. During these cyclic reactions, NAD\textsuperscript{+} and Thio-NADH are produced. The concentration of total ketone bodies in each sample is determined by measuring the rate of Thio-NADH production. Thio-NADH is measured spectrophotometrically at 405 nm using a calibration curve previously performed by plotting the absorbance corresponding to 3-HB standards of known concentrations. 

**Western Blot Analysis**

Whole protein cell extracts (WCE) were obtained from infected HepG2 cells or liver of mice. Briefly, cells were homogenized in NP-40 Lysis buffer (NaCl 150 mM, TrisHCl 50 mM, NP40 1%) supplemented with a cocktail of protease inhibitors (Sigma Aldrich) and 0.1mM phenylmethylsulfonyl fluoride (Sigma Aldrich). Proteins from mouse liver were obtained by homogenization in RIPA buffer (1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 10 mM Tris 7.0) containing protease inhibitor cocktail (Sigma Aldrich). Protein extracts were subjected to centrifugation at 16,000 x g for 15 min. The supernatant (WCE) was quantified following Bradford and stored at -80ºC. WCE were loaded in a 8% SDS-PAGE gel and then transferred to Immobilon-P membranes (Millipore, Bedford, MA) and probed with different antibodies. The antibodies used were: Human HMGCS2 polyclonal antibody obtained as described previously (24) or mHMGCS from Santa Cruz Biotechnologies (SCB) (1:500, cat#: sc-33828); SIRT1 antibody from Upstate Biotechnologies (1:1000, cat#: 07-131) or from SCB (1:500, cat#: sc-15404); PPARα antibody from SCB (1:1000, cat#: sc-1985); RXRα SCB antibody (1:500, cat#: sc-553); actin antibody (1:1000, Sigma Aldrich, cat#: A2066) and tubulin antibody (1:1000, Calbiochem, cat#: CP06). Detection was carried out using an ECL kit Chemiluminiscence Detection Kit for HRP (Biological Industries).

**Immunocytochemistry**

HepG2 were seeded onto cover slips (6x10\textsuperscript{5} cell/p60) 24 h before infection. HepG2 cells were infected with adenovirus, as indicated in the figure legends, and after 48 h of infection they were fixed with 4% paraformaldehyde. Antibody staining on fixed cells was done using a standard protocol. Cells were permeabilized using 0.1% Triton X (Sigma Aldrich). The primary antibodies used were goat anti-PPARα (1:1000, sc-1985-Santa Cruz Biotechnologies) and rabbit anti-HMGCS2 (1:100, SantaCruz Biotechnologies, sc-33828). Secondary antibodies were obtained from Invitrogen: Alexa Fluor® 488 donkey anti-goat IgG (H+L) *2mg/mL (1:1000), Alexa Fluor® 647 chicken anti-rabbit (H+L) *2mg/mL (1:500). DAPI (6.25µg/mL; Sigma) was used to label DNA. Fluorochrome-labeled samples were analyzed and captured using a Leica SP2 confocal microscope. Final artwork was processed using ImageJ software.

**Pull-down assay**

GST and GST-PPARα fusion proteins were produced in *Escherichia Coli* and purified on glutathione-sepharose beads (Amersham Pharmacia Biotech), as described previously (17). The amount and integrity of GST was checked by SDS-PAGE and Coomassie Blue staining. 250µg of whole cell protein extracts from HepG2 infected cells were incubated in the presence of equivalent amounts of immobilized GST or GST-PPARα (12.5 µg) in 1 mL of binding buffer NETN (20mM Tris pH 8, 100mM NaCl, 1mM EDTA 0.5% Nonidet 40), supplemented with 0.5% non fat milk, protease inhibitor cocktail (Sigma) and 1mM DTT, for 4h at 4ºC with agitation. The samples were then centrifuged for 1 min at 500x g and the resin was washed twice in NETN at room temperature. After that the samples were boiled, mixed with 2x Laemlli Buffer and resolved by SDS-PAGE in a 8% polyacrylamide gel. Bound proteins were detected by immunoblot using anti-HMGCS2 (24) or RXRα antibody from SCB (1:500, cat#: sc-553).

**Real time RNA analysis**

Total RNA was extracted from cells or liver by Tri-Reagent (Ambion) and was further treated with DNase I (Ambion). For real-time PCR analysis, cDNA was synthesized from total RNA by MLV reverse transcriptase (Invitrogen) with random hexamers (Roche Diagnostics). cDNA was subjected to PCR real time analysis using TaqMan universal PCR master mix (Invitrogen Cat#:11743) and the specific gene expression primer pair Taqman probes from Applied Biosystems (For HepG2 human gene probes were used: FGF21 Hs00173927_m1; CPT1A Hs00157079_m1; HMGS2 000840165_g1; CPT1A Mm00550438_m1; PCK1 Hs00159918_m1; CPT2 Hs00988962_m1; RXRα Hs00231882_m1; CPT1A Mm00157079_m1; HMGS2 Hs00085427_m1; PCK1 Hs00159918_m1; CPT2 Hs00988962_m1; RXRα Hs00231882_m1; For mice experiments mouse probes were used: HMGS2 Mm000550050_m1; FGF21 Mm00840165_g1; CPT1A Mm00550438_m1;
Measurement of serum FGF21

Mouse FGF21 enzyme-linked immunosorbent assay (ELISA) kit was obtained from Millipore (Cat.#: EZRMFGF21-26K) for the quantification of FGF21 in mice serum. The assay was conducted according to the manufacturer's protocol. Briefly, a calibration curve was constructed by plotting the difference of absorbance values at 450 nm and 590nm versus the FGF21 concentrations of the calibrators, and concentrations of unknown samples (performed in duplicate) were determined by using this calibration curve.

Statistical Analysis

Data are expressed as means ± standard deviation. The significance of differences was determined using SPSS statistical software (SPSS Worldwide Headquarters, Chicago, IL). P < 0.05 was considered statistically significant. *p<0.05; **p<0.01; ***p<0.001.

Results

Human HMGCS2 expression induces fatty acid oxidation and ketogenesis in HepG2 cell line. Wild type or a mutant form of HMGCS2 enzymes and human PPARα were expressed in HepG2 cell line by using adenoviral vector systems. The HMGCS2 C166A mutant was predicted as a dead enzyme since an equivalent cysteine residue 129 has been characterized as the catalytic site in the homologous HMGCS1, the cytosolic enzyme involved in cholesterol synthesis (25, 26). Figure 1 shows recombinant expression of human HMGCS2 in terms of activity (Fig 1A) and protein (Fig 1C, bottom) levels. The HMGCS2 enzymatic activity (approx. 0.5 mU/mg) that was achieved from adenoviruses-mediated expression was similar than observed in liver mitochondria from feed mouse [data not shown and (27)]. Figure 1A also shows that the C166A mutant (AdHMGCS2 MUT) expresses an inactive HMGCS2 enzyme. Figure 1B shows that expression of HMGCS2 was sufficient to induce ketone body production by the HepG2 cells and, as expected, the expression of the dead enzyme lacks this ability. Interestingly, Figure 1C shows that recombinant expression of human HMGCS2 was also sufficient to induce fatty acid oxidation. Figure 1C also shows that the induction of β-oxidation mediated by HMGCS2 expression is similar to the induction mediated by PPARα expression which, as expected (6), also induces HMGCS2 protein levels (Figure 1C bottom) and HMGCS2 mRNA levels (Table I). These data suggest that expression of HMGCS2 in HepG2 cell line is sufficient to restore the HMG-CoA cycle (28) and that activation of ketogenesis from acetyl-CoA, in turn, activates fatty acid β-oxidation.

Human HMGCS2 expression is necessary for PPARα-mediated induction of fatty acid oxidation. PPARα expression in HepG2 cells induces both HMGCS2 and mitochondrial fatty acid oxidation (6). Therefore, we hypothesized that active ketogenesis could be necessary for PPARα-mediated induction of fatty acid β-oxidation. Figure 2A shows that a specific shRNA abrogated HMGCS2 induction mediated by PPARα. Figure 2B shows that β-oxidation induction mediated by PPARα was blunted by down-regulation of HMGCS2 expression. These data suggest that PPARα induces fatty acid oxidation but, importantly, that the expression of the ketogenic key enzyme HMGCS2 is needed for the induction.

HMGCS2 regulates FGF21 expression in HepG2 cell line. The effect of HMGCS2 on PPARα-mediated-stimulation of fatty acid oxidation could be mediated by direct HMGCS2 co-activation (11, 12) or a metabolic effect driven by stimulation of fatty-acid degradation (Figure 2). Therefore, we analyzed the effect of HMGCS2 expression on PPARα target genes. Figure 3A shows that over-expression of HMGCS2 induced FGF21 expression and that catalytic activity of the enzyme was needed for this mRNA induction. Figure 3B shows that knock-down of HMGCS2 down regulated FGF21 mRNA levels in the absence (Fig. 3B right) or presence (Fig. 3B left) of PPARα. This effect was specific for FGF21, since the mRNA levels of other PPARα target genes were not affected by HMGCS2 expression (Table I).

To follow the activator effect of HMGCS2, we performed luciferase promoter assays in which no co-activation by HMGCS2 was observed on PPARα mediated-induction of CPT1α or HMGCS2 promoters (data not shown). These data are consistent with the lack of nuclear co-localization of recombinant PPARα and endogenous HMGCS2 shown by immunohistochemistry analysis in HepG2 cells (Figure 4A). In addition, pull-down experiments showed no interaction of HMGCS2 over-expressed in HepG2 with the GST-PPARα purified protein that can interact with its RXRα partner (Figure 4B). These results indicate that the effect on FGF21 expression observed in HepG2 cells was not related to co-activation of PPARα by HMGCS2.
FGF21 is a SirT1 target gene during fed-to-fast transition. An alternative metabolic hypothesis for HMGCS2 stimulation of FGF21 is that the effect of HMGCS2 on ketogenesis and FGF21 expression could be correlated throughout the NAD⁺ intracellular levels since: i) ketone body production implies the reduction of acetoacetate to β-hydroxybutyrate with the concomitant generation of NAD⁺ (2), and ii) the effect of PPARα ligands on FGF21 expression are dependent on SirT1 activity (14). Therefore we hypothesize that HMGCS2 could affect FGF21 expression via a mechanism relaying on ketogenesis stimulation of SirT1 activity. To confirm this hypothesis we studied the mRNA expression FGF21 in wild-type or SirT1 liver knockout mice during the fed-to-starved transition. Figure 5A shows that FGF21 induction mediated by starvation was dependent of SirT1. Figure 5A also shows a specific effect for FGF21, since the starvation-associated induction of other PPARα target genes, like CPT1A and HMGCS2, was not affected (black bars compared to dark grey bars). Figure 5B shows that circulating levels of FGF21 protein were impaired in starved SirT1 LKO, indicative of the physiological relevance of SirT1 dependence. Figure 5C shows that SirT1 is specifically knocked out in the livers of SirT1 LKO mice (18).

Acetoacetate modulates FGF21 mRNA expression by a SirT1 dependent mechanism. Next, to pursue the hypothesis about NAD⁺ levels we treated HepG2 cells with the oxidizing (acetoacetate) or reducing (β-hydroxybutyrate) partners of ketone bodies. Figure 6A shows that acetoacetate induced FGF21 expression in a dose dependent manner while β-hydroxybutyrate did so to a lesser extent. Figure 6B (left) shows that acetoacetate mediated induction of FGF21 expression was dependent on SirT1 expression. Figure 6B (right) shows that acetoacetate did not affect endogenous SirT1 levels and that the siRNA treatment was efficient. These results suggest that the products of ketogenesis can stimulate gene expression through the SirT1 activity and therefore that HMGCS2 could control metabolic processes other than ketogenesis in this cell line.

Discussion

Fatty acid β-oxidation and ketogenesis are induced during fasting or lactation (1) and also in pathological situations such as diabetes (reviewed in 29). PPARα mediates the induction of genes responsible for controlling both processes. The HMGCS2 gene controls ketogenesis (2) and is a PPARα target (4-6). Studies with the homologous HMGCS1 (19, 20) (an isotype that catalyzes the same reaction in the cytosol, where it controls the mevalonate pathway) suggested that Cysteine 166 is part of the active site of the mitochondrial HMGCS2 enzyme. Therefore, in this paper, we generated two adenoviruses expressing wild type and a catalytically inactive mutant (C166A, see Figure 1A) of the human HMGCS2. When HMGCS2 activity was expressed, HepG2 cells become ketogenic (Fig 2B). Interestingly, we also observed that these cells had a greater capacity to oxidize long chain fatty acids. The β-oxidation induction was similar to that seen in PPARα-infected cells (Fig. 2C). These results suggest that expression of the gene not only controls ketogenesis, but that it can also control the β-oxidation pathway in certain circumstances.

Recombinant PPARα expression in HepG2 cells induces β-oxidation of fatty acids and the expression of genes such as FGF21, HMGCS2, and CPT1A ([6] and Table I). It has been proposed that HMGCS2 is a co-activator of PPARα (11) through a mechanism involving the HMGCS2 palmitoylation of Cys166 (12). In fact, we found that expression of HMGCS2 was involved in the induction of fatty acid oxidation mediated by PPARα (Fig. 2). However, our data do not support the role of HMGCS2 as a PPARα co-activator. We did not observe an interaction between PPARα and HMGCS2 nor a nuclear co-localization of both proteins (Fig. 4). In addition, no effect of co-activation was observed at the level of reporter gene even for HMGCS2, which was proposed (12) as the target of PPARα co-activation (data not shown). Therefore, we have sought an alternative hypothesis to explain the role of HMGCS2 in controlling fatty acid oxidation.

Liver synthesizes acetoacetate through the 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) cycle, which is driven by the three mitochondrial located enzymes acetoacetyl-CoA thiolase (EC 2.3.1.9), hydroxymethylglutaryl-CoA synthase [HMGCS2, (EC 4.1.3.5)], and hydroxymethylglutaryl-CoA lyase (EC 4.1.3.4) and produces 1 mol of acetoacetate, 1 mol of acetyl-CoA, and 2 mol of free CoA from three molecules of acetyl-CoA (2). Acetoacetate is further reduced to β-hydroxybutyrate through mitochondrial β-hydroxybutyrate dehydrogenase (EC 1.1.1.30), driven by high levels of NADH in hepatic mitochondria. Interestingly, in humans (30) and other mammals (31) a cytosolic isotype of β-hydroxybutyrate dehydrogenase has been characterized, suggesting a reversible conversion between acetoacetate and β-hydroxybutyrate depending on the [NAD⁺]/[NADH] ratio. In addition, diet manipulation, like caloric restriction, affects the
FGF21 expression in a mechanism dependent on cytosolic [NAD]/[NADH] generation, could modulate the certain circumstances HMGCS2 activity, through To explain our data, we propose that under conditions of fasting, the [NAD]/[NADH] ratio and therefore SirT1 activity. Consistent with this hypothesis we observed that addition of acetoacetate, but not β-hydroxybutyrate, to the HepG2 cell medium mimics FGF21 expression in a mechanism dependent on SirT1 (Fig. 6). Therefore, HMGCS2 seems to have a specific effect on gene expression, which does not depend on PPARα co-activation. However, our results do not fully explain why HMGCS2 expression is necessary for the induction of β-oxidation mediated by PPARα. It is possible that changes in the mitochondrial [NAD]/[NADH] ratio are also partly responsible for this phenomenon. However, it is also possible that removal of acetyl-CoA, as acetoacetate, accelerates the catabolic process; and that both FGF21 mRNA expression and β-oxidation are stimulated by HMGCS2 through independent mechanisms.

Proliferating cells do not express HMGCS2 activity (10, 15). The human gene is a target of c-Myc and its expression is characteristic of differentiated cells of the colon (24). The role of oxidative metabolism in cell transformation has recently been highlighted (reviewed in 32). It has been found that p53 upregulates fatty acid oxidation induced by glucose starvation (33) and it is proposed that this effect would be part of its role as a tumor suppressor gene since active β-oxidation would protect the cell from Warburg effect (reviewed in 34). We have not observed a clear effect of HMGCS2 gene expression on cell proliferation, a target gene of p53 (data not shown). However, we have shown how HMGCS2 expression produces metabolic effects capable of inducing the expression of genes such as FGF21 and altering the oxidative flux of long-chain fatty acids.

In conclusion, our results suggest that HMGCS2 expression affects the PPARα-mediated response. However, we propose an alternative mechanism to the previously proposed co-activation of PPARα (11, 12). Our mechanism would be related to changes in the metabolites of the cell induced by the acceleration of ketogenesis, although these changes could be related to factors other than SirT1 activity.

REFERENCES

FOOTNOTES

We are truly grateful to Dr. Leonard Guarente and Dr. Hung-Chun Chang (Department of Biology, Massachusetts Institute of Technology) for liver specific SirT1 KO mice. This project was supported by grants BFU2007-67322/BMC (to PFM) and SAF2010-15217 (to DH) from Spain’s Ministerio de Educació n y Ciencia and RCMNCO3/08 (to DH), and also by funding from the Catalan government (Ajut de Suport als Grups de Recerca de Catalunya 2005SGR0857 and 2009SGR163). AVB was supported by Scholarship from Catalan government (Ajut al Personal Investigador –FI 2007-2011), ALDSC was supported by"Fundação para a Ciência e a Tecnologia (FCT)" from Portuguese Government.

The abbreviations used are: HMGCS2, hydroxymethylglutaryl CoA synthase 2; PPARα, peroxisome proliferator activated receptor alpha; FGF21, fibroblast growth factor 21; SirT1, sirtuin 1; CPT1A, carnitine palmitoyltransferase-1A; CPT2, carnitine palmitoyltransferase-2; PCK1, Cytosolic phosphoenolpyruvate carboxykinase
TABLE I: mRNA induction of different PPARα target genes. HepG2 cells were infected with adenovirus expressing human PPARα or human wild type or C166A mutant of HMGCS2. The mRNA levels were determined by real time PCR. Data represent the fold of induction of the specific mRNA after adenovirus infection of at least three independent experiments. N.d.: not determined

<table>
<thead>
<tr>
<th></th>
<th>AdPPARα</th>
<th>AdHMGCS2 WT</th>
<th>AdHMGCS2 MUT</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMGCS2</td>
<td>45.0±15.7***</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>CPT1A</td>
<td>2.74±0.9*</td>
<td>1.27±0.7</td>
<td>0.86±0.2</td>
</tr>
<tr>
<td>CPT2</td>
<td>7.35±5.1</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>PCK1</td>
<td>5.95±3.3*</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>FGF21</td>
<td>15.4 ± 5.8*</td>
<td>3.3 ± 1.03*</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>PPARα</td>
<td>n.d.</td>
<td>1.41±0.2</td>
<td>1.61±0.3</td>
</tr>
</tbody>
</table>

FIGURE LEGENDS

Figure 1.- Effect of wild type or mutant HMGCS2 expression on activity, ketogenesis, and fatty acid β-oxidation. HepG2 cells were infected with adenovirus control (AdGFP) or expressing wild type (AdHMGCS2-wt) or C166A mutant (AdHMGCS2-mut) human HMGCS2 for 48h. (A) Enzymatic activity was measured in dialyzed mitochondria preparation. (B) Total ketone bodies were determined in the media. (C) Total [14C]-palmitate oxidation was measured in HepG2 cells infected with adenoviruses expressing human HMGCS2 variants or human PPARα (AdPPARα). The average of 3 independent experiments is shown. The bottom panel shows a representative Western blot of recombinant PPARα and recombinant (left) or endogenous (right) HMGCS2. 10µg of whole cell extract were loaded to show recombinant HMGCS2 expression, and 70µg to show endogenous HMGCS2.

Figure 2.- Effect of HMGCS2 expression on PPARα-mediated induction of fatty acid β-oxidation. HepG2 stable cell lines expressing a shRNA control (scrambled shRNA) or a shRNA specific for HMGCS2 were infected with human PPARα expressing adenovirus (AdPPARα). (A) Representative Western blot analysis of recombinant PPARα, endogenous HMGCS2 and actins as loading control. 70µg of whole cell extract were loaded (B) Total [14C]-palmitate oxidation was measured in HepG2 cells expressing (scrambled shRNA) or not expressing (HMGCS2 shRNA) HMGCS2. Results are expressed in PPARα fold induction versus GFP infection. The average of 3 independent experiments is shown.

Figure 3.- HMGCS2 regulates FGF21 expression in HepG2 cell line. (A) FGF21 mRNA levels of cells infected with adenoviruses expressing GFP (AdGFP), wild type (AdHMGCS2-wt) or C166A dead mutant (AdHMGCS2-mut) human HMGCS2. (B) FGF21 mRNA levels of HepG2 transfected with siRNA control siRNA (non targeting) or specific siRNA against HMGCS2 and infected with adenoviruses expressing GFP (AdGFP) (right) or human PPARα (AdPPARα) (left) is shown. Results are expressed as the percentage of induction by PPARα in presence of HMGCS2 (100%) or in absence of HMGCG2. The average of 5 independent experiments is shown The average of 3 independent experiments is shown.

Figure 4.- Recombinant PPARα does not co-localize nor interact with endogenous HMGCS2. (A) Immunohistochemical analysis of recombinant PPARα and endogenous HMGCS2 expression in HepG2 cell. Cells infected with adenovirus control (AdLacZ) or adenovirus encoding human PPARα (AdPPARα) were fixed and stained with DAPI and incubated with anti-PPARα or anti-HMGCS2 antibody followed by Alexa-Fluor 488 or Alexa-Fluor 647, respectively, and viewed with a confocal microscope. (B) Pull-down experiment of recombinant GST-PPARα. HepG2 cells were infected with adenoviruses expressing GFP (AdGFP), wild type (AdHMGCS2 WT) or C166A dead mutant (AdHMGCS2 MUT) human HMGCS2 were
treated (+) or not (-) with palmitate 0.5mm for 16 hours. Whole protein cell extracts of the infected HepG2 cells were incubated with GST-PPARα generated in *E.coli*. GST recombinant protein was captured by glutation-sepharose beads, and the recovered beads were immunoblotted with anti-HMGCS2 and RXRα antibodies. Inputs show endogenous RXRα and recombinant HMGCS2 variants in whole protein cells extracts.

*Figure 5.* FGF21 is a SIRT1 target gene during fed-to-fast transition. (A) Quantitative RT-PCR analysis of FGF21 (left), HMGCS2 and CPT1A (right) gene expression in liver from mice fed *ad libitum* or fasted for 15 h normalized to 18S expression; (B) ELISA measurement of FGF21 in serum. (C) Western blot of liver extracts from mice WT and LKO between 5 and 10 animals per group were used. Open bars represent WT mice fed *ad libitum*, pale grey bars represent SIRT1 LKO fed *ad libitum*, dark grey represent 15h fasted WT mice and closed bars represent 15h fasted SIRT1 LKO.

*Figure 6.* FGF21 is induced by acetoacetate in HepG2 cell line. (A) HepG2 cells were treated with acetoacetate 1 (low) or 10mM (high) or with 3-hydroxybutyrate 2 (low) or 20mM (high) for 5h in regular growth media. Relative expression of FGF21 mRNA was assessed by real time PCR. The average of 3 independent experiments is shown. (B) Levels of mRNA of FGF21 in cells transfected with a siRNA control or siRNA specific for SIRT1 in the absence (open bars) or 10 mM acetoacetate (closed bars) (Left). Levels of SIRT1 protein cells transfected with a control siRNA or specific SIRT1 siRNA, in the absence (-) or the presence (+) of 10 mM acetoacetate. The average of 3 independent experiments is shown.
Figure 1

A) HMGCS2 activity (mU)

B) Ketone Bodies (µM 3HB)

C) Palmitate oxidation (nmol/h/mg prot)

HMGCS2

PPARα
Figure 2

(A) Western blot analysis of HMGCS2, PPARα, and ACTIN expression levels with scrambled shRNA and HMGCS2 shRNA treatments. AdPPARα treatment is indicated.

(B) Bar graph showing the fold change in palmitate oxidation compared to GFP levels. AdGFP and AdPPARα treatments are compared with scrambled shRNA and HMGCS2 shRNA treatments.
Figure 6

A) 

[Graph showing FGF21 mRNA relative expression (fold induction) for Acetoacetate and 3-Hydroxybutyrate under control, Low, and High conditions.]

B) 

[Graph showing FGF21 mRNA relative expression (fold induction) for control and AcAc 10mM conditions with siRNA control and SIRT1 siRNA.]

[Additional inset showing Western blot images for Control siRNA and SIRT1 siRNA with TUBULINS as a control.]
Human HMGCS2 regulates mitochondrial fatty acid oxidation and FGF21 expression in HepG2 cell line

Anna Vila-Brau, Ana Luisa De Sousa-Coelho, Cristina Mayordomo, Diego Haro and Pedro F. Marrero

J. Biol. Chem. published online April 18, 2011

Access the most updated version of this article at doi: 10.1074/jbc.M111.235044

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2011/04/18/jbc.M111.235044.full.html#ref-list-1