Conclusions: The mechanisms underlying impaired energy sensing signaling differ with the duration of HFD feeding. In the early phase of HFD feeding, LKB1 and SIRT1 were not impaired, while in the later phase of HFD feeding, decreased SIRT1 expression and LKB1 phosphorylation may be involved in the development of severe glucose and lipid intolerance.

Introduction

AMP-activated protein kinase (AMPK) is a key sensor of cellular energy status that regulates glucose and lipid metabolism. AMPK is activated through an increase in the intracellular AMP/ATP ratio induced by metabolic stresses such as starvation, hypoxia, or exercise [1], as well as stimulation with leptin [2], adiponectin [3] and the anti-diabetic agents metformin [4–6] and thiazolidinediones [6–8]. The process of AMPK activation requires phosphorylation at threonine residue (Thr) 172 within the catalytic α subunit by LKB1 [9]. The phosphorylation of AMPK is reportedly suppressed in the livers [10,11] and skeletal muscles [12] of HFD-fed rats.

LKB1, an upstream kinase for AMPK and AMPK-related kinases including the salt-inducible kinase (SIK) cascade, is localized mainly in the nucleus under basal conditions. LKB1 is reportedly phosphorylated at serine residue (Ser) 431 in mice (Ser428 in humans) by protein kinase A (PKA) [13], p90 ribosomal S6 kinase [14] or PKC-ζ [15], and is translocated from the nucleus to the cytoplasm. In the cytoplasm, LKB1 forms a heterotrimeric complex with two adaptor proteins termed STE20-related adaptor (STRAD) and MO25 [16,17]. The binding and interaction of LKB1 with STRAD and MO25 increase the catalytic activity of LKB1, resulting in the phosphorylation and activation of AMPK [18]. Activated AMPK then induces the phosphorylation and inactivation of acetyl coenzyme A carboxylase (ACC), decreasing malonyl-CoA and thereby relieving inhibition of carnitine palmitoyl-transferase 1, and leading to an increase in fatty acid oxidation in the liver and skeletal muscle [19].

In addition, activated AMPK phosphorylates and inactivates the transducer of regulated cyclic AMP responsive element binding protein (CREB) 2 (TORC2, or CREB-regulated transcription coactivator, CRTC2), identified as a critical molecular switch in the regulation of hepatic gluconeogenesis [20]. TORC2 phosphorylated at Ser171 translocates from the nucleus to the cytoplasm, leading to its poly-ubiquitination and degradation [21,22], thereby reducing the expressions of gluconeogenic genes, including peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), phosphoenolpyruvate carboxykinase and glucose-6-phosphatase [20].

Sirtuin 1 (SIRT1), a mammalian homologue of Sir2 (silent information regulator 2), is an NAD^+-dependent histone deacetylase, and its expression and enzymatic activity are enhanced by an increase in the NAD^+/NADH ratio [23]. In HFD-fed rodents, SIRT1 protein levels are reportedly decreased in the liver [24] and skeletal muscle [25]. Moreover, resveratrol, an activator of SIRT1, improves HFD-induced fat accumulation and insulin resistance in the liver by enhancing AMPK phosphorylation [10,26].

Thus, there exist multiple key molecules including AMPK and SIRT1 in the liver, which regulate glucose tolerance and insulin sensitivity in response to the nutrient conditions. In this study, we attempted to investigate how AMPK and SIRT1-mediating pathways are impaired after the different duration of high-fat diet feeding, and prepared the mice treated with short (2 weeks) or long-term (8 weeks) control or HFD feeding. Herein, we discuss the pathophysiological significance of our observations on HFD-induced insulin resistance.

Materials and methods

Animals and diets

Male 7-week-old ddY mice were purchased from Shimizu Laboratory Supplies (Kyoto, Japan) and fed a standard diet (SD, $n=8$) or a HFD ($n=8$) for 2 weeks and then the SD ($n=4$) or HFD ($n=4$) for 8 weeks. The nutrient composition of the SD was 54.4% carbohydrate, 23.6% protein, 5.3% fat, and a vitamin and mineral mixture, while that of the HFD purchased from Oriental Yeast (Tokyo, Japan) was 7.5% carbohydrate, 24.5% protein, 60% fat, and a vitamin and mineral mixture, as in our previous studies [27,28]. All mice were fed *ad libitum* and maintained on 12-h light and dark cycles under controlled environment settings. All mice were fasted for 6 h and then killed. The protocols for this animal

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study were approved by the Animal Experimental Committee of Hiroshima University.

Liver tissue samples

All mice were anesthetized with pentobarbital sodium (60 mg/kg body weight) intraperitoneally. The livers were removed and homogenized in ice-cold lysis buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride] and centrifuged at 16,000 × *g* for 30 min at 4 °C. The supernatants were used as liver lysates. The liver lysates were added to Sodium Dodecyl Sulfate (SDS) sample buffer [200 mM Tris–HCl (pH 6.8), 4% SDS, 10% glycerol, 0.1% bromophenol blue, 5% 2-mercaptoethanol], boiled for 5 min, and analyzed by Western blot assays.

Western blot assays

For Western blot assays, the liver lysates were analyzed with antibodies against: LKB1 (Molecular Weight 51 kDa), SIRT1 (110 kDa) (Upstate, NY), phosphorylated LKB1-Ser431 (52 kDa), TORC2 (87 kDa) (Santa Cruz Biotechnology, CA), STRAD (20 kDa) (Imgenex, CA), MO25α (39 kDa), phosphorylated AMPKα-Thr172 (62 kDa), phosphorylated ACC-Ser79 (280 kDa), phosphorylated TORC2-Ser171 (85 kDa) (Cell Signaling Technology, MA), or Actin (Sigma–Aldrich, MO), followed by the secondary antibodies: anti-rabbit or anti-mouse immunoglobulin-horseradish peroxidase-linked whole antibody, and also enhanced chemiluminescent reagents (GE Healthcare, UK).

Quantitative real-time PCR

Total RNA from mouse liver tissue was extracted using Sepasol-RNA I (Nacalai Tesque, Kyoto, Japan), and cDNA was generated using a Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany). Expression level of the *TORC2* gene were analyzed by quantitative real-time PCR using SYBR Premix Ex Taq™ (Takara Bio, Shiga, Japan) and an ABI PRISM 7700 Sequence Detector (Applied Biosystems, CA). Primer sequences were as follows: *TORC2*, 5′-AGTGAGGGATCAATGAGTGG-3′ (forward) and 5′-AGAGTCTCCTGTGAGTATGA-3′ (reverse). All PCR data were normalized to expression levels of *GAPDH* in the corresponding sample.

Statistical analysis

The data are expressed as means ± standard deviation. The difference in the mean of any parameter between the two groups was tested using the unpaired *t* test. A $P < 0.05$ was considered statistically significant.

Results

Parameters of mice fed HFD for 2 or 8 weeks

As shown in the Table 1, after 2 weeks of HFD feeding, body weights and fasting blood glucose levels were higher than those of mice fed the SD, but the differences were not significant ($P = 0.549$ and 0.068, respectively). However, HFD feeding for 8 weeks significantly increased body weight and fasting blood glucose as compared with SD feeding.

LKB1-AMPK-ACC/-TORC2 pathways in livers of mice fed HFD for 2 weeks

Regarding the LKB1-AMPK-ACC and -TORC2 pathways in the liver, we compared protein expressions and phosphorylation levels, by Western blotting, between mice fed the SD versus the HFD for 2 weeks. As shown in Fig. 1, AMPKα and TORC2 protein levels were significantly increased, but phosphorylated AMPKα-Thr172 and phosphorylated ACC-Ser79 protein levels were markedly decreased in HFD-fed mice as compared with SD-fed mice. Phosphorylated LKB1-Ser431 was slightly, but not significantly ($P = 0.132$), decreased in HFD-fed mice. Levels of two adaptor proteins of LKB1, STRAD and MO25, did not differ between SD and HFD-fed mice. Thus, these data show that HFD feeding for 2 weeks suppresses hepatic AMPK-ACC

Table 1 Body weight and fasting blood glucose in ddY mice fed SD or HFD for 2 or 8 weeks.

	2 weeks		8 weeks	
	SD	HFD	SD	HFD
Body weight (g)	38.2 ± 3.0	39.3 ± 4.1	48.9 ± 1.7	66.5 ± 4.7*
Blood glucose (mg/dl)	164 ± 31	205 ± 49	154 ± 18	363 ± 36*

Data are expressed as means ± S.D. Male 7-week-old ddY mice were fed a standard diet (SD, $n = 8$) or a high-fat diet (HFD, $n = 8$) for 2 or 8 weeks (SD, $n = 4$; HFD, $n = 4$). Body weight and fasting blood glucose were measured at 9 and 15 weeks of age.
* $P < 0.05$; SDvsHFD.

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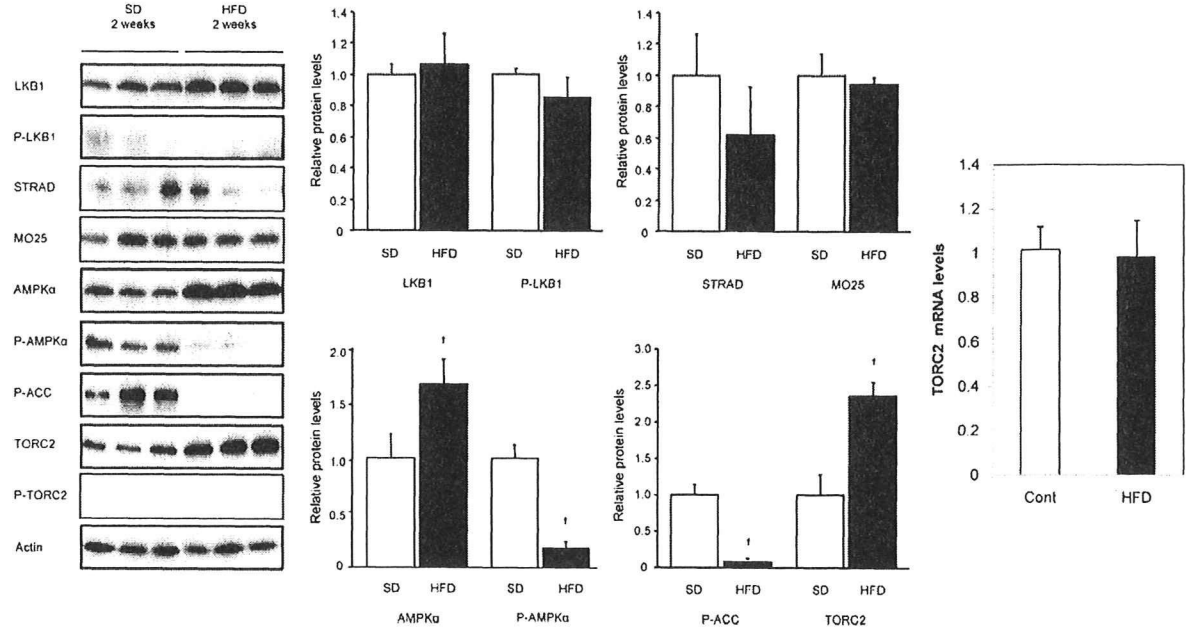

Figure 1 LKB1-AMPK-ACC/-TORC2 pathways in livers of mice fed HFD for 2 weeks. Western blot analysis of LKB1, phosphorylated LKB1-Ser431 (P-LKB1), STRAD, MO25α, AMPKα, phosphorylated AMPKα-Thr172 (P-AMPKα), phosphorylated ACC-Ser79 (P-ACC), TORC2 and phosphorylated TORC2-Ser171 (P-TORC2) proteins as well as real-time PCR analysis of TORC2 mRNA level in mice fed the SD (white columns; $n=4$) or HFD (black columns; $n=4$) for 2 weeks ($^{\dagger}P<0.05$; vs SD). The blots shown are representative of three blots from each group of mice.

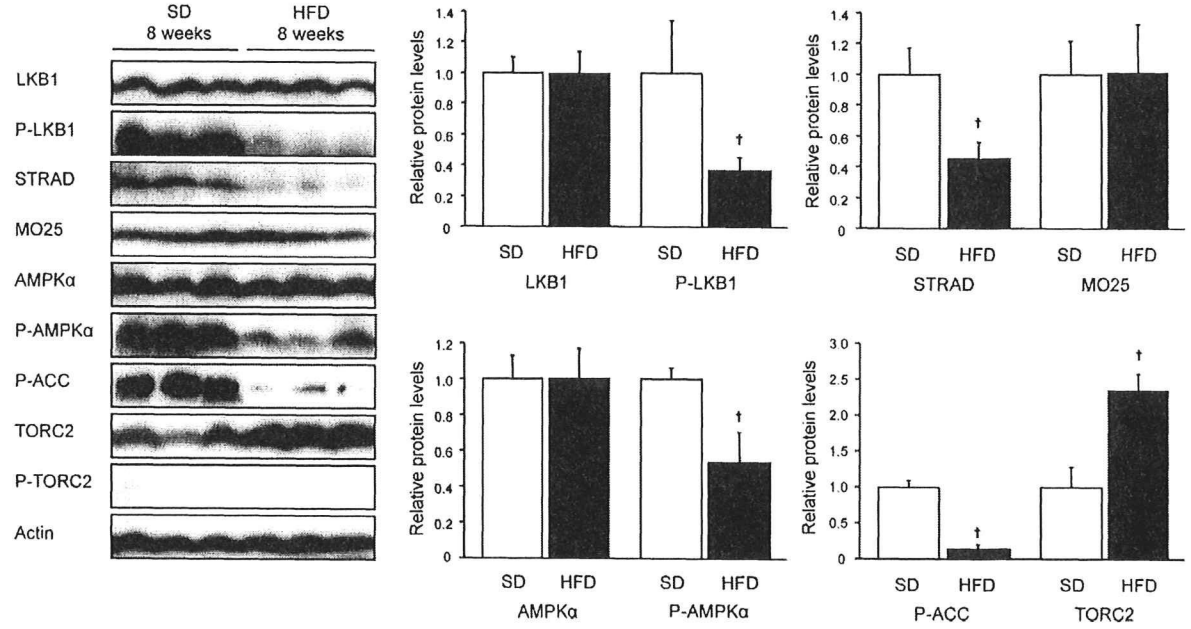

Figure 2 LKB1-AMPK-ACC/-TORC2 pathways in livers of mice fed HFD for 8 weeks. Western blot analysis of LKB1, phosphorylated LKB1-Ser431 (P-LKB1), STRAD, MO25α, AMPKα, phosphorylated AMPKα-Thr172 (P-AMPKα), phosphorylated ACC-Ser79 (P-ACC), TORC2 and phosphorylated TORC2-Ser171 (P-TORC2) proteins in mice fed the SD (white columns; $n=4$) or HFD (black columns; $n=4$) for 8 weeks ($^{\dagger}P<0.05$; vs SD). The blots shown are representative of three blots from each group of mice.

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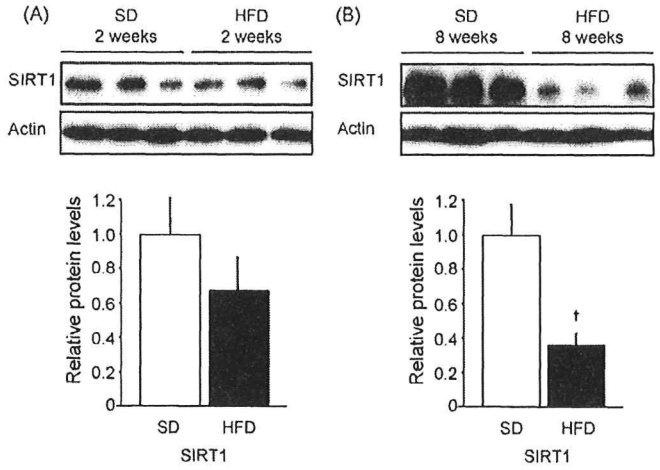

Figure 3 SIRT1 expressions in livers of mice fed HFD for 2 or 8 weeks. Western blot analysis of SIRT1 proteins in mice fed the SD (white columns; $n=4$) or HFD (black columns; $n=4$) for 2 weeks (A) or 8 weeks (B) ($^{\dagger}P<0.05$; vs SD). The blots shown are representative of three blots from each group of mice.

signaling without changing LKB1 phosphorylation while upregulating TORC2 expression.

LKB1-AMPK-ACC/-TORC2 pathways in livers of mice fed HFD for 8 weeks

As shown in Fig. 2, phosphorylated LKB1-Ser431, STRAD, phosphorylated AMPKα-Thr172 and phosphorylated ACC-Ser79 protein levels were significantly decreased in mice fed the HFD for 8 weeks as compared with SD-fed mice. TORC2 protein levels were significantly increased in HFD-fed mice, while TORC2 mRNA levels were not significantly altered, which suggests increased TORC2 protein not via the transcriptional regulation but rather via the increased translational regulation or the decreased degradation velocity. Thus, reduced hepatic LKB1 phosphorylation was observed after 8 weeks of HFD feeding, while suppressed AMPK-ACC signaling was similar to that with 2 weeks of HFD feeding.

SIRT1 expression in livers of mice fed HFD for 2 or 8 weeks

It was recently reported that SIRT1 functions as an upstream regulator for LKB1 in cultured cells and in the livers of mice and rats [29,30]. As shown in Fig. 3A, in mice fed the HFD for 2 weeks, SIRT1 protein levels were slightly decreased as compared with SD-fed mice, but the difference was not significant ($P=0.117$). However, SIRT1 protein levels were drastically decreased in mice fed the HFD for 8 weeks (Fig. 3B). These data indicate that long-term HFD feeding gradually reduces SIRT1 expression, which would then contribute to the declining LKB1 phosphorylation.

Discussion

In the energy deficient state, increased AMP binds to AMPK, and structurally modified AMPK is thereby rendered accessible to phosphorylation by LKB1. Thus, at the cellular level, increases in both the AMP/ATP ratio and LKB1 activity are considered to be responsible for AMPK phosphorylation. In this study, LKB1 phosphorylation was not significantly suppressed after 2 weeks of HFD feeding, although AMPK phosphorylation had already been attenuated. This suggests AMPK impairment in the relatively early phase of HFD feeding to be due to the decreased hepatic AMP/ATP ratio (Fig. 4A). However, after long-term HFD feeding, decreased LKB1 phosphorylation becomes overt. The phosphorylation of LKB1 at Ser431 reportedly does not affect the kinase activity itself, but is necessary for the formation of a complex with STRAD and MO25 and its translocation to the cytoplasm [16,17]. In addition, SIRT1 expression was also markedly reduced after long-term HFD feeding. Thus, it is reasonable to speculate that not only a decreased AMP/ATP ratio but also decreased cytoplasmic LKB1 activities are involved in the reduced AMPK phosphorylation seen after long-term HFD feeding (Fig. 4B).

According to recent studies, SIRT1 regulates LKB1 deacetylation and induces translocation of LKB1 to the cytosol from the nucleus [29,30]. Translocated LKB1 associates with STRAD and MO25 and exhibits kinase activity leading to the phosphorylation of AMPK [18]. Moreover, SIRT1 also reportedly deacetylates TORC2 and promotes its ubiquitin-dependent degradation in the mouse liver after prolonged fasting and in primary hepato-

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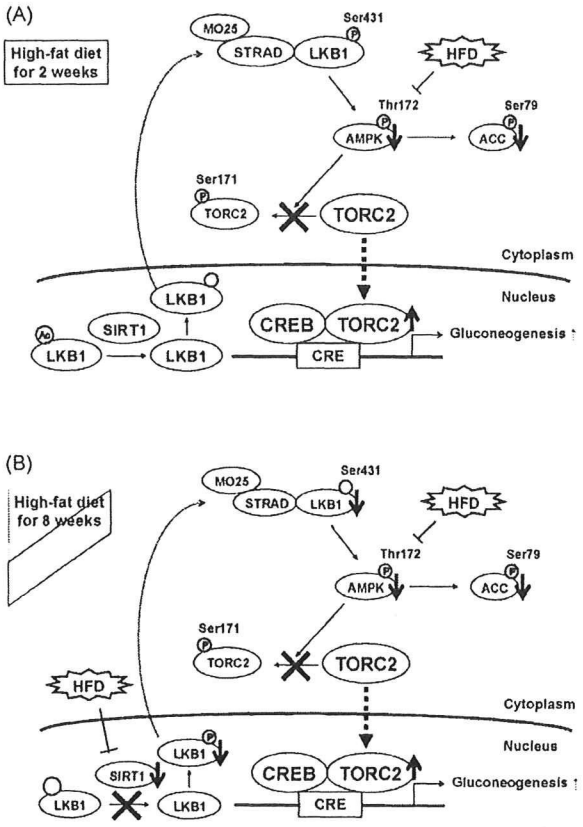

Figure 4 Proposed hepatic LKB1-AMPK-ACC/-TORC2 pathways in mice fed HFD for 2 weeks (A) or 8 weeks (B). (A) In mice fed the HFD for 2 weeks, AMPK phosphorylation is decreased, without changing SIRT1 and LKB1, followed by reduced ACC phosphorylation, resulting in the suppression of fatty acid oxidation. TORC2 phosphorylation and degradation are reduced by the inactivation of AMPK, which in turn raises the de-phosphorylated form of TORC2 and enhances gluconeogenesis. (B) In mice fed the HFD for 8 weeks, a decrease in SIRT1 expression suppresses LKB1 deacetylation and the phosphorylations of LKB1, AMPK and ACC. TORC2 phosphorylation and degradation are reduced by the inactivation of AMPK, and in addition, TORC2 deacetylation may be impaired by the decrease in SIRT1 expression [32], leading to an extreme increase in de-phosphorylated TORC2 proteins and even greater enhancement of gluconeogenesis. P, phosphorylation; Ac, acetylation.

cytes exposed to glucagon [32]. Taken together, our results suggest that SIRT1 expression reduction observed after long-term HFD feeding would suppress hepatic LKB1-AMPK-ACC signaling and TORC2 degradation. As a result, increased gluconeogenesis and the resultant hyperglycemia may become increasingly evident, depending on the duration of overnutrition. In fact, with prolonged HFD feeding from 2 to 8 weeks, hyperglycemia was markedly exacerbated as compared with SD-fed controls.

AMPK and SIK are kinases located downstream from LKB1. These two kinases have conserved kinase domains, but SIK is phosphorylated by LKB1 without being affected by the AMP/ATP ratio [31]. In addition, SIK phosphorylates TORC2 very efficiently, but probably not ACC. Thus, while fatty acid oxidation through ACC phosphorylation is mediated by AMPK, increased hepatic gluconeogenesis mediated by the TORC2 pathway is induced by both SIK and AMPK. Taking these observations together, decreased LKB1 phosphorylation after long-term overnutrition would appear to impair both AMPK and SIK phosphorylation, possibly leading to more markedly reduced TORC2 phosphorylation.

In conclusion, we revealed that short-term HFD feeding suppressed hepatic AMPK-ACC signaling, while prolonged HFD feeding decreased SIRT1 expression and LKB1 phosphorylation. These clearly different impairments in energy sensing mechanisms may also develop gradually in the livers of human diabetic and obese subjects, possibly affecting the efficiency of AMPK activators including metformin and SIRT1 activators such as polyphenols like resveratrol.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

We are grateful to the Analysis Center of Life Science, Hiroshima University for the use of their facilities.

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