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Prevention and reversal of hepatic steatosis with a high-protein diet in mice

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ABSTRACT

The hallmark of NAFLD is steatosis of unknown etiology. We tested the effect of a high-protein (HP)[2] diet on diet-induced steatosis in male C57BL/6 mice with and without pre-existing fatty liver. Mice were fed all combinations of semisynthetic low-fat (LF) or high-fat (HF) and low-protein (LP) or HP diets for 3 weeks. To control for reduced energy intake by HF/HP-fed mice, a pair-fed HF/LP group was included. Reversibility of pre-existing steatosis was investigated by sequentially feeding HF/LP and HF/HP diets. HP-containing diets decreased hepatic lipids to ~40% of corresponding LP-containing diets, were more efficient in this respect than reducing energy intake to 80%, and reversed pre-existing diet-induced steatosis. Compared to LP-containing diets, mice fed HP-containing diets showed increased mitochondrial oxidative capacity (elevated *Pgc1α*, *mAco*, and *Cpt1* mRNAs, complex-V protein, and decreased plasma free and short-chain acyl-carnitines, and $[C_0]/[C_{16}+C_{18}]$ carnitine ratio); increased gluconeogenesis and pyruvate cycling (increased PCK1 protein and fed plasma–glucose concentration without increased *G6pase* mRNA); reduced fatty-acid desaturation (decreased *Scd1* expression and $[C_{16:1n-7}]/[C_{16:0}]$ ratio) and increased long-chain PUFA elongation; a selective increase in plasma branched-chain amino acids; a decrease in cell stress (reduced phosphorylated eIF2α, and *Fgf21* and *Chop* expression); and a trend toward less inflammation (lower *Mcp1* and *Cd11b* expression and less phosphorylated NFκB). Conclusion: HP diets prevent and reverse steatosis independently of fat and carbohydrate intake more efficiently than a 20% reduction in energy intake. The effect appears to result from fuel-generated, highly distributed small, synergistic increases in lipid and BCAA catabolism, and a decrease in cell stress.

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[2] AA: arachidonic acid; Acac: acetyl-CoA carboxylase; Acox: acyl-coenzyme A oxidase; Akt: thymoma viral proto-oncogene 1 (a.k.a. PKB-protein kinase B); Alas1: aminolevulinic acid synthase 1; Arntl (a.k.a. Bmal1): aryl hydrocarbon receptor nuclear translocator-like protein; BCAA: branched-chain amino acids; BHB: β-hydroxybutyrate; ITGAM: Integrin-αM; Chop/Ddit3: C/EBP-homologous protein/DNA damage-inducible transcript 3 protein; Chrebp: carbohydrate-responsive element-binding protein; Cpt1: carnitine palmitoyltransferase 1; DHA: docosahexaenoic acid; eIF2α: eukaryotic translation–initiation factor 2α; Elovl: elongation of very long-chain fatty acids; en%: energy percent; EPA: eicosapentaenoic acid; Fasn: fatty acid synthase; Fgf21: fibroblast growth factor 21; FFA: free fatty acids; G6Pase: glucose-6-phosphatase; GCN2: general control nonrepressed 2; HF: high fat; HF/LPres: high fat, low protein restricted; HP: high protein; LF: low fat; LP: low protein; Mcp1: monocyte chemotactic protein 1; Mlxipl (a.k.a. Chrebp): Mlx-interacting protein-like; mAco: mitochondrial aconitase; mmBCFA: monomethyl branched-chain fatty acid; NAFLD: non-alcoholic fatty liver disease; NASH: non-alcoholic steatohepatitis; Nfil3: nuclear factor interleukin-3-regulated protein; NFκB: nuclear factor κB; Nr1d1 (a.k.a. rev-erbα): nuclear receptor subfamily 1, group D, member 1; PCK1: phospho*enol*pyruvate carboxykinase 1; PERK: protein kinase RNA-like endoplasmic reticulum kinase; Pgc1α: ppar-γ-coactivator1-α, PL: choline-containing phospholipids; Ppar: peroxisome proliferator-activated receptor; r_s: Spearman correlation coefficient; Scd1: stearoyl-CoA desaturase 1; Srebf: sterol regulatory element-binding transcription factor; TC: total cholesterol; TG: triglycerides.

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1. Introduction

Non-alcoholic fatty-liver disease (NAFLD), the most common chronic liver disease in affluent societies, is the hepatic manifestation of the metabolic syndrome [1]. The hallmark of NAFLD is hepatic lipid accumulation (steatosis) of unknown etiology. Non-alcoholic steatohepatitis (NASH) develops when a fatty liver becomes invaded by inflammatory cells. Although steatosis itself is generally considered to be benign, lipotoxic by-products of fatty-acid metabolism, such as free fatty acids, (lyso-)phosphatidic acids, lysophosphatidylcholines, ceramides and diacylglycerols, also accumulate [2,3]. Furthermore, steatosis co-occurs with increased plasma insulin and VLDL, and decreased plasma adiponectin levels, but not necessarily with higher body-mass index or visceral adiposity [4]. Finally, steatosis is an established risk factor for patients undergoing major liver surgery [5]. Steatosis-reducing therapies should benefit patients with NAFLD but, apart from changes in life style, currently no nutritional recommendations or specific treatment options for NAFLD or NASH [6] exist.

Steatosis results from an imbalance between lipid supply (uptake or *de novo* lipogenesis) and lipid disposal (via free fatty-acid oxidation or triglyceride-rich lipoprotein secretion). Since high-protein (HP) diets may have anti-lipogenic effects [7], we tested if a HP diet prevents the development of steatosis in mice. Mice were fed all four combinations of a low-fat (LF) or a high-fat (HF) and a low-protein (LP) or a high-protein (HP) diet. Our findings show that HP-containing diets decrease hepatic lipid content independently of dietary fat and carbohydrate intake, due to increased fatty-acid oxidation and gluconeogenesis, and reduction of cellular stress. We also tested and confirmed that a HP diet can reverse a pre-existing fatty liver.

2. Methods

Detailed protocols (mRNA quantitation, Western blotting, plasma and liver analysis) and details on materials, antibodies, diets, and primers used (Appendix A: Supplemental Tables A.1–A.4, respectively), and RNA normalization (Supplemental Table A.5) are provided in online Supplemental materials, Appendix A.

2.1. Animals

Eight-10 male C57BL/6J mice (12 weeks) were kept in groups of 2 per cage in a temperature-controlled facility with fixed 12 h light–dark cycles and free access to food and water. Twice weekly, food intake and body weight were measured. After three weeks on the specified diet, mice were sacrificed in the morning (10:00 h–12:30 h) to avoid chronobiological effects. The study was approved by the Committee for Animal Care and Use of Maastricht University.

2.2. Diets

Four semi-synthetic diets (Research Diets, New Brunswick, NJ, USA; Supplemental Table A.3) were designed to test the effect of a high-protein (HP) content in a low-fat (LF) or high-fat (HF) diet. The low-protein (LP) diet contained 11 en% protein, which meets the daily protein requirement of adult mice [8]. The LF/LP diet contained 8 en% fat and 11 en% protein; the LF/HP diet 8 en% fat and 35 en% protein; the HF/LP diet 42 en% fat and 11 en% protein; and the HF/HP diet 42 en% fat and 35 en% protein. We previously showed that the source of dietary fat does not affect the development of fatty liver in C57BL/6 mice [9]. To control for the reduced energy intake of mice on a HF/HP diet (~80% of that of mice on the HF/LP diet; Table 1), an additional group (HF/LP restricted or "HF/LPres") received the same (reduced) amount of calories as consumed by the HF/HP group. To determine if a HP diet also reduced the fat content of a pre-existing steatotic liver, 12 mice were fed a HF/LP diet for three weeks and then either the same diet ("HF/LP4w"; n = 6) or a HF/HP diet ("HF/LP → HP"; n = 6) during the 4th week.

2.3. Statistical analysis

Error bars represent standard errors of the mean (SEM). A two-way ANOVA (computed with SPSS 15) was used to test for differences between the main effects (LP versus HP and LF versus HF) and their interactions. Subsequently, pair-wise comparisons between groups (LF/LP, LF/HP, HF/LP, HF/HP or HF/LPres diet) were performed. The outcome of the reversion experiment (HF/LP4w versus HF/LP → HP) was analyzed with a *t*-test. P values < 0.05 were considered significant and <0.10 as indicating a trend. Details of the analyses are shown in Supplemental Tables A.6–A.9.

3. Results

3.1. A HP content in the diet abolishes weight gain due to a HF diet

Biometric data of mice that were fed the respective experimental diets for 3 weeks are summarized in Table 1. All diets were accepted well. Triglyceride concentration in feces did not exceed 140 μg/100 g fecal dry mass and did not differ between the diets (not shown), demonstrating that all dietary fat was absorbed. Mice on a HP diet gained less body weight (P = 0.016) and had smaller epididymal fat pads (P = 0.006) than mice on the corresponding LP diet, but liver weight had not changed. Mice on the HF/LP diet had a higher energy intake (+20%) than mice on the HF/HP diet (P = 0.001). If the HF/LP group was pair-fed to the HF/HP group (HF/LPres), mice lost 3.6% of their initial body weight (P = 0.107), and had lower epididymal fat-pads

Table 1
Biometric data of WT mice fed a LF/LP, LF/HP, HF/LP, HF/HP, HF/LPres, HF/LP4w, or HF/LP → HP diet.

						ANOVA (P value)		
	LF/LP	LF/HP	HF/LP	HF/HP	HF/LPres	HP	HF	HPxHF
Body weight start [g]	24.7 ± 0.3	24.1 ± 0.7	24.7 ± 0.6	25.4 ± 0.5	25.1 ± 0.5	n.s.	n.s.	n.s.
Weight change [% BW start]	4.0 ± 1.2^{a}	1.8 ± 1.5^{b}	6.2 ± 2.3^{c*}	−0.7 ± 1.3^{d*}	−3.6 ± 2.1	0.016	n.s.	0.1
Epididymal fat pad [g]	0.36 ± 0.02^{a}	0.31 ± 0.02^{a}	0.50 ± 0.05^{b*}	0.36 ± 0.03^{a*}	0.39 ± 0.04	0.006	<0.001	n.s.
Liver weight [% BW]	5.4 ± 0.1^{ab}	5.6 ± 0.1^{a}	5.0 ± 0.1^{b*}	5.1 ± 0.1^{ab*}	4.5 ± 0.1	n.s.	<0.001	n.s.
Energy intake [kcal/cage/day]	12.5 ± 0.1^{a}	12.3 ± 0.9^{a}	14.1 ± 0.2^{b*}	11.7 ± 0.1^{a}	11.8 ± 0.0	0.001	n.s.	<0.001
Reversion experiment:				HF/LP4w				HF/LP → HP
Body weight start [g]				26.9 ± 0.7				26.8 ± 0.6
Weight change [% BW start]				9.2 ± 2.9				2.3 ± 1.8$^{\#}$
Epididymal fat pad [g]				0.74 ± 0.1				0.56 ± 0.04$^{\#}$
Liver weight [% BW]				4.9 ± 0.1				5.0 ± 0.3

Mean ± SEM; n = 8–10 mice per group. Different letters (a, b, or c) above the bars indicate a significant difference between the diets fed ad libitum. * indicates P<0.05 when compared to HF/LPres; # indicates P<0.05 when HF/LP4w versus HF/LP → HP.

(P = 0.070) and liver weights (P = 0.021) than mice on the HF/LP diet fed ad libitum.

3.2. A HP content in the diet prevents hepatic lipid accumulation

The HF/LP diet effectively induced hepatic steatosis, as demonstrated by Oil-Red O staining (Fig. 1A) and an ~1.8-fold increase of hepatic triglyceride (TG) concentration relative to the LF/LP diet (P<0.001; Fig. 1B). In mice on HP diets, hepatic fat amounted to only 35–40% of that in mice fed with LP diets, irrespective of the fat intake (see also Supplemental Fig. B.1). HP-containing diets had a comparable lowering effect on hepatic free fatty acid (FFA), total cholesterol (TC) and phospholipid (PL) concentrations (P<0.001; Fig. 1B), again irrespective of the fat intake. In fact, at the same dietary fat or carbohydrate intake, the hepatic lipid content was ~3-fold lower on diets with a HP than a LP content (Supplemental Fig. B.1), showing that the anti-steatotic effect of dietary protein was independent of dietary carbohydrate and lipid content. Concentrations of hepatic TG, FFA and TC were similar in mice in the HF/LPres and the HF/LP groups, and higher than in the HF/HP-fed mice (all P≤0.017; Fig. 1), despite the same energy intake.

The HP-containing diets decreased the hepatic content of all saturated C_{14}–C_{22} fatty acids to a similar degree (Supplemental Table A.6). The $[C_{16:1n7}]/[C_{16:0}]$, $[C_{18:1n9}]/[C_{18:0}]$, and $[C_{16:0}]/[C_{18:2n6}]$ ratios in mice on a HF diet were decreased to 35–45% of that in mice on a LF diet, indicating decreased stearoyl-CoA desaturase [10] and *de-novo* lipogenesis [11] activity (all P<0.001). The protein content of the diet had no effect. The $[C_{18:1}]/[C_{16:1}]$ ratio (a measure of elongase activity [10]) was not different between the diets. The hepatic content of the essential PUFAs linoleic ($C_{18:2n6}$) and α-linolenic acids ($C_{18:3n3}$) was, as expected, higher in the HF than the LF diets, but >2-fold reduced in mice fed the HF/HP compared to the HF/LP diet (P = 0.002 and P = 0.034, respectively). The concentration of the functionally important PUFAs arachidonic acid (AA; $C_{22:4n6}$), eicosapentaenoic acid (EPA; $C_{20:5n3}$), and docosahexaenoic acid (DHA; $C_{22:6n3}$) was not different between the diets, that is, preserved in mice fed the anti-steatotic HP diets (Supplemental Fig. B.2). The $[C_{22:4n6}]/[C_{18:2n6}]$ ratio, a parameter of ELOVL5 fatty-acid elongase activity [12], was 1.3–1.6-fold increased in mice fed a HP compared to a LP diet (P = 0.004), while the $[C_{20:4}]/[C_{20:3}]$ and $[C_{18:3n6}]/[C_{18:2n6}]$ ratios, reflecting Δ5 and Δ6 desaturase activities, respectively [10], were similar. Collectively, these data suggest an increase in long-chain PUFA synthesis.

3.3. The effects of a HP content in the diet on markers of hepatic lipid metabolism

3.3.1. Lipogenesis

Expression of the lipogenic transcription factors *Srebf1* and *Mlxipl* (a.k.a. *Chrebp*) was not affected by the diets, but that of *Pparγ* was decreased to ~50% in mice fed a HF/HP compared to a HF/LP diet (P = 0.048; Fig. 2A). The expression of fatty-acid synthase (*Fasn*), stearoyl-CoA desaturase 1 (*Scd1*) and acetyl-CoA carboxylase-α (*Acacα*) was lower in mice on a HF than a LF diet (P = 0.046), irrespective of protein intake (Fig. 2A). The HP-containing diets consistently resulted in a lower mRNA abundance of these markers than the LP-containing diets, but these differences did not reach significance. The hepatic concentration of ACACα and -β proteins was also lower in mice fed a HF than a LF diet (P = 0.011). ACACα increased with an increasing dietary carbohydrate content ($R^2 = 0.64$; P<0.001), whereas ACACβ decreased with increasing dietary fat content ($R^2 = 0.72$; P<0.001; Fig. 2B and Supplemental Fig. B.3). The activation status of both ACAC isoforms (P-ACAC/total ACAC; phosphorylation activates ACAC), and that of their kinase AMP-activated protein kinase (AMPK) (Supplemental Fig. B.4) was not affected by any of the diets. None of the measured genes involved in cholesterol metabolism (*Cyp7a1*, *Hmgcr*, *Srebf2*) was changed in expression (Supplemental Table A.7).

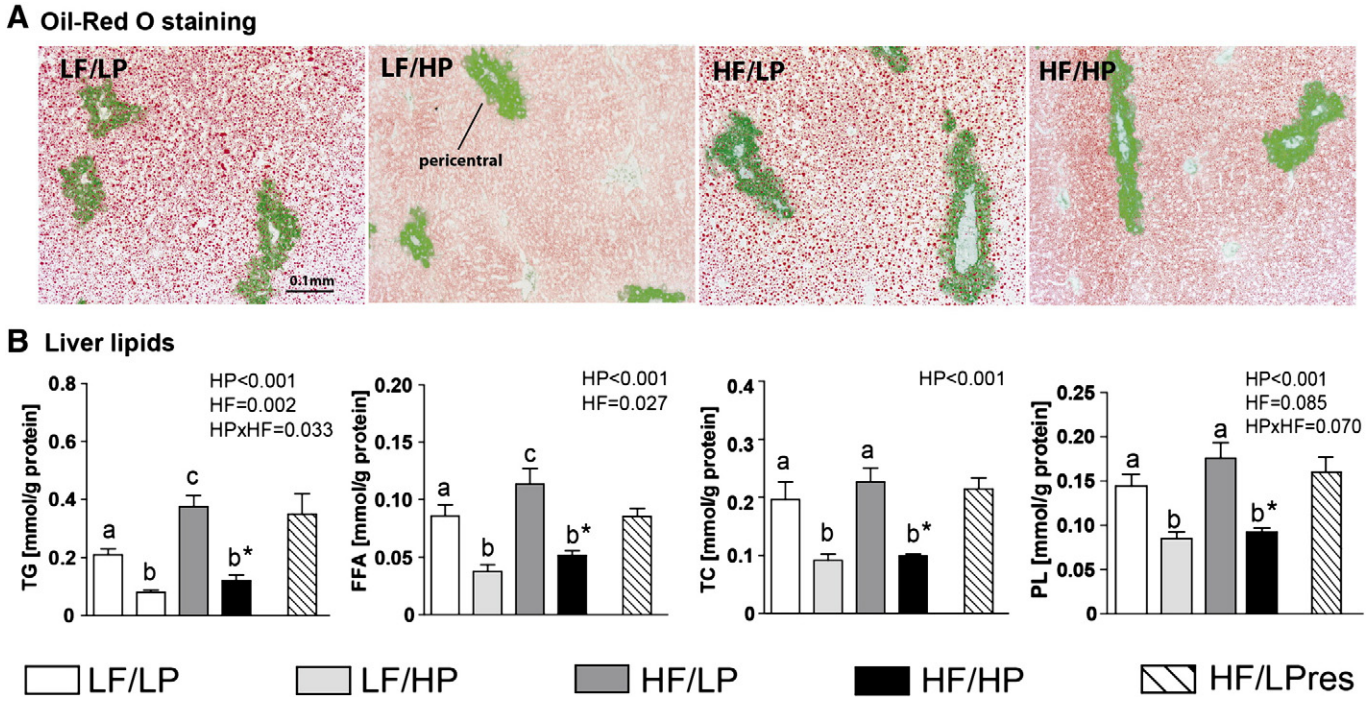

Fig. 1. Hepatic lipid distribution and concentration in mice fed diets differing in protein and fat content for 3 weeks. Panel A shows Oil-Red O (red) and glutamine synthetase (green; located pericentrally) double staining of livers from mice on the indicated diets; panel B, from left to right, liver triglyceride, free-fatty acid, cholesterol and phospholipid content. The coding of the bars is shown at the bottom of the Figure. The "HF/LPres" bars indicate mice pair-fed the HF/LP diet to match caloric intake of HF/HP mice. Bars depict mean ± SEM, with n = 8–10. Different letters (a, b, or c) above the bars indicate a significant difference between the diets fed ad libitum; * indicates a significant difference (P<0.05) between the isocaloric HF/LPres and HF/HP diets (the HF/LP and HF/LPres diets did not differ in their effect on liver lipids).

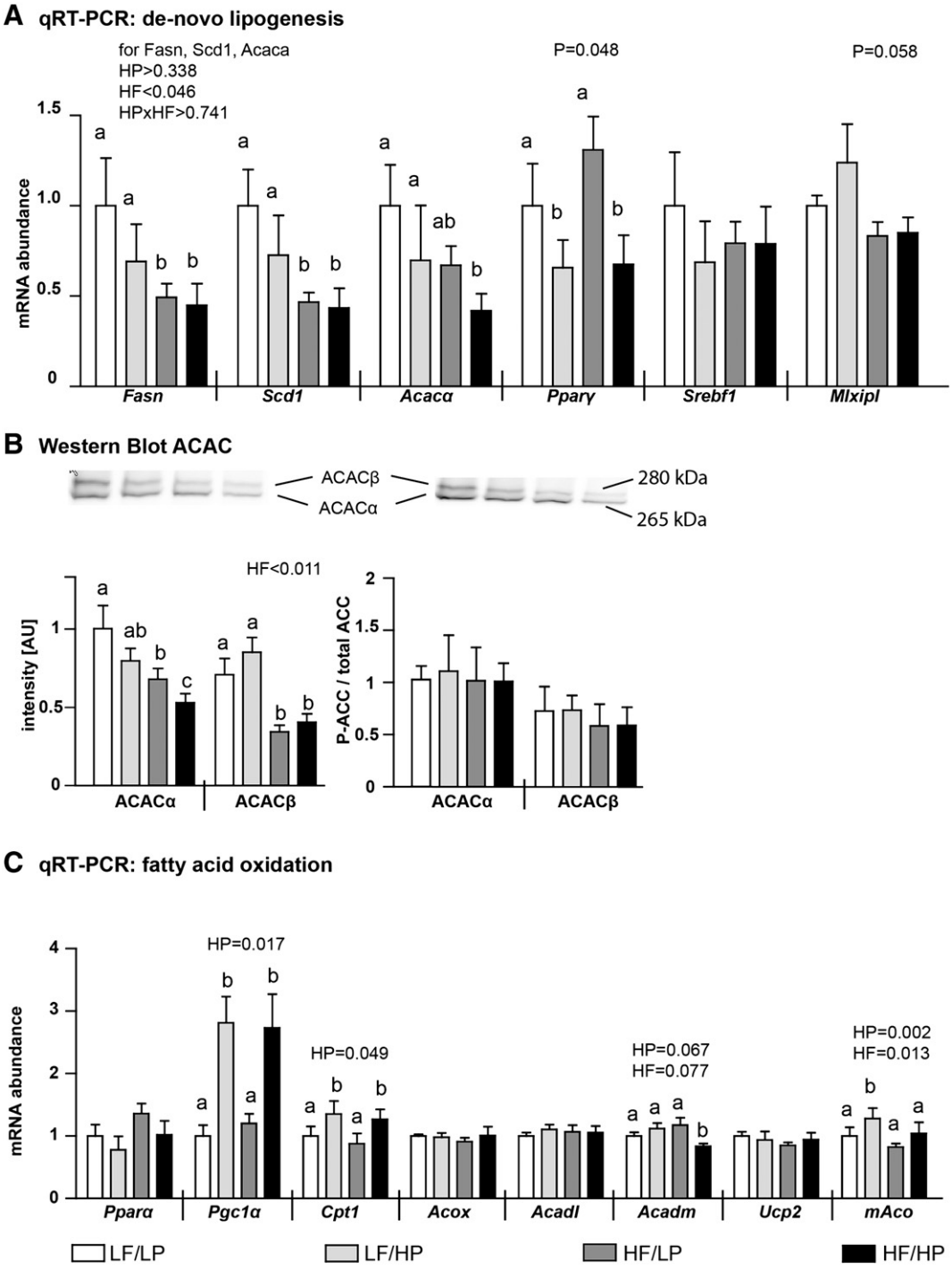

Fig. 2. Differences in gene expression and metabolite concentrations in livers of mice fed diets differing in protein and fat content for 3 weeks. Panel A shows hepatic mRNA abundance of lipogenic genes; panel B hepatic ACAC protein content (left subpanel) and phosphorylation status (right subpanel); and panel C mRNA abundance of genes involved in fatty-acid oxidation. For bar coding, sample size and symbols, see Fig. 1.

3.3.2. Fatty-acid oxidation

A HP-containing diet increased the hepatic mRNA content of the transcription factor *Pgc1α* ~2.5-fold (P = 0.017; Fig. 2C), and that of *Cpt1*, a marker for long-chain fatty-acid transport into the mitochondria, and the TCA-cycle enzyme aconitase2 (*mAco*) ~1.3-fold (P = 0.002). In contrast, a HF-containing diet decreased *mAco* mRNA levels to ~80% (P = 0.013). The expression of other genes involved in fatty-acid oxidation, such as *Acadl*, *Acadm*, and *Acox*, and *Ucp2*, a marker for thermogenesis, was not significantly affected by any of the diets (Fig. 2C). Since mitochondria play a key role in the oxidation of fatty acids, we also measured the expression complexes I–V of the respiratory chain, the concentration of mitochondrial DNA in hepatocytes, and the activity of citrate synthase. Apart from complex V protein, which was elevated by a HP diet, none of these parameters were affected by any of the diets (Supplemental Fig. B.5). Moreover, analysis of the ultrastructure of the hepatocyte mitochondria of mice fed either the HF/LP or HF/HP diet revealed a very similar hepatocyte make-up, except that there were fewer fat globules in the HP-treated livers and larger, more intensely staining mitochondria (Supplemental Fig. B.6).

3.4. A HP content of the diet increases plasma glucose and hepatic PCK1 levels

Plasma glucose concentrations (Fig. 3A) were ~20% higher in 4 h-fasted mice on a HF than on a LF diet (P = 0.010), but ~15% higher in mice fed on a HP- than a LP-containing diet (P = 0.025). The mRNA levels of the catalytic subunit of glucose-6-phosphatase (*G6pc*) and of phospho*enol*pyruvate carboxykinase (*Pck1*) were similar in mice with a LP and a HP content of the diet, but PCK1 protein content was increased ~3-fold on a HP-containing diet (P<0.001; Fig. 3B). Liver glycogen concentrations were unchanged (P = 0.93; Fig. 3C). As a surrogate test for insulin sensitivity, we measured AKT phosphorylation in liver and muscle ([13]; Supplemental Fig. B.4), but did not observe an effect of any of the diets. As expected, the HP diets increased the mRNA levels of the urea-cycle enzymes argininosuccinate synthetase and arginase-1 (Supplemental Table A.7).

3.5. A HP content of the diet reduces cell stress in liver

3.5.1. Endoplasmic-reticulum stress

Activation of the integrated stress response has been observed in fatty liver [14]. The phosphorylation of the translation–initiation factor eIF2α, a central component of this stress response, was lower in livers of mice fed a HP- than a LP-containing diet (P = 0.019; Fig. 4A), implying a reduction of cellular stress. This effect was most pronounced in combination with a LF diet. In line, *Clec2* mRNA, a target that is downregulated upon activation of the eIF2α kinases PERK and GCN2 [15], was increased in mice on a HP diet (P = 0.025), while the abundance of *Asns* mRNA, which is upregulated by GCN2 [15], was decreased on a HF diet (P<0.001). However, the protein and mRNA concentrations of the ER-stress markers ATF4, *Ddit3* (a.k.a. *Chop*), *Gadd34* and *sXbp1* were not different (Fig. 4B, C). The validity of our cell-stress assays was confirmed in livers of tunicamycin-treated mice (Supplemental Fig. B.7). The notion of a decrease in cell stress in livers of mice fed HP-containing diets was further supported by the reduced expression of the inflammatory markers *Mcp1* (P = 0.022) and *Itgam* (*Cd11b*; P = 0.067) (Supplemental Table A.7), and the decrease in NFκB phosphorylation (P = 0.068; Supplemental Fig. B.4, Supplemental Table A.7).

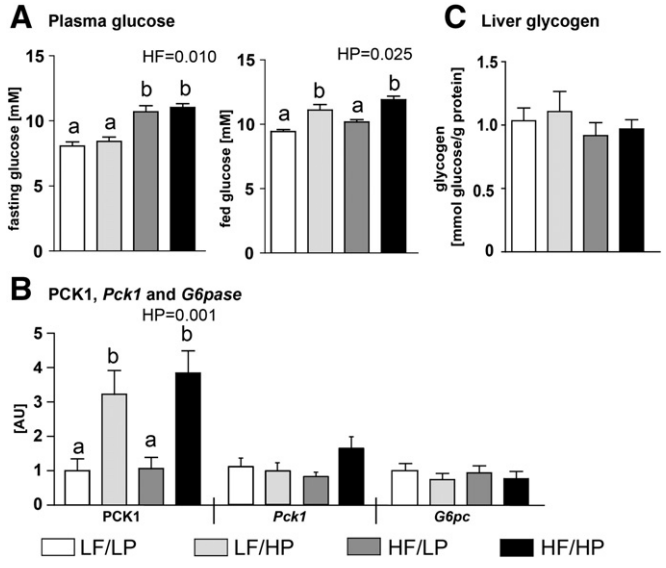

Fig. 3. Differences in glucose metabolism in mice fed diets differing in protein and fat content for 3 weeks. Panel A shows plasma glucose levels in 4h-fasted and fed mice; panel B depicts phosphoenolpyruvate carboxykinase (PCK1) protein and mRNA levels of *Pck1* and the catalytic subunit of glucose-6-phosphatase (*G6pc*). Panel C shows liver glycogen content. For bar coding, sample size and symbols, see Fig. 1.

3.5.2. Fibroblast growth factor 21

FGF21 regulates glucose, lipid, and energy homeostasis and appears to be a marker of cell stress [16], with diet-induced obesity in mice possibly representing a FGF21-resistant state [17]. Both hepatic *Fgf21* mRNA (P = 0.031) and plasma FGF21 concentration (P = 0.036) were reduced in mice on a HP-containing diet (Fig. 4D), without effect of the dietary fat content. Plasma FGF21 concentrations correlated tightly with hepatic TG concentrations ($R^2 = 0.94$; P<0.001; Fig. 4E). Since a HP diet induced *Pgc1α* and suppressed *Fgf21* expression and since PGC1α is a negative regulator of *Fgf21* expression [18], we also measured the expression of other modulators of *Fgf21* expression, namely *Alas1*, *Arnt1* (*Bmal1*), and *Nfil3* (*E4bp4*) [18,19]. However, their mRNA abundance was not changed by any of the diets (Supplemental Fig. B.5).

3.6. The effects of a HP content in the diet on plasma parameters of lipid metabolism

3.6.1. Hormones and lipids

Plasma insulin, glucagon and leptin concentrations in the fed state were not different between the groups (Table 2). A HP content of the diet tended to increase plasma β-hydroxybutyrate (BHB) concentrations (P = 0.096; Table 2). Plasma FFA and TG concentrations were not different between groups, while a HF diet increased TC concentration (P = 0.039). Mice on the HF/LPres diet had unchanged plasma TG and FFA concentrations, but a lower TC concentration compared to mice on an *ad-libitum* HF diet (P = 0.02).

3.6.2. Acyl-carnitines

Since changes in plasma acyl-carnitines reflect changes in the hepatic mitochondrial acyl-CoA pool [20], the concentration of plasma acyl-carnitines (Fig. 5A, Supplemental Table A.8) is a parameter to assess the balance between fatty-acid catabolism and acetyl-CoA metabolism in the TCA cycle. The ratio between total acyl-carnitines and free carnitine was similar for all diets. In mice fed a HP-containing diet, the plasma concentration of free carnitine and short-chain ($\leq C_4$) acyl-carnitines was reduced to ~55% (all P ≤ 0.013) and that of malonyl-carnitine (C_3-DC) to ~75% (P = 0.014) of that in mice on a LP diet (Fig. 5A), suggesting an enhanced fatty-acid oxidation. Plasma isovaleryl-carnitine, a product of leucine catabolism, was the only acyl-carnitine that was elevated in mice fed a HP diet (P = 0.001). Plasma long-chain acyl-carnitines were unaffected, except that palmitoyl-carnitine was 2-fold lower in mice fed a LF/HP than a HF/HP diet (P = 0.02; Supplemental Table A.8). In agreement with the increased *Cpt1* expression in mice fed the HP diets (Fig. 2C), the $[C_0]/[C_{16}+C_{18}]$ acyl–carnitine ratio, which correlates with carnitine palmitoyltransferase-1 activity [21], decreased 1.4–1.8-fold (P = 0.075).

3.6.3. Amino acids

Despite a >3-fold increased dietary supply, the HP diets did not change the plasma concentration of most amino acids (Supplemental Table A.9). However, plasma branched-chain amino acids (BCAA) were increased ~1.3-fold in mice fed a HP diet (P<0.001; Fig. 5B) and showed a strong inverse correlation with hepatic TG ($R^2 = 0.72$; P<0.001) and a positive correlation with *Pgc1α* mRNA levels ($R^2 = 0.55$, P<0.001; Fig. 5C, D). Because these correlations suggested a role for BCAAs in mediating the effect of a HP diet and because the liver is a major contributor in the irreversible step in BCAA catabolism [22], we investigated the concentrations of their monomethyl branched-chain fatty-acid (mmBCFA) products in liver (Supplemental Fig. B.8). Plasma leucine concentration correlated very well with hepatic 15-methylhexanoic acid (15-MHDA) concentration ($R^2 = 0.63$, P = 0.006), but the correlations of mmBCFAs with hepatic TG contents were weak (Supplemental Fig. B.8).

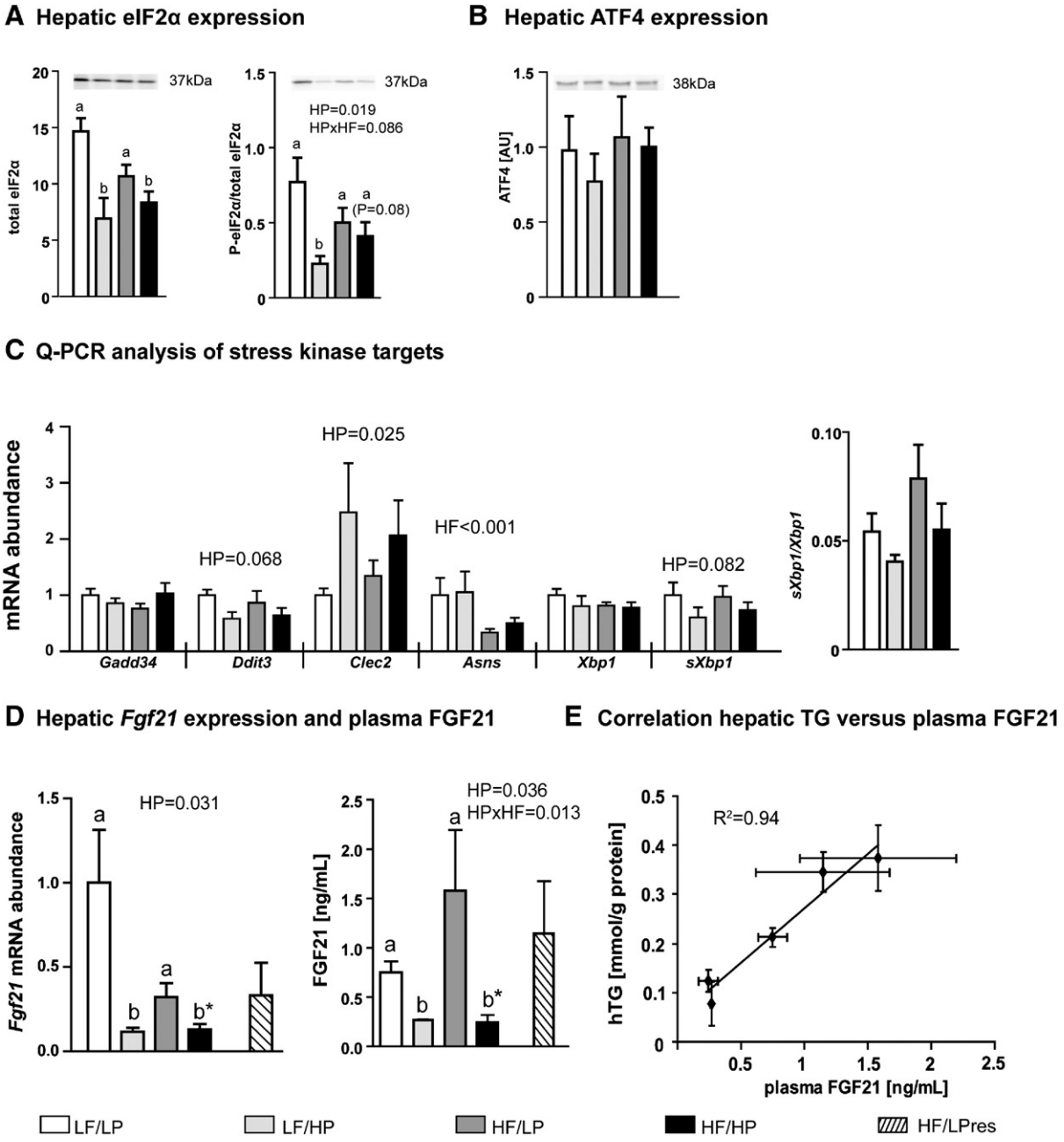

Fig. 4. Expression of ER-stress markers in mice fed diets differing in protein and fat composition for 3 weeks. Panel A shows total eIF2α protein concentration and phosphorylation (inactivation) status; panel B ATF4 protein concentration; panel C mRNA abundance of the indicated ER-stress genes; panel D hepatic *Fgf21* mRNA abundance and plasma FGF21 protein concentration; panel E correlation between hepatic triglyceride concentration and plasma FGF21 concentration. For bar coding, sample size and symbols, see Fig. 1.

3.7. Feeding a HP-containing diet for one week reverses pre-existing steatosis

To test if a HP diet could reverse pre-existing steatosis, mice were fed a HF/LP diet for three weeks to induce a fatty liver (Fig. 1) and then maintained on this diet for another week ("HF/LP4w") or switched to a HF/HP diet ("HF/LP→HP"). Mice on the HF/LP→HP regimen had lower body and epididymal fat-pad weights than mice on the HF/LP4w regimen ($P=0.024$ and 0.006, respectively, Table 1). Furthermore, this intervention reduced hepatic TG, FFA and TC concentrations to ~40% of that in the HF/LP4w group (Fig. 6A; all: $P<0.001$; Supplemental Fig. B.9), that is, to almost the same level as present in mice fed a HF/HP diet *ab initio*. No differences in plasma TG, FFA, or BHB concentrations were observed, but plasma TC was ~20% lower in the HF/LP→HP than the HF/LP4w group (Table 2; $P=0.001$). Markers of lipogenesis (total ACACα, *Acacα*, *Fasn*, *Scd1*) declined, with the reduction of *Acacα* mRNA and ACAC protein concentrations reaching statistical significance ($P=0.002$ and $P=0.006$, respectively; Fig. 6B, C). *Pgc1α* mRNA was increased in HF/LP→HP fed mice ($P=0.001$; Fig. 6D). Fasting and fed plasma glucose concentrations were similar in both groups (Fig. 6E). Although *Pck1* mRNA concentrations were unchanged, PCK1 protein concentration was >3-fold increased (Fig. 6E; $P<0.001$). In contrast, *G6pc* mRNA concentration was reduced ($P=0.019$, Fig. 6E). Furthermore, cell stress markers declined: eIF2α phosphorylation was decreased to ~50% ($P=0.014$; Fig. 6F) and *Fgf21* mRNA to ~15% ($P=0.031$) of the control, while plasma FGF21 level tended to be lower ($P=0.085$; Fig. 6F). These data show that the effects of increased protein content in the diet did not depend on the previously consumed diet and became effective within one week.

Table 2
Plasma hormones and lipids of mice fed a LF/LP, LF/HP, HF/LP, HF/HP, HF/LPres, HF/LP4w, or HF/LP→HP diet.

						ANOVA (P value)		
	LF/LP	LF/HP	HF/LP	HF/HP	HF/LPres	HP	HF	HP×HF
Hormones								
Insulin [pM]	175±23	189±58	197±48	115±19	307±48	n.s.	n.s.	n.s.
Leptin [pM]	239±40	200±40	316±54	235±26	345±54	n.s.	n.s.	n.s.
Glucagon [pM]	14±1	17±2	12±2	12±2	15±1	n.s.	n.s.	n.s.
Lipids								
Triglyceride [mM]	0.58±0.09	0.68±0.09	0.43±0.06	0.51±0.03	0.44±0.06	n.s.	n.s.	n.s.
Free fatty acid [mM]	0.50±0.14	0.47±0.09	0.40±0.04	0.44±0.14	0.34±0.11	n.s.	n.s.	n.s.
Cholesterol [mM]	2.36±0.06	2.54±0.22	2.90±0.26	2.81±0.10	2.39±0.20	n.s.	0.039	n.s.
α-Hydroxy butyrate [mM]	0.05±0.01	0.08±0.01	0.06±0.02	0.09±0.02	0.06±0.01	0.096	n.s.	n.s.
				HF/LP4w				HF/LP→HP
Reversion experiment								
Triglyceride [mM]				0.65±0.21				0.64±0.09
Free fatty acid [mM]				0.37±0.08				0.32±0.02
Cholesterol [mM]				3.23±0.11				2.57±0.08#
α-Hydroxy butyrate [mM]				0.03±0.01				0.03±0.01

For sample size and symbols, see Table 1.

4. Discussion

The present study shows that a HP-containing diet effectively reduces hepatic TG, FFA, TC, and PL concentrations and that this intervention reverses a pre-existing steatosis within just one week. Thirty-five en% protein in the diet, which is feasible and safe for humans [23], suffices to bring about this anti-steatotic effect. Of further clinical relevance, a HP diet also counteracts steatosis in humans [24]. The fact that a 3-fold increase in protein intake affected far more parameters than a 5-fold increase in fat intake (Supplemental Table A.7, HP versus HF column) further underscores the pronounced effect of dietary protein on hepatic metabolism.

4.1. The effect of HP content of the diet on hepatic lipid balance

The hepatic content of lipids depends on the balance between uptake plus synthesis and export plus oxidation. We observed quantitatively the same effects of a HP diet in *APOE2ki* mice that suffer from an impeded uptake of lipids into the liver (unpublished observations), indicating that the HP effect is not mediated by a reduced fat uptake into the liver. Very recent data indicate that inhibition of the lipogenic marker PNPLA3 prevents fatty liver [25], but markers of lipogenesis, such as *Fasn*, *Scd1*, ACACα, and metabolite-based lipogenic parameters, such as the $[C_{16:1n7}]/[C_{16:0}]$ and $[C_{16:0}]/[C_{18:2n6}]$ ratios [10], were not affected by the HP diets in our study. Instead, the lipogenic markers correlated, as expected [26], with dietary carbohydrate content. These data show that the inevitable decrease in dietary carbohydrate content due to the increase in protein and/or fat content did not materially contribute to the anti-steatotic effect of HP diets.

The capacity of the liver to oxidize fatty acids is another determinant of the hepatic lipid balance. After three weeks on the respective diets, none of the marker genes involved in fatty-acid oxidation (e.g. *Pparα*, *Acox*, *Acadl* or *Acadm*) was changed in expression, but plasma acyl-carnitine concentrations, which reflect changes in the hepatic mitochondrial acyl-CoA pool [20,21], were informative. The increase of *Cpt1* mRNA and the decrease of the plasma $[C_0]/[C_{16}+C_{18}]$ acyl–carnitine ratio, which correlates with CPT1 activity [21], and the reduction in plasma free carnitine and short-chain (≤4C) acyl-carnitine levels indicated that the import and oxidation of fatty acids into the mitochondria increased in mice fed a HP diet. The (small) increase in circulating ketone bodies also argued in favor of an increased hepatic fatty-acid oxidation.

Female *Pparα*-deficient mice show no reversion of their (pre-existing) steatosis after three weeks on a HF/HP diet, while male *Pparα*-deficient mice even die within 3 days on this diet [IC Gaemers and WH Lamers, unpublished observations]. This finding, which implies a role for PPARα in the anti-steatotic effect of dietary protein, virtually mimics the sex-dependent effects of pharmacologically blocking CPT1 activity in *Pparα*-deficient mice [27]. Since *Pparα*-deficient mice depend on amino-acid catabolism for energy supply [20], carnitine availability can apparently become a limiting factor in the oxidation of amino acids. The increased plasma concentration of isovaleryl-carnitine, a fingerprint intermediate of BCAA catabolism, in mice fed the HP-containing diets [28] confirms that hepatic amino-acid catabolism was increased. The hypothesis that CPT1 function is an important point of control in the adaptation to a HP-containing diet is also supported by the decrease in plasma malonyl-carnitine concentration of mice fed a HP diet, because malonyl-CoA acts as an inhibitor of CPT1 if synthesized via ACACβ [29]. The strongly increased hepatic PCK1-protein content, the unchanged *G6pc* mRNA and hepatic glycogen levels, the higher plasma glucose (in fed, but not 4h-fasted mice), and the decreased plasma alanine concentration in mice on a HP-containing diet support an enhanced diversion of TCA-cycle intermediates to pyruvate cycling and gluconeogenesis. The unchanged degree of AKT phosphorylation in liver and muscle suggests that the HP diets did not induce insulin resistance, as was observed earlier in rats [30,31].

Mitochondria play a key role in fatty-acid oxidation. The increased expression of *Pgc1α*, a co-factor of PPARα and a key regulator of mitochondrial biogenesis and the coordinated functions of the TCA cycle and gluconeogenesis [32], may, therefore, be crucial in mediating the anti-steatotic effect of a HP diet. A HP content of the diet also increased *Pgc1α* mRNA abundance in epididymal fat tissue, but not in muscle, in agreement with the reportedly opposite effect of PGC1α on insulin sensitivity in liver and muscle [33]. In addition to the increase in *Pgc1α* mRNA, we observed an increase in the diameter and electron density of the mitochondria (cf. [34]) and an increased mRNA expression of aconitase, but not of other markers of mitochondrial biogenesis (citrate synthase, OXPHOS proteins, and mtDNA-copy number (Supplemental Fig. B.5)). In aggregate, the data, therefore, suggest that increased substrate cycling in the TCA cycle arises from a substrate supply-driven increase in enzyme activities.

The strong mutual correlations between hepatic *Pgc1α* mRNA abundance, plasma BCAA, and hepatic triglyceride content suggest a specific role for BCAA metabolism in the anti-steatotic effect of a HP-containing diet. In support of this hypothesis, supplementation of a diet with BCAAs, lysine, and threonine was also shown to reduce

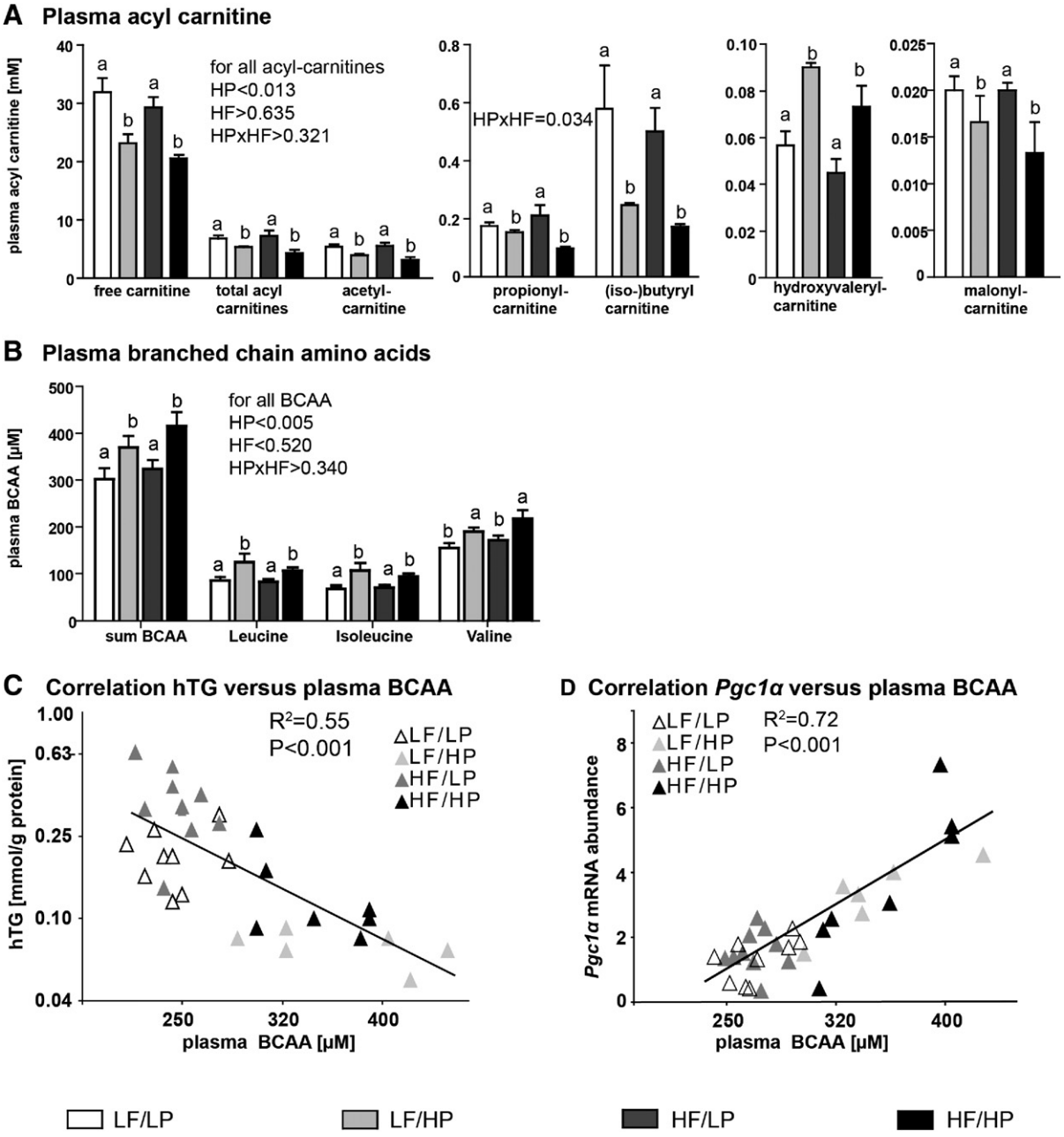

Fig. 5. Differences in plasma metabolites in mice fed diets differing in protein and fat content for 3 weeks. Panel A shows plasma concentration of free carnitine and the indicated acyl-carnitines; panel B plasma branched-chain amino acid (BCAA) concentration; panels C and D the correlation between plasma BCAA level and hepatic triglyceride content (TG) and *Pgc1α* mRNA levels, respectively. For bar coding, sample size and symbols, see Fig. 1. Panels A-C: n = 8–10 mice/group; panel D: n = 6/group; panels E and F: n = 6–8/group.

liver TG content [35]. Since our mice were not hyperinsulinemic and had an increased plasma hydroxyvaleryl-carnitine concentration, the high plasma BCAA levels in HP-fed mice arose from an excess supply rather than a limited catabolic capacity [36–38]. The weak correlation of the lipid content of the liver with hepatic monomethyl branched-chain fatty acids (mmBCFAs), despite the strong correlation with plasma BCAAs, probably arises from the fact that the activity state of branched-chain keto-acid dehydrogenase complex is much higher in males than in females [39], so that monomethyl branched-chain acyl-CoAs do not accumulate sufficiently to stimulate the synthesis of mmBCFAs. In agreement, we found a strong correlation between hepatic lipid content and mmBCFAs in female mice (Garcia-Caraballo, unpublished results).

4.2. Reduced cell stress in mice fed a HP diet

Cell stress leads to activation of stress kinases like PERK and GCN2, which phosphorylate the translation–initiation factor eIF2α. Mice that are deficient in one of the ER stress-sensing pathways are prone to develop steatosis in response to cell stress [40,41], which implies that the capacity to cope with cell stress is necessary to avoid or reverse steatosis. Maintaining the translation–initiation factor eIF2α in the non-phosphorylated state is probably crucial for this effect [40]. The decreased phosphorylation status of eIF2α in mice fed a HP-containing diet, therefore, indicates that a HP content in the diet resolves cell stress. The absence of a transcriptional response in the downstream recovery pathway for cell stress (ATF4-*Ddit3*-*Gadd34*)

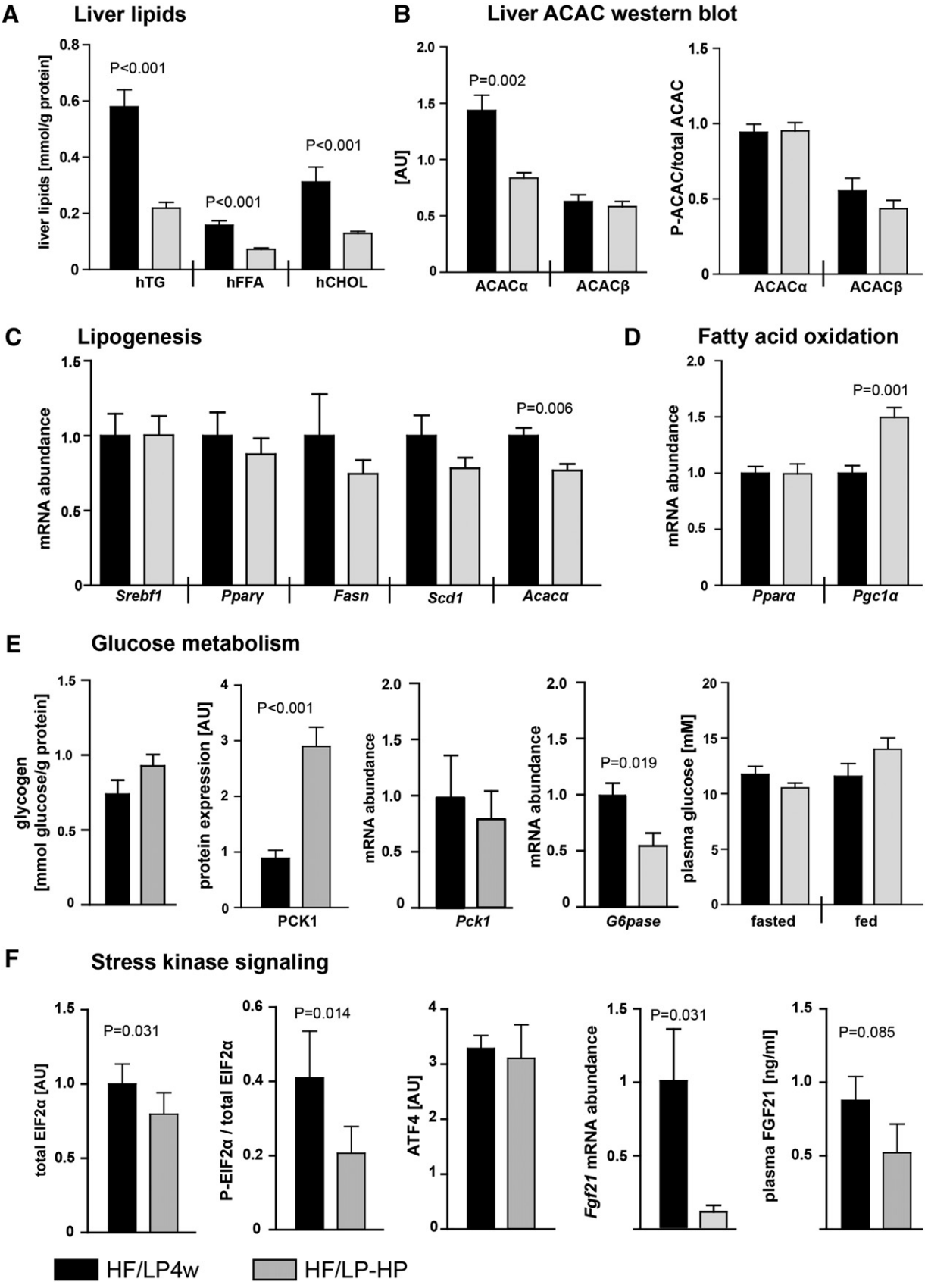

Fig. 6. Response to a HF/HP diet in mice with pre-existing steatosis. Panel A shows hepatic triglyceride, free fatty acid and cholesterol content; panel B hepatic ACAC protein levels and their activation status (phosphorylation = inactivation); panel C markers of lipogenic genes; panel D *Pparα* and *Pgc1α* mRNA levels; panel E changes in glucose metabolism; and panel F the changes in ER-stress markers. For bar coding, sample size and symbols, see Fig. 1. Panels A–C: n = 8–10 mice/group; panel D: n = 6/group; panels E and F: n = 6–8/group.

that we observed was also reported for human fatty liver [42] and concurs with earlier findings that a weak activation of PERK need not fully activate the unfolded-protein response [40,41]. The abundance of *Clec2* mRNA, a negatively regulated marker of PERK activity [15], increased on a HP-containing diet, also indicating that a HP content in the diet suppressed PERK activity even in the context of a HF diet.

One of the more striking findings was the strong positive correlation between hepatic fat content and hepatic *Fgf21* expression or plasma FGF21 levels. *FGF21* is also highly expressed in the liver of obese patients [43] and rodents [17,35]. FGF21 reduces fasting blood glucose and insulin levels, and increases, like PPARα, TCA-cycle flux, gluconeogenesis, ketogenesis and β-oxidation, and O_2 consumption [44,45] and is, in view of the increased hepatic *Fgf21* mRNA abundance in tunicamycin-treated mice (Supplemental Fig. B.7), probably a marker for cell stress [16]. A strong suppression of plasma FGF21 levels was also seen upon increasing the dietary protein content from 5.5 via 12 to 19 en% in carbohydrate-free diets [46], supporting earlier findings that *Fgf21* is also a sensitive target of GCN2 stress-kinase activity [15,47].

4.3. Conclusion

A HP-containing diet prevented and reversed hepatic steatosis independently of dietary fat and carbohydrate intake, and was more efficient than reducing the daily energy intake to 80%. Remarkably, this clear-cut effect could not be attributed to activation of one or a few metabolic or signaling pathways (see Supplemental Table A.7). The apparently highly distributed regulation of small, synergistic increases in lipid and BCAA catabolism, TCA cycling and decrease in cell stress that are induced by a HP-containing diet, together reduce the fat content of the liver in a very effective manner. Since recent studies have taken away concerns about the most feared side effects of HP diets, that is, adverse effects on bone health [48,49], renal function [50], and insulin sensitivity [51], a moderately HP-containing diet (30–40 en%) appears to be an attractive treatment option for both short-time (e.g. to reverse steatosis prior to liver surgery [5]) and longer-time (weight reduction [52]) dietary interventions.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2013.02.003.

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