Nutritional stress exacerbates hepatic steatosis induced by deletion of the histidine nucleotide-binding (Hint2) mitochondrial protein

Juliette Martin,1 Maria L. Balmer,1 Saranya Rajendran,1 Olivier Maurhofer,1 Jean-François Dufour,1,2 and Marie V. St-Pierre1

1Department of Clinical Research, University of Bern, Bern, Switzerland; and Department of Hepatology, Gastroenterology; and 2Visceral Surgery, University Hospital, Inselspital, Bern, Switzerland

Submitted 4 June 2015; accepted in final form 7 January 2016

Martin J, Balmer ML, Rajendran S, Maurhofer O, Dufour JF, St-Pierre MV. Nutritional stress exacerbates hepatic steatosis induced by deletion of the histidine nucleotide-binding (Hint2) mitochondrial protein. Am J Physiol Gastrointest Liver Physiol 310: G497–G509, 2016. Published 1 January 2016; doi:10.1152/ajpgi.00178.2015.—The histidine nucleotide-binding protein, Hint2, is a mitochondrial phosphoromidase expressed in liver, brown fat, pancreas, and muscle. The livers of Hint2 knockout (Hint2−/−) mice accumulate triglycerides and show a pattern of mitochondrial protein lysine hyperacetylation. The extent and nature of the lysine acetylation changes and the response of Hint2−/− mice to nutritional challenges that elicit a modification of protein acetylation have not been investigated. To compare the adaptation of Hint2−/− and control (Hint2+/+) mice with episodes of fasting and high-fat diet (HFD), we subjected animals to either feeding ad libitum or fasting for 24 h, and to either a HFD or control diet for 8 wk. Triglyceride content was higher in Hint2−/− than in Hint2+/+ livers, whereas plasma triglycerides were fourfold lower. Malonyl-CoA levels were increased twofold in Hint2−/− livers. After 24 h fasting, Hint2−/− displayed a decrease in body temperature, commensurate with a decrease in mass of brown fat and downregulation of uncoupling protein 1. HFD-treated Hint2−/− livers showed more steatosis, and plasma insulin and cholesterol were higher than in Hint2+/+ mice. Several proteins identified as substrates of sirtuin 3 and 5 and active in intermediary and ketone metabolism were hyperacetylated in liver and brown fat mitochondria after both HFD and fasting regimes. Glutamate dehydrogenase activity was downregulated in fed and fasted livers, and this was attributed to an increase in acetylation and ADP-ribosylation. The absence of Hint2 deregulates the posttranslational modification of several mitochondrial proteins, which impedes the adaptation to episodes of nutritional stress.

histidine nucleotide-binding protein; lysine acetylation; hepatic steatosis; fasting; high-fat diet

THE HISTIDINE TRIP nucleotide-binding protein, Hint2, belongs to the histidine triad family of enzymes that share the sequence motif, H-Φ-H-Φ-H-Φ, where Φ is a hydrophobic amino acid. Hint2 catalyzes the hydrolysis of phosphoromidase bonds, which yield a nucleotide product (19, 25). Hint2 has been crystallized in its homodimeric form bound to AMP (25). Hint2 is a mitochondrial protein expressed in the liver, pancreas, brown fat (27), skeletal muscle, and heart (26). We have shown previously that the genetic deletion of Hint2 in mice (Hint2−/−) is associated with an age-dependent accumulation of hepatic triglycerides and structural anomalies in liver mitochondria, as well as a decrease in activity of the mitochondrial matrix enzyme glutamate dehydrogenase (GDH). Moreover, Hint2−/− mice display an increase in lysine acetylation of several mitochondrial proteins, including GDH (27).

Lysine acetylation is a reversible posttranslational modification that regulates the activity of selected enzymes mediating the intermediary metabolism of carbohydrates, fat, and proteins. Hence, lysine acetylation permits a targeted tissue-specific response to changes in energy supply. A low energy supply resulting from acute fasting or an extended caloric restriction regulates acetylation of certain mitochondrial enzymes that govern fatty acid metabolism and electron transport in the liver, but not in brown fat or heart (21, 33). An oversupply of energy resulting from a chronic high-fat diet (HFD) also promotes global changes in hepatic lysine acetylation (18). One basis for a reversible change in acetylation patterns relates to the upregulation or downregulation of sirtuin 3, a mitochondrial deacetylase (16, 18, 34). However, in some instances, such as after long-standing caloric restriction (33), sirtuin 3 upregulation occurs concomitantly with an increase in acetylated proteins. A second physiological basis for acetylation changes, independent of sirtuin 3 expression, relates to the availability of acetyl-coenzyme A (acCoA), the cosubstrate required by acetyltransferases, and NAD+, the cosubstrate used by sirtuin deacetylases. Intracellular concentrations of acCoA increase under conditions of fasting or caloric restriction (39). A third basis for changes in global protein acetylation could be linked to the actions of acetyltransferases. The GCSN5L1 protein, whose genetic knockdown reduces protein acetylation, was identified as a mitochondrial acetyltransferase (35), although a regulatory role has yet to be confirmed. It is likely that several factors interact to reversibly regulate protein acetylation and thereby influence the physiological response to changes in nutritional status. On the basis of the changes in mitochondrial lysine acetylation observed so far in Hint2−/− mice, the Hint2 protein should join the list of agents capable of influencing protein acetylation.

The extent and nature of the acetylation changes in mitochondrial proteins in the absence of Hint2 have not been studied. Moreover, the response of Hint2−/− mice to nutritional changes that elicit a modification of protein acetylation has not been investigated. We postulated that the absence of Hint2 protein perturbs the normal metabolic response to changes in the nutritional status and renders the Hint2 knockout mice more vulnerable than control mice to episodes of fasting and HFD. Therefore, we subjected age-matched groups of Hint2+/+ and Hint2−/− mice to dietary changes. To generate nutrient deficiency, we either fed mice ad libitum or imposed a 24-h fasting period. To generate nutrient excess, we fed mice either a standard control diet or a HFD for 8 wk. The metabolic
parameters and acetylation profiles were compared. Our findings show that the Hint2 protein wields influence in the overall adaption to nutritional stress. Moreover, our findings support the notion that Hint2 modulates the posttranslational modification of several proteins by an indirect mechanism.

METHODS

Animals. As described previously, the genetic deletion of Hint2 by homologous recombination in embryonic stem cells generated Hint2−/− mice in a mixed C57BI6/J129Sv background (27). Mice were housed and cared for in accordance with protocols approved by the Institutional Animal Care and Use Committee, University of Bern. For the fed/fasting experiments, adult male (20–25 wk) Hint2+/+ and Hint2−/− mice were housed individually and either fed a Teklad Global Rodent 2918 diet (Harlan, Madison, WI) ad libitum or fasted for 24 h. The 20- to 25-wk age group was chosen because hepatic steatotic changes had previously been detected in Hint2−/− mice of this age (27). Mice were killed between 0900 and 1200. Brown adipose tissue (BAT) and plasma were frozen immediately at −80°C. The antibody complexes were captured with Dynabeads M-280 sheep anti-rabbit IgG (Dynal) for 2 h at 4°C and then washed four times in 0.1% detergent. The bound proteins were diluted to a final detergent concentration of 0.5% and incubated with 1% -D-maltoside (Sigma, St. Louis, MO), 0.5 mM EDTA, 4°C and then washed four times in 0.1% detergent. The bound proteins were diluted to a final detergent concentration of 0.5% and incubated with 1% -D-maltoside (Sigma, St. Louis, MO), 0.5 mM EDTA, 0.5 mM nicotinamide, 50 mM Tris-HCl, pH 7.4, 10 mM nicotinamide, 50 mM Tris-HCl, pH 7.4, 10 mM nicotinamide, 50 mM trichostatin A, and a protease inhibitor cocktail (Roche). The lysate was diluted to a final detergent concentration of 0.5% and incubated with an anti-acetylated-lysine rabbit antibody (10 µl) (Cell Signaling Technology, Beverly, MA) overnight at 4°C. The antibody complexes were captured with Dynabeads M-280 sheep anti-rabbit IgG (Dynal) for 2 h at 4°C and then washed four times in 0.1% detergent. The bound proteins were extracted with SDS-PAGE loading buffer (Thermo Fisher Scientific, Rockford, IL).

Immunoprecipitation. Isolated mitochondria (650 µg) were lysed in 1% n-dodecyl-β-D-maltoside (Sigma, St. Louis, MO), 0.5 mM EDTA, 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 10 mM nicotinamide, 50 mM trichostatin A, and a protease inhibitor cocktail (Roche). The lysate was diluted to a final detergent concentration of 0.5% and incubated with an anti-acetylated-lysine rabbit antibody (10 µl) (Cell Signaling Technology, Beverly, MA) overnight at 4°C. The antibody complexes were captured with Dynabeads M-280 sheep anti-rabbit IgG (Dynal) for 2 h at 4°C and then washed four times in 0.1% detergent. The bound proteins were extracted with SDS-PAGE loading buffer (Thermo Fisher Scientific) and subjected to immunoblotting.

Quantification of triglycerides, free fatty acids, ammonia, glycogen. For triglyceride measurements, frozen liver and BAT tissues were minced, lysed in buffer containing 5% Triton X-100, and homogenized. For free fatty acid (FFA) quantification, both liver and BAT tissues were homogenized in chloroform-Triton X-100 and then centrifuged. The organic phase was dried under N2 and suspended in assay buffer. Triglycerides and FFA in liver, BAT, and plasma were measured colorimetrically by means of a quantification kit (Biovison, Mountainview, CA). Ammonia was measured in 20 µl plasma by means of a colorimetric assay (Abcam, Cambridge, UK). Glycogen in liver homogenate was quantified by means of a colorimetric assay (Glycogen Assay Kit II; Biovison). Protein quantities of 50–60 µg from fasted livers and 1–2 µg from fed livers were used to fall within the linear range of the glycogen assay.

Isolation of mitochondria from BAT. Interscapular BAT from five mice were pooled and homogenized in 250 mM sucrose. A mitochondrial-enriched fraction was isolated, as described (4).

Sirtuin 3 activity. Sirtuin 3 activity was measured fluorometrically in Hint2−/− and Hint2+/+ mitochondrial fractions. The assay buffer contained 25 mM Tris, 150 mM NaCl, 1 mM DTT, 2 mM NAD+, and the fluorogenic peptide substrate Ac-Arg-Gly-Lys(Ac)-7amino-4-methylcoumarin (R&D Systems, Minneapolis, MN). The reactions were terminated in a stop solution (50 mM Tris, 100 mM NaCl, 30% isopropanol, and 4 mM nicotinamide) containing 0.2 ng/µl recombinant mouse Sirtuin 3. To test whether sirtuin 3 activity was affected by the presence of Hint2 protein, we resuspended recombinant human His-sirtuin 3 (1 µg) (R&D Systems) in assay buffer containing equimolar recombinant glutathione-S-transferase (GST)-Hint2 or GST, the fluorogenic peptide substrate, and varying concentrations of NAD+/NADH (0.1–2 mM). The reaction was terminated after 30 min.

Quantification of CoA esters. CoA and its short-chain esters were quantified by HPLC-MS. The method described by Demoz et al. was adapted (8). Liver pieces (100 mg), previously frozen in liquid N2 and stored at −150°C, were pulverized in liquid N2 and then homogenized at 4°C in 5% sulfosalicylic acid containing 50 µM diethyrythrol. After centrifugation (16,000 g), the protein-free supernatant was passed through a 0.45-µm PTFE filter and then injected onto an Atlantis T3 3-µm column (Waters, Milford, MA). The protein pellet was dissolved in 40 mM Tris-10% SDS for estimation by means of the Lowry method. The separation of acetyl-CoA, malonyl-CoA, methylmalonyl-CoA, succinyl-CoA, propionyl-CoA, and free CoA was achieved by means of a gradient elution with 100 mM ammonium formate, pH 6.38, and methanol (95:5 to 45:55).

Liver histology. Liver specimens from 10 Hint2+/+ and 10 Hint2−/− from each diet group were cryosectioned, fixed in 4% buffered formaldehyde solution, and stained with hematoxylin and eosin. The pathological features of the liver were assessed microscopically and coded (0–2, 3–4, >4) according to the nonalcoholic fatty liver disease (NAFLD) Activity Score (NAS) criteria described by Kleiner et al. (22). NAS values for the HFD groups were compared by means of the 2 × 3 Fisher’s exact probability test with the Freeman–Halton extension and two-tailed P values (13).

Additional sections were incubated in 100% propylene glycol and then in 0.5% Oil Red O, which stains the neutral lipids. Images were captured at ×100 magnification by means of a Nikon DN100 camera mounted on an Olympus BHS-PC microscope. The areas positive for Oil Red O were quantified with the imaging software MetaMorph (Molecular Devices, Sunnyvale, CA). Data from individual sections (three fields on each section) were pooled and are presented as means ± SD expressed as pixels per square millimeter. For statistical comparison, a one-way ANOVA and Bonferroni’s multiple-comparison tests were applied.

RESULTS

Response of Hint2+/+ and Hint2−/− mice to 24 h fasting. In 25-wk-old mice, the mean body weight of Hint2−/− was 23% higher than that of Hint2+/+ (P < 0.05) (Table 1). Likewise, the ratio of liver weight to body weight after fasting was higher in Hint2−/− than in Hint2+/+ mice (P < 0.05). These differences were not evident in younger mice. The loss of body weight after 24 h of fasting was not different between the

| Table 1. Comparison of body and liver weights, blood glucose, and ketones in Hint2+/+ and Hint2−/− mice |
|---|---|---|---|---|
|   | 20 wk | 25 wk |   |   |
| +/+ | −/− | +/+ | −/− |   |
| Body wt, g | 34.2 ± 1.9 | 38.9 ± 5.3 | 32.4 ± 3.5 | 40.0 ± 6.3* |
| Loss body wt after 24-h fast, % | 10.3 ± 1.5 | 10.5 ± 2.1 | 11.6 ± 3.6 | 10.6 ± 1.8 |
| Fasting liver wt, %body wt | 3.7 ± 0.5 | 3.6 ± 0.2 | 3.2 ± 0.5 | 4.0 ± 0.4* |
| Interlobar blood glucose, mM | 8.5 ± 1.6 | 10.2 ± 2.6 | 7.7 ± 1.4 | 8.7 ± 2.1 |
| Fasting blood glucose, mM | 3.5 ± 0.6 | 3.7 ± 0.8 | 3.6 ± 1.7 | 3.8 ± 0.9 |
| Fasting plasma β-hydroxybutyrate, mM | 1.0 ± 0.3 | 0.9 ± 0.3 | 0.7 ± 0.1 | 0.9 ± 0.2 |

Values are means ± SD. Groups (n = 10; age 20 wk) (n = 5; age 25 wk) of histidine nucleotide-binding protein (Hint2) wild-type (Hint2+/+) and knockout (Hint2−/−) mice were fed ad libitum or fasted for 24 h. *P < 0.05, unpaired t-test, Hint2+/+ vs. Hint2−/−.
groups. No significant differences in blood glucose or plasma ketone levels were detected between Hint2+/+ and Hint2−/− mice (Table 1).

We have shown previously that, as Hint2−/− mice age, their livers exhibit steatotic changes (27). To determine whether steatosis persisted after fasting, we measured triglyceride levels in the liver and plasma of 25-wk-old Hint2+/+ and Hint2−/− mice. Triglyceride content was 2.9-fold higher in Hint2−/− than in Hint2+/+ livers (Fig. 1A). The 24-h fast induced triglyceride accumulation in both groups. Commensurate with the increase in liver triglycerides, plasma triglycerides were decreased in Hint2−/− under fed conditions (Fig. 1B). FFA levels in fasting livers were not different between Hint2−/− and Hint2+/+ (Fig. 1C). Liver glycogen content was depleted by the 24-h food deprivation in both groups (Fig. 1D).

In addition to the liver, Hint2 is also expressed in BAT, but not white fat (27). BAT is a thermogenic tissue, by virtue of the actions of the highly expressed uncoupling protein (UCP1). The body temperatures of 20-wk-old mice were compared under fed and fasting conditions. After fasting, body temperature was significantly lower in Hint2−/− than in Hint2+/+ mice (Fig. 2A). The weight of interscapular BAT was equal between the fed groups but tended to decrease after fasting in Hint2−/− mice (Fig. 2B). The UCP1 protein and mRNA levels in mitochondria isolated from pooled BAT were compared. The UCP1 protein was downregulated in fasting Hint2−/− BAT (Fig. 2C), without a corresponding decrease in mRNA (Fig. 2D). Levels of FFA decreased in BAT of fasted Hint2−/− mice (Fig. 2E), which implies an acceleration of fatty acid mobilization. This was supported by the increase in phosphorylated perilipin in the fasted BAT (Fig. 2F).

Response of Hint2+/+ and Hint2−/− mice to HFD. Excess dietary fat constitutes a nutritional stress that can promote hepatic steatosis, a manifestation of the metabolic syndrome. To gauge the response of Hint2−/− mice to this stress, we imposed an 8-wk course of either a HFD or a control diet. Regardless of regimen, Hint2−/− mice gained more body weight than did Hint2+/+ mice, without a commensurate increase in food consumption (Table 2). However, the HFD provoked a higher weight gain in Hint2+/+ (210% increase over control diet) than in Hint2−/− (130% increase). Fasting insulin concentrations were higher in Hint2−/− mice than in Hint2+/+ mice under both feeding regimens, although fasting blood glucose concentrations were not different. Plasma cholesterol tended to be higher in Hint2−/− than in Hint2+/+. The HFD provoked an increase in alanine transaminase in both groups (Table 2).

Fig. 1. Metabolic effects of fasting in histidine nucleotide-binding protein (Hint2) knockout (Hint2−−) and wild-type (Hint2+/+) mice. A: hepatic triglycerides were measured in fed and 24-h-fasted (n = 5) mice (25 wk of age). The 24-h fast induced triglyceride accumulation in both groups. Triglyceride levels were significantly higher in Hint2−/− than in Hint2+/+ livers. B: plasma triglyceride concentrations were significantly lower in Hint2−/− than in Hint2+/+ fed mice. The 24-h fast induced a significant decrease only in Hint2+/+ mice. C: free fatty acid levels in fasting livers were not significantly different. D: glycogen content was not different between Hint2−/− and Hint2+/+ livers under fed conditions. Glycogen was depleted by the 24-h food deprivation. Values are means ± SD. A, B, and D display significant changes tested by one-way ANOVA and Bonferroni’s multiple comparisons, P < 0.05. *Hint2+/+ vs. Hint2−/− fed; **Hint2+/+ vs. Hint2−/− fasting; #Hint2+/+ fed vs. fasted; and ##Hint2−/− fed vs. fasted.
Hint2 DELETION AND NUTRITIONAL STRESS

Fig. 2. Comparison of temperature and brown fat (BAT) in Hint2+/- and Hint2++/+ mice. A: body temperature of Hint2+/- and Hint2++/+ mice after fasting. Body temperature decreased after 24 h fasting. Fasted Hint2+/- mice (filled bars) maintained a lower body temperature than did Hint2++/+ mice (open bars). Mice (n = 16 for the fed group, n = 19 for the fasted group) were aged 20 wk. Kruskal-Wallis test with Dunn’s multiple comparisons, P < 0.05. *Hint2++/+ vs. Hint2+/- fasted; #Hint2++/+ fed vs. fasted; ##Hint2+/- fed vs. fasted. Means ± SD are shown. B: ratio of interscapular BAT to body weight (BATW/BW). The ratio was equal in the two groups under fed conditions. The decrease in BAT weight in fasting Hint2+/- mice was not statistically significant (Kruskal-Wallis test). Mice (n = 6–8/group) were aged 20 wk. C: immunoblotting of uncoupling protein (UCP1) in BAT mitochondria. BAT mitochondria isolated from 3 mice were pooled and tested by SDS-PAGE. Cytochrome c oxidase IV (COXIV) was used as a loading control. After fasting, the expression of COXIV relative to COXIV increased in Hint2++/+ (open bars) but decreased in Hint2+/- (filled bars) BAT. D: quantitative PCR of UCP1 mRNA in BAT. RNA was extracted from 3 pools of BAT tissue harvested from fed and fasted Hint2+/- and Hint2++/+ mice and analyzed in triplicate. UCP1 mRNA was 2.6-fold higher in fed Hint2+/- BAT (filled bars) than in Hint2++/+ BAT (open bars). *P < 0.05, unpaired t-test. E: free fatty acids (FFA) in BAT lysate. FFA was lower in fasted Hint2+/- than in Hint2++/+ BAT (n = 4). *P < 0.05, unpaired t-test. F: immunoblotting of phosphorylated perilipin in Hint2+/- and Hint2++/+ BAT. Phosphorylation of perilipin was higher in fasted Hint2+/- mice. Actin was used as a loading control.

After HFD, steatosis was more severe and more frequent in Hint2+/- livers (Fig. 3A). Hint2+/- livers presented steatotic changes in 70% of cases, whereas fewer Hint2++/+ livers were affected. Although plasma triglycerides were not different between the groups (data not shown), the Oil Red O staining of liver specimens confirmed that excess lipid accumulated in Hint2+/- livers (Fig. 3, B and C). When HFD-treated livers were evaluated according to the histological scoring system for NAFLD, the features of hepatocyte ballooning, lobular inflammation, microgranulomas, and megamitochondria were occasionally detected in both Hint2+/- and Hint2++/+ groups. However, the NAS values were not significantly different between the groups (Fig. 3D).

Changes in protein acetylation after fasting and HFD. Because mitochondrial lysine acetylation changes in response to nutritional status, but the global acetylation pattern in Hint2+/- livers is upregulated even without nutritional stress (27), we asked whether the hyperacetylation patterns of Hint2+/- mitochondria would increase further when exposed to nutrient excess or deprivation. The global lysine acetylation

Table 2. Effect of high-fat diet on body/liver weights and metabolic parameters in Hint2++/+ and Hint2+/- mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control Diet</th>
<th>High-Fat Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+/+</td>
<td>–/-</td>
</tr>
<tr>
<td>Body wt gain, g</td>
<td>3.2 ± 0.9</td>
<td>6.4 ± 2.0a</td>
</tr>
<tr>
<td>Food consumption, kcal</td>
<td>5,091 ± 537</td>
<td>4,806 ± 20</td>
</tr>
<tr>
<td>Liver wt, %body wt</td>
<td>4.6 ± 0.4</td>
<td>4.0 ± 0.4</td>
</tr>
<tr>
<td>Fasting blood glucose, mM</td>
<td>5.4 ± 1.0</td>
<td>4.9 ± 1.3</td>
</tr>
<tr>
<td>Fasting plasma insulin, ng/mL</td>
<td>0.5 ± 0.2</td>
<td>0.9 ± 0.4a</td>
</tr>
<tr>
<td>Plasma cholesterol, mM</td>
<td>2.4 ± 0.2</td>
<td>3.1 ± 0.5a</td>
</tr>
<tr>
<td>Alanine transaminase, U/A</td>
<td>12.3 ± 4.3</td>
<td>17.0 ± 5.5</td>
</tr>
</tbody>
</table>

Values are means ± SD. Groups (n = 10) of Hint2++/+ and Hint2+/- mice (aged 8 wk) were fed a control diet or high-fat diet for 8 wk. *P < 0.05, unpaired t-test, Hint2++/+ vs. Hint2+/-; **P < 0.01, unpaired t-test, high-fat vs. control diet.
patterns of mitochondrial protein under control, fasting, and HFD were compared (Fig. 4). Under conditions of control diet and HFD (Fig. 4A), as well as after 24 h fasting (Fig. 4B), the mitochondrial proteins in Hint2+/− livers were hyperacetylated. The greatest differences in global acetylation were observed between the Hint2+/− and Hint2+/+ groups, and these differences were not further amplified or attenuated by the dietary manipulation. A range of liver proteins was affected, primarily those between 30 and 150 kDa (Fig. 4, A and B). To determine whether acetylation was affected in tissues other than the liver, we examined BAT mitochondria in fed and fasted groups. BAT mitochondrial proteins from Hint2+/− mice showed a similar pattern of hyperacetylation under both fed and fasted conditions (Fig. 4C). The Hint2 status was a greater determinant of the lysine acetylation pattern than was the dietary manipulation.

To determine whether the upregulation of lysine acetylation affected proteins associated with hepatic intermediary metabolism, we adopted the candidate approach. Lysine-acetylated proteins were immunoprecipitated from liver mitochondria of control/HFD groups (Fig. 5) and fed/fasted groups of mice (Fig. 6A). Proteins previously described as regulating the adaptation to nutritional status were screened. The extent of acetylation of carbamoyl phosphate synthase 1 (CPS1), which catalyzes the first step of the urea cycle and is a substrate for sirtuin 5 (28), was higher in Hint2+/− than in Hint2+/+ mitochondria under all conditions (Figs. 5 and 6A). Acyl-CoA dehydrogenase long-chain (ACADL), a substrate of sirtuin 3 dehydrogenase long-chain (ACADL), a substrate of sirtuin 3 (16), showed increased acetylation in both control and HFD-treated Hint2+/− mice (Fig. 5) but not in the fasted Hint2+/− group (data not shown). The acyl-CoA dehydrogenase medium-chain (ACADM) showed a marginal increase in control, HFD, and fasted Hint2+/− mice (Figs. 5 and 6A). The 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMG-CoA), which catalyzes the conversion of acetyl-CoA and acetoacetyl-CoA into HMG-CoA and participates in ketogenesis, was hyperacetylated in Hint2+/− mice from the control and HFD groups (Fig. 5). A slight increase was observed after fasting in Hint2+/− mice compared with Hint2+/+ mice (Fig. 6A). Acetylation of HMG-CoA lyase was increased in control/HFD Hint2+/− mitochondria (Fig. 5). Pyruvate carboxylase was hyperacetylated in Hint2+/−, more so after HFD. The chaperone protein, heat shock protein (HSP)-60, showed an increase in acetylation in...
Hint2<sup>−/−</sup> in both control and HFD (Fig. 5), although no difference was detected in fasted mice (data not shown). The peptidylprolyl cis-trans isomerase cyclophilin D, which is a substrate for sirtuin 3 (37), showed an increase in acetylation under both HFD (Fig. 5) and fasting (Fig. 6A) conditions. The acetylation of cyclophilin D increases its isomerase activity and its interaction with adenine nucleotide translocase (ANT), which in turn regulates the opening of the mitochondrial permeability transition pore (PTP) (11). The acetylation of ANT in Hint2<sup>−/−</sup> was not changed (data not shown). The increase in acetylation of cyclophilin D can perhaps explain the augmented response to repetitive Ca<sup>2+</sup> spikes with premature opening of mitochondrial PTP exhibited previously in mitochondria from isolated Hint2<sup>−/−</sup> hepatocytes (31).

The acetylation of ATP synthase α, one subunit of the ATP-synthase complex V, was upregulated in Hint2<sup>−/−</sup> liver.
mitochondria after feeding ad libitum, but not after fasting (Fig. 6A). Similarly, the acetylation of urate oxidase, which catalyzes uric acid to allantoin in rodents and is a substrate for sirtuin 5 (30), was upregulated in Hint2+/− mitochondria from fed but not fasted mice (Fig. 6A). The expression of sirtuin 5 itself did not differ between Hint+/+ and Hint2−/− mitochondria (Fig. 6B). To determine whether increased acetylation of uric acid oxidase resulted in concomitant metabolite changes, we measured liver and plasma uric acid levels. Uric acid levels were higher in liver but lower in plasma of Hint2−/− mice fed ad libitum, although the differences did not reach statistical significance (data not shown).

Cyclophilin D interacts with p53 protein that has translocated to the mitochondria, and this interaction can regulate the mitochondrial PTP (38). p53 itself is acetylated by the acetyltransferase p300/CBP. We tested whether differences in p53 acetylation could be detected on immunoblots probed with an anti-acetyl (Lys389) p53 antibody. Acetylated p53 was detected only in Hint2−/− mitochondrial fractions of fed and fasting mice, but not after HFD (Fig. 6C). Because p53 is deacetylated by sirtuin 1, which in turn translocates to the mitochondria (2), we tested whether differences in expression of sirtuin 1 could account for the observed changes in acetyl-p53. Sirtuin 1 was expressed similarly in Hint2+/+ and Hint2−/− mitochondria (Fig. 6C).

To determine whether a similar set of proteins was affected in BAT, we isolated and pooled BAT mitochondria from four fasting Hint2+/+ and Hint2−/− mice and immunoprecipitated the acetylated proteins. The UCPI protein was absent from the input of fasted Hint2−/− mitochondria (Fig. 7A). An acetylated version of UCPI at the requisite size of 33 kDa was pulled down in Hint2+/+ but not Hint2−/− mitochondria. An acetylated product of a lower size was pulled down in both Hint2+/+ and Hint2−/− preparations. Because BAT functions as a site of fatty acid β-oxidation, the acetylation status of ACADL and 3-hydroxyacyl coenzyme A dehydrogenase short chain (Hadhsc) was investigated. Both showed increased acetylation in BAT mitochondria from fasted Hint2−/− mice. Changes in the acetylation status of GDH and complex V ATP synthase β and α were also detected (Fig. 7A). No change in the expression of the deacetylase sirtuin 3 was detected (Fig. 7B).

Mitochondrial sirtuins 3 and 4 in Hint2−/− mice. Unlike sirtuin 3, sirtuin 4 is not considered to exert its major function as a deacetylase, but rather as an NAD+ requiring ADP-ribosyltransferase of GDH (1, 15). We have previously shown that the activity of GDH is lower in Hint2−/− than in Hint2+/+ mice, due in part to an increase in its lysine acetylation (27). We have reexamined GDH activity and its regulation by ADP-ribosylation in mitochondria under fed and fasted conditions (Fig. 8, A and B). Mitochondrial GDH activity was lower...
In addition to the deacetylase sirtuin enzymes, the acetylation status of proteins is also a function of the acetylation reaction, which in mitochondria is partly mediated by the GCN5L1 protein. GCN5L1 binds to acetyl-CoA, likely resides in the matrix and intermembrane space fractions of the mitochondria, and interacts with and mediates the acetylation of complex V ATP5α (35). To determine whether the expression of GCN5L1 differed between Hint2+/+ and Hint2−/− mice, we probed immunoblots of mitochondria from control and HFD groups with an anti-GCN5L1 antibody. No differences were detected (data not shown).

The mechanism of hepatic steatosis in Hint2−/− mice. We reasoned that the additive consequences of a moderate increase in ACADL and ACADM (Figs. 5 and 6) acetylation were insufficient to produce the degree of hepatic steatosis observed in Hint2−/− mice, since neither is the rate-limiting step in fatty acid β-oxidation. Therefore, we examined the rate-limiting carnitine palmitoyltransferase 1α (Cpt1α), which is allosterically regulated by intracellular concentrations of malonyl-CoA, and is subject to lysine acetylation. Mice from the fasted group were chosen for this comparison because their hepatic triglyceride levels were highest. Hint2−/− mitochondria showed a slight increase in Cpt1α acetylation (Fig. 10A). Moreover, the total malonyl-CoA concentration was twofold higher in Hint2−/− livers, whereas acetyl-CoA concentrations were not significantly different (Fig. 10B). Therefore, a decrease in the acyl-carnitine transferase step likely contributed, in part, to the accumulation of triglycerides in the Hint2−/− livers. To determine whether a change in the expression or acetylation state of the mitochondrial or cytosolic form of the malonyl-CoA decarboxylase (MlyCD) enzyme is responsible for the twofold increase in malonyl-CoA, we examined the level of acetylation of MLYCD in immunoprecipitated preparations of liver mitochondria and cytoplasm. No change was detected (Fig. 10A). No significant changes were detected in the cytoplasmic levels of acetyl-CoA carboxylase (ACC), p-ACC, or the p-ACC-to-ACC ratio (Fig. 10C). However, a minor increase in the levels of fatty acid synthase (FAS) was detected, implicating an increase in fatty acid synthesis (Fig. 10D).

The elevated liver triglyceride levels coupled with the reduced plasma triglycerides in Hint2−/− mice raise the possibility that a reduction in the export of triglycerides from the liver could have contributed to the hepatic steatosis. To determine whether the export of lipids was affected in Hint2−/− livers, we measured the activity of the microsomal triglyceride transfer protein (MTP) under fed conditions. MTP activity was reduced by 20% in Hint2−/− livers (Fig. 11A). This decrease in triglyceride transfer activity was not related to a reduction in expression of either the MTP protein or its heterodimeric partner, protein disulfide isomerase (Fig. 11B).

DISCUSSION

The major phenotype exhibited by our mice bearing a constitutive deletion of Hint2 was the increased accumulation of hepatic lipids along with the hyperacetylation of mitochondrial proteins in liver and brown fat. When the Hint2−/− mice were stressed with a nutritional challenge, the hepatic steatosis was aggravated. Hence, the underlying propensity for steatosis
rendered Hint2−/− mice more vulnerable than control mice to episodes of fasting and HFD.

Several proteins whose acetylation was modified by the absence of Hint2 likely contributed to the accumulation of triglycerides in the liver. The additive effects of an increase in acetylation of ACADL and ACADM predict a reduction in fatty acid oxidation capability. Likewise, the increase in acetylation of malonyl-CoA decarboxylase (Fig. 10). In skeletal muscle and white adipose tissue, sirtuin 4 deacetylates and represses the activity of malonyl-CoA decarboxylase, thereby favoring an increase in malonyl-CoA levels and lipogenesis de novo (23). However, the actions of sirtuin 4 are tissue specific. No changes in hepatic malonyl-CoA levels or hepatic lipogenesis were observed in sirtuin 4 knockout mice (23), which was not mediated by sirtuin 4 or malonyl-CoA decarboxylase.

In mice, the liver-specific knockout of FAS leads to a threefold increase in hepatic malonyl-CoA levels (6). However, FAS is thought to make a minor contribution to liver triglyceride storage, since FAS knockout mice show normal levels of liver lipids and remain subject to fasting-induced hepatic steatosis (20). In Hint2−/− livers, FAS expression tended to increase, but the difference was not significant. This suggests that the increase in hepatic malonyl-CoA levels (6) was not mediated by sirtuin 4 or malonyl-CoA decarboxylase. Malonyl-CoA levels can be influenced by the activity of FAS. In mice, the liver-specific knockout of FAS leads to a threefold increase in hepatic malonyl-CoA levels (23). However, the actions of sirtuin 4 are tissue specific. No changes in hepatic malonyl-CoA levels or hepatic lipogenesis were observed in sirtuin 4 knockout mice (23), which supports the notion that the increase in lipids of Hint2−/− mice is not mediated by sirtuin 4 or malonyl-CoA decarboxylase. Malonyl-CoA levels can be influenced by the activity of FAS.

In mice, the liver-specific knockout of FAS leads to a threefold increase in hepatic malonyl-CoA levels (6). However, FAS is thought to make a minor contribution to liver triglyceride storage, since FAS knockout mice show normal levels of liver lipids and remain subject to fasting-induced hepatic steatosis (20). In Hint2−/− livers, FAS expression tended to increase,
which is consistent with a decrease rather than increase in malonyl-CoA (Fig. 10), although FAS activity was not measured. The increase in FAS could have been secondary to the high insulin levels in Hint2+/− mice (Table 2) (27), since FAS is transcriptionally upregulated by insulin (32).

An experimental disturbance in microsomal triglyceride transfer activity, either by pharmacological inhibition or by liver-specific knockout of the MTTP protein, produces changes in triglyceride levels similar to our Hint2+/− mice (9). However, the marginal decrease in MTTP activity observed in Hint2+/− livers, coupled with a slight increase rather than decrease in plasma cholesterol (Table 2), discounts a dysfunction in MTTP as a primary determinant of lipid accumulation in Hint2+/− livers.

Despite the exacerbation of steatosis and the decrease in body temperature, Hint2+/− mice could withstand a fasting period of 24 h. An extended fasting period of 48 h was previously shown to provoke the complete deacetylation of CPS1 with a consequent increase in enzymatic activity (28). In our studies, Hint2+/− mice responded to the 24-h fasting protocol by an incomplete deacetylation of CPS1 (Fig. 6A), but this did not impair urea production (data not shown) nor increase the circulating ammonia levels. In fact, the plasma ammonia levels decreased after fasting in the Hint2+/− group (Fig. 8E). Likewise, HMG-CoA synthase, which should respond to fasting by deacetylation (36), showed residual acetylation in the Hint2+/− fasted group. However, the production of ketones, as estimated by plasma levels of β-hydroxybutyrate, was not significantly different between Hint2+/− and Hint2+/+ mice. The Hint2+/− mouse was also able to elicit the expected response to fasting of a decrease in acetylation of urate oxidase (30).

The BAT tissue in Hint2−/− but not Hint2+/+ mice responded to fasting by a downregulation of UCP1, which can explain the decrease in body temperature. Previous reports have described starvation-mediated BAT atrophy and UCP1 downregulation in mice and have emphasized that changes are time and age dependent and occur in specific mitochondrial subfractions (3). Because mRNA levels of UCP1 remained constant, a posttranslational modification leading to an increase in UCP1 turnover in Hint2+/− is the most likely explanation. UCP1 is posttranslationally modified by acetylation (Fig. 7) and ubiquitination and degraded by the cytoplasmic proteosome (7). In the BAT of 24-h-fasted rats, genes related to the ubiquitin and proteasome system are upregulated (29). We speculate that fasting could have upregulated a relevant E3 ubiquitin ligase that targets UCP1, and that the absence of Hint2 may have increased the accessibility of UCP1 to subsequent ubiquitination and degradation. In doing so, Hint2 would mimic the actions of another Hint family member, Hint1, which is expressed in cytosolic and nuclear compartments and interacts with the Scf-Skp2 E3 ubiquitin ligase complex to regulate the levels of the cyclin-dependent kinase inhibitor p27 (5). Alternatively, the accelerated downregulation of UCP1 in Hint2+/− may have occurred indirectly. UCP1 is activated upon direct interaction with FFA (12) and then undergoes a conformational change that increases its susceptibility to proteolysis (10). The levels of FFA in the BAT of fasted Hint2+/− mice were decreased, which implies an acceleration of fatty acid mobilization. Mobilized fatty acids in BAT are destined for local oxidation as an energy source or for release to other tissues (14). The higher acetylation state of the fatty acid oxidation enzymes Hadhsc and ACADL (Fig. 7) in Hint2−/− BAT predicts a decrease rather than an increase in local β-oxidation, but we have not further investigated the fate of BAT-mobilized fatty acids. Nevertheless, we speculate that the mobilization of fatty acids was accelerated in fasted Hint2−/−.
Hint2 may operate by means of a similar mechanism. Disruption of protein-protein interactions can be inferred (41). Identified as a component of the TIP60 histone acetyltransferase whose activity is most affected in both enzymes could be affected. In support of this is the such an interaction, downstream modification of substrates for ADP-ribosylation likely by means of an indirect mechanism. Experimental evidence supports an interaction between sirtuin Experimental evidence supports an interaction between sirtuin and Hint2 influences the state of protein acetylation and posttranslation modifications other than acetylation are affected by an increase in ADP-ribosylation, which shows that fatty acid synthase (FAS) in Hint2” and Hint2”” livers. An increase in the level of FAS was detected in Hint2” and Hint2”” livers. The expression of ACC and phosphorylated ACC (p-ACC) was not different between the groups. Actin served as the loading control.

BAT, which in turn was linked to the shortened half-life of the UCP1 protein.

The proteins whose acetylation status is affected by the loss of Hint2 include confirmed substrates both of sirtuin 3 (ACADL, ACADM, ATP synthase α, HSP-60, pyruvate carboxylase, HMG-CoAs, HMG-CoAl, GDH) and sirtuin 5 (CPS, urate oxidase). Moreover, the sirtuin 4 substrate GDH was affected by an increase in ADP-ribosylation, which shows that posttranslation modifications other than acetylation are affected in the absence of Hint2. Taken together, our findings show that Hint2 influences the state of protein acetylation and ADP-ribosylation likely by means of an indirect mechanism. Experimental evidence supports an interaction between sirtuin 3 and 4 within the mitochondria (1). If Hint2 were to disturb such an interaction, downstream modification of substrates for both enzymes could be affected. In support of this is the observation that GDH, a substrate for both sirtuins, is the enzyme whose activity is most affected in Hint2””-livers.

Like Hint2, the ubiquitous Hint1 protein present in the cytosol and nucleus of cells influences the acetylation of specific proteins. Unlike Hint2, a Hint1 deficiency impairs rather than augments the acetylation of ataxia telangiectasia mutated and the histone γ-H2AX (24). Because Hint1 has been identified as a component of the TIP60 histone acetyltransferase complex, an indirect mechanism secondary to the disruption of protein-protein interactions can be inferred (41). Hint2 may operate by means of a similar mechanism.

An alternative explanation for the indirect effect of Hint2 is an alteration of the mitochondrial milieu to promote autoacetylation, which in turn impacts the histone acetyltransferase activity and thus the acetylation of specific targets.
lation of proteins. Scott et al. showed that denaturation of proteins by boiling was sufficient to cause global hyperacetylation, regardless of the presence of the GCN5L1 acetyltransferase (35). Moreover, nonenzymatic lysine acetylation increases as a function of acetyl-CoA concentration and pH (39). The Hint2−/− mice showed no increase in acetyl-CoA when total liver extracts were tested although this does not exclude a change at the mitochondrial level. Hint2−/− mice have not yet been studied with respect to status and regulation of intramitochondrial pH.

In summary, the deletion of Hint2 elicits changes in the posttranslational modification of substrate proteins of sirtuin 3, 4, and 5 in both liver and brown fat. Hint2−/− mice are subject to a perpetual state of hyperacetylation of metabolic enzymes in the mitochondria, which is characteristic of nutritional stress. The ensuing disturbance of fatty acid oxidation renders Hint2−/− mice susceptible to hepatic lipid accumulation, which is aggravated when mild experimental regimens of short-term HF diet are imposed. Hint2−/− mice may lack the metabolic capacity to adapt to more severe nutritional challenges such as long-standing HF diet or starvation because of the inability to fine tune physiological responses to the burden of nutritional excess or deprivation. Our findings show that the Hint2 status is an even more important determinant of the pattern of lysine acetylation of mitochondrial proteins than is the nutritional state.

ACKNOWLEDGMENTS

We thank Philipp Kellmann and Jürg Müller for technical assistance. We thank Dr. Gisèle Ferrand at the animal facility, EPFL, Lausanne, Switzerland, for breeding and diet management of the animals.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


