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Ketogenic essential amino acids replacement diet ameliorated hepatosteatosis with altering autophagy-associated molecules

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ABSTRACT

Ketogenic amino acid (KAA) replacement diet has been shown to cure hepatic steatosis, a serious liver disease associated with diverse metabolic defects. In this study, we investigated the effects of KAA replacement diet on nutrition sensing signaling pathway and analyzed whether induction of hepatic autophagy was involved. Mice are fed with high fat diet (HFD) or KAA replacement in high-fat diet (30% fat in food; HFD)-fed (HFD^{KAAR}) and sacrificed at 8, 12, 16 weeks after initiation of experimental food. Hepatic autophagy was analyzed in protein expression of several autophagy-associated molecules and in light chain-3 green fluorescent protein (LC-3 GFP) transgenic mice. HFD^{KAAR} showed increased AMP-activated protein kinase (AMPK) phosphorylation and enhanced liver kinase B1 (LKB1) expression compared to control HFD-fed mice. The KAA-HFD-induced activation of AMPK was associated with an increased protein expression of sirtuin 1 (Sirt1), decreased forkhead box protein O3a (Foxo3a) level, and suppression of mammalian target of rapamycin (mTOR) phosphorylation compared with the HFD-fed mice. The intervention study revealed that a KAA-replacement diet also ameliorated all the established metabolic and autophagy defects in the HFD-fed mice, suggesting that a KAA-replacement diet can be used therapeutically in established diseases. These results indicate that KAA replacement in food could be a novel strategy to combat hepatic steatosis and metabolic abnormalities likely involvement of an induction of autophagy.

1. Introduction

Hepatic steatosis is associated with serious metabolic disorders, such as obesity, metabolic syndrome, dyslipidemia, and type 2 diabetes. Non-alcoholic fatty liver disease, also known as non-alcoholic steatohepatitis, is a highly studied hepatic disease research topic [1–3]. Therefore, knowledge of the precise pathomechanisms in these liver diseases is essential, and molecular pathway-based therapeutic strategies have emerged as important tools to combat these devastating diseases.

Autophagy is the system that maintains cellular homeostasis by degrading and recycling unnecessary, aged, damaged or dysfunctional components within cells [4]. The targets of autophagy are long-lived proteins and malfunctioning organelles [4]. Originally, autophagy was observed as an energy-utilizing strategy in starvation or when the amount of food was insufficient to maintain body homeostasis [4]. However, the importance of autophagy is not limited to starvation conditions. Defects in the autophagy system have been associated with diseases, such as cancer [5], neurodegenerative diseases, including Alzheimer's disease and Huntington's disease [6,7], atherosclerosis [8], and age-related macular degeneration [9]. Enhanced autophagy in calorie restriction has been reported as an effective nutritional intervention under pathological conditions [10–13], suggesting that autophagy could play an essential role in tissue homeostasis. Furthermore, autophagy has been reported to be essential in the protection from alcohol- and non-alcohol-mediated liver injury [14,15]. Therefore, autophagy normalization is likely a potent therapeutic strategy for hepatic diseases; however, except for severe calorie restriction, no established tool has reported inducing autophagy for disease intervention.

Food containing ketogenic essential amino acids (KAAs), which include high ratios of branched-chain essential amino acids, has been shown to be beneficial for metabolic abnormalities in humans [16] and rodents [17–19]. However, contradictory data has been reported showing negative correlations between ketogenic amino acids and

Abbreviations: KAA, ketogenic amino acids; HFD, high fat diet; LC-3, light chain-3; LKB1, liver kinase B1; AMPK, adenine monophosphate-activated protein kinase; mTOR, mammalian target of rapamycin; Foxo, forkhead homeobox type O; Atg, autophagy-related protein

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diabetes onset [20–22]. We have reported that KAA-modulated lipid synthesis pathways, together with increased oxygen consumption and respiratory quotient, prevented hepatic steatosis [23]. In this study, we analyzed the involvement of autophagy induction in the high KAA diet-associated alteration of nutritional sensing pathways.

2. Materials and methods

2.1. Antibodies

LKB1, p-AMPK, AMPK, p-mTOR, mTOR, and Foxo3a antibodies were obtained from Cell Signaling Technology (Danvers, MA). β-Actin, LC3, Atg7, Atg13, and beclin antibodies were obtained from Sigma (St. Louis, MO), and the anti-P62/SQSTM1 antibody was purchased from MBL (Nagoya, Japan).

2.2. Diets

For the C57/Bl6 mouse experiments, the control diet (control), high-fat diet (HFD) and KAA-fortified HFD (HFD^{KAAR}) were prepared based on our previous report [23]. The casein-mimic free amino acid mixture (CAAM) or KAA mixture was used to replace a portion of the protein component. Using partial protein replacement by free KAA, the essential/non-essential amino acids (E/N) ratio in the diet was 1.8. Additionally, to avoid changes in the amounts of dietary total amino acids, fat and carbohydrates, the total amino acids, including protein/amino acids, were equalized among the groups by the addition of up to 23% of CAAM (Supplementary Table 1).

2.3. Animals

All studies were reviewed and approved by the Institutional Animal Care Committee of Kanazawa Medical University. Eight-week-old male C57/Bl6 mice were obtained from CLEA Japan, Inc. (Tokyo, Japan). All mice were housed in colony cages and maintained on a 12:12-hour light and dark cycle with free access to water. Male LC3-GFP mice (C57/Bl6 background) were obtained from Riken Bio Resource Center (Ibaraki, Japan). Mice were fed a control diet, HFD or HFD^{KAAR} from the age of 8 weeks. The metabolic status and histological/biochemical evaluations were performed at 8, 12 or 16 weeks after the initiation of the indicated diets. For the interventional protocol, mice were fed an HFD for 8 weeks. Then, 8 weeks after HFD initiation, the HFD-fed mice were divided into two groups: one group of mice was fed the same HFD, and the other group was fed HFD^{KAAR} for 4 or 8 weeks.

2.4. Glucose tolerance

Mice were starved for 6 h before the injection of glucose (1 g/kg BW) intraperitoneally (intraperitoneal glucose tolerance test: IPGTT). Blood glucose levels were measured at 0, 15, 30, 60, 90 and 120 min after the injection of glucose. In the 12-week protocol, we measured insulin levels via an ELISA (Morinaga Institute of Biological Science, Inc.). The insulin resistance index (IRI) was calculated by a previously reported method (IRI = glucose (mmol/L) × insulin (mU/L)/22.5) [24]. The levels of ketone bodies in the plasma were evaluated by an ELISA (Bioassay System, Hayward, CA).

2.5. Oil Red O staining

Frozen liver sections were used for the evaluation of hepatic steatosis, and Oil Red O staining was performed. 8-μm fresh frozen liver sections were fixed in 10% formalin for 15 min, rinsed in distilled water, and placed in 60% isopropylene for 1 min. They were then stained in an Oil Red O solution (0.5 g of Oil Red O dissolved in 100 mL of isopropylene) for 20 min at 37 °C, placed in 60% isopropylene for 1 min, washed with distilled water three times, and mounted with 70% glycerin jelly. The red lipid droplets were observed using microscopy.

2.6. LC3-GFP detection

Mice were anesthetized by procaine and perfused with a fixative (4% paraformaldehyde in PBS). Fixed samples were then stored at −80 °C until evaluation. Frozen sections (5 μm) were observed by microscopy to detect LC3-positive puncta. LC3-GFP-positive cells, which were observed in 200× visual fields, and then counted and quantified.

2.7. Macrophage detection

Five-micrometer sections of frozen liver samples were used for macrophage labeling by F4/80. Samples were fixed with −20° acetone for 10 min; after blocking with PBS containing 2% BSA, the sections were incubated with an anti-F4/80 antibody (AbD Serotec, Oxford, UK) in PBS containing 2% BSA for 1 h. The sections were washed three times and incubated with a TRITC-conjugated secondary antibody (1:200 dilution; Jackson ImmunoResearch, West Grove, PA) at room temperature for 30 min. After washing three times and mounting, which included DAPI staining (Vector Laboratories, Inc. Burlingame, CA), the F4/80-positive macrophages were visualized by fluorescence microscopy (Biozero, Keyence, Osaka, Japan). The number of F4/80 positive macrophages per field of view (×400 magnification) was assessed by counting positively labeled cells in 5 random fields of view per slide, with 5 slides per experimental group. The results were evaluated by two independent investigators.

2.8. Western blotting

All the tissue samples were harvested without starvation. Protein lysates were boiled in sample buffer containing sodium dodecyl sulfate (SDS) at 100 °C for 5 min. After centrifugation at 17,000 ×*g* for 10 min at 4 °C, the supernatant was separated on 6% or 12% SDS-polyacrylamide gels and blotted onto PVDF membranes (Immobilon, Bedford, MA) with a semidry method. After blocking with TBS-T (Tris-buffered saline containing 0.05% Tween 20) containing 5% non-fat dry milk or bovine serum albumin (BSA), the membranes were incubated with each primary antibody in TBST containing 5% BSA at 4 °C overnight. The membranes were washed three times and incubated with a 1:2000 dilution of horseradish peroxide (HRP)-conjugated secondary antibody (Cell Signaling Technology) at room temperature for 1 h. The immunoreactive bands were detected with an enhanced chemiluminescence (ECL) detection system (Pierce Biotechnology, Rockford, IL).

2.9. Statistical analysis

The data are expressed as the mean ± s.e.m. in a graph. The Mann–Whitney *U*-test was used to detect statistical significance (defined as $P < 0.05$). GraphPad Prism 5.0 was used for statistical analysis.

3. Results

3.1. The effect of KAA replacement diet on weight of body and organs

When used for 8 weeks, a KAA replacement diet has been shown to ameliorate high fat diet-induced obesity, glucose intolerance, and lipotoxicity in mice [23]. In our study, we first proposed the use of a longer treatment, such as 12 or 16 weeks, of a KAA diet. When the body weights of the mice were analyzed, all groups of mice exhibited similar body weights, even in the HFD-fed groups, until 12 weeks after the induction of nutritional intervention (Fig. 1A). The HFD-fed group of mice displayed significantly heavier body weight at 16 weeks after

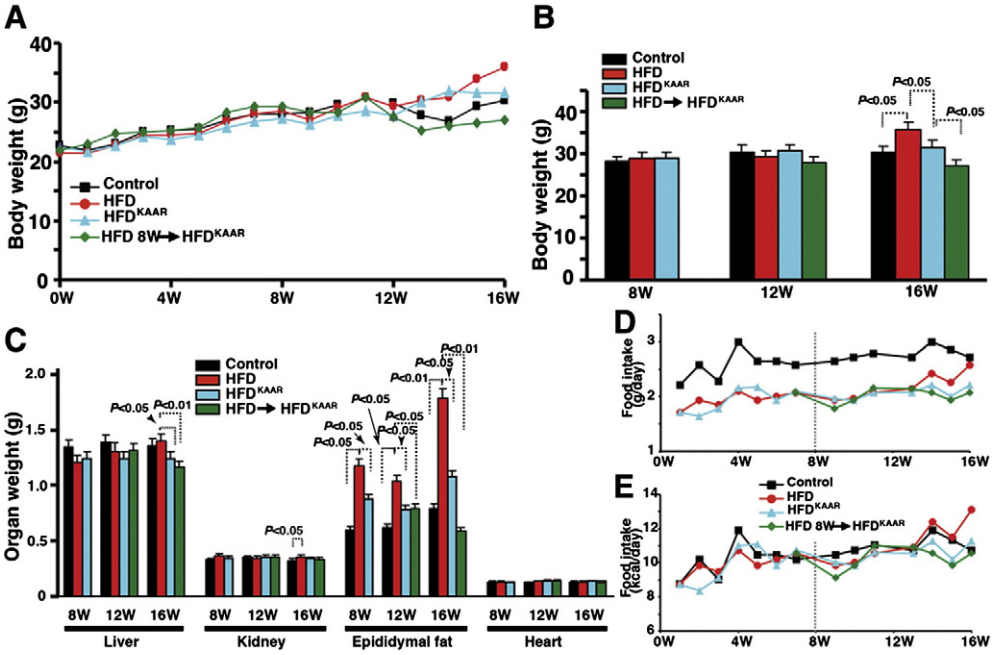

Fig. 1. Body and organ weights and food intake of the mice. A. Time course of body weight changes in the mice. Eight-week-old C57/Bl6 mice were fed the indicated diets (0 W on the graph). The body weight of each mouse was evaluated each week, and the averages are plotted. B. The body weights of the mice at the indicated weeks after the indicated diet was initiated: 8 W (control n = 10, HFD n = 8, HFD^{KAAR} n = 10), 12 W (control n = 12, HFD n = 11, HFD^{KAAR} n = 12, HFD → HFD^{KAAR} n = 12), and 16 W (control n = 4, HFD n = 4, HFD^{KAAR} n = 5, HFD → HFD^{KAAR} n = 5). C. The liver, kidney, epididymal fat and heart weights: 8 W (control n = 4, HFD n = 5, HFD^{KAAR} n = 5), 12 W (control n = 8, HFD n = 7, HFD^{KAAR} n = 7, HFD → HFD^{KAAR} n = 7), and 16 W (control n = 4, HFD n = 4, HFD^{KAAR} n = 5, HFD → HFD^{KAAR} n = 5). D, E. 3–4 mice are housed in the cage. The food intake was evaluated each week, and the average level per mouse was analyzed for each indicated group after the initiation of the protocol. The food intakes at 8 and12 weeks after experimental food initiation were not analyzed due to IPGTT, which may affect the amount of food taken. The number of mice analyzed was the same as shown in B. Data are expressed as the mean + s.e.m.

the induction of a HFD (Fig. 1A, B). Under dietary conditions of identical calories, nitrogen content and fat, the KAA replacement diet (HFD^{KAAR})-fed mice weighed less compared to the HFD-fed group of mice (Fig. 1A, B). We evaluated the weight of several organs. The liver weights were the same in all of the groups until 12 weeks after the induction of nutritional treatment (Fig. 1C). Sixteen weeks after protocol initiation, the livers of HFD-fed mice displayed heavier trend when compared to the livers of control diet-fed mice, whereas livers from mice fed the HFD^{KAAR} diet were significantly lighter compared to the HFD-fed mice (Fig. 1C). The kidney weights were slightly but significantly heavier in HFD-fed mice compared to control diet-fed mice at 16 weeks after the initiation of the experimental protocol (Fig. 1C). The epididymal fat weight was heavier in the HFD-fed mice compared with the control diet-fed mice in the time period analyzed, and the HFD^{KAAR}-fed mice exhibited a significantly lower epididymal fat weight compared with the HFD-fed mice (Fig. 1C). The heart weight remained constant in all groups of mice during the experimental protocol (Fig. 1C). An analysis of the daily food intake indicated that the mice did not prefer to eat food containing high fat compared with normal chow and that the HFD^{KAAR} diet did not affect the food intake until 12 weeks after initiation of experimental food initiated (Fig. 1D). After 12 weeks, mice in HFD group started to eat more foods when compared to HFD^{KAAR} (Fig. 1D). Per calorie-based analysis revealed that all groups of mice ate almost identical calories until 12 weeks after the protocol (Fig. 1E). Similar to the food amount data, mice in HFD group took more calories when compared to other groups from 12 weeks after protocol initiation (Fig. 1E).

3.2. KAA replacement diet inhibit HFD-induced glucose tolerance defects

The effect of a KAA replacement diet on glucose metabolism was analyzed by IPGTT. The HFD-fed mice exhibited a significant deterioration of glucose tolerance at 12 and 16 weeks after the initiation of the experimental protocol (Fig. 2A–C) in addition to higher insulin levels (Fig. 2D, E). The HFD^{KAAR}-fed mice exhibited significantly ameliorated glucose tolerance compared to the HFD-fed mice (Fig. 2A–C), as similar to the observation we reported [23]. This amelioration of glucose tolerance by the KAA diet was associated with decreased insulin levels (Fig. 2D, E). When IRI was analyzed using previously reported formula during IPGTT [24] it demonstrated that the HFD^{KAAR} diet significantly ameliorated IRI compared to the HFD-fed mice (Fig. 2F).

3.3. KAA replacement diet prevent HFD-induced hepatosteatosis

Upon analysis with Oil Red O staining, we found that both 12 and 16 weeks of treatment with the KAA-containing diet significantly ameliorated hepatic steatosis in mice (Fig. 3A–E). Twelve weeks of treatment with HFD suppressed LKB1 expression, which was associated with decreased AMP-activated protein kinase (AMPK) phosphorylation (Fig. 3F, G). In contrast, treatment with the HFD^{KAAR} diet increased LKB1 expression, which was associated with increased levels of AMPK phosphorylation compared to the HFD-fed mice (Fig. 3F, G). The HFD-fed mice were also observed to have suppressed levels of the sirt1 protein, whereas the KAA diet increased the expression levels of sirt1 (Fig. 3F, G). Mammalian target of rapamycin (mTOR) levels and their phosphorylation levels were not significantly altered in mice fed the HFD in our analysis compared to the control diet-fed mice, although the KAA-containing diet significantly suppressed mTOR phosphorylation levels (Fig. 3F, G). The analysis of the effect of the KAA diet on forkhead box protein O3a (Foxo3a) levels revealed similar trends to those observed for mTOR (Fig. 3F, G). Mice treated for 16 weeks with an HFD displayed similar molecular defects in the livers when compared to 12 weeks of treatment and HFD^{KAAR} diet rescued such molecular defects (data not shown).

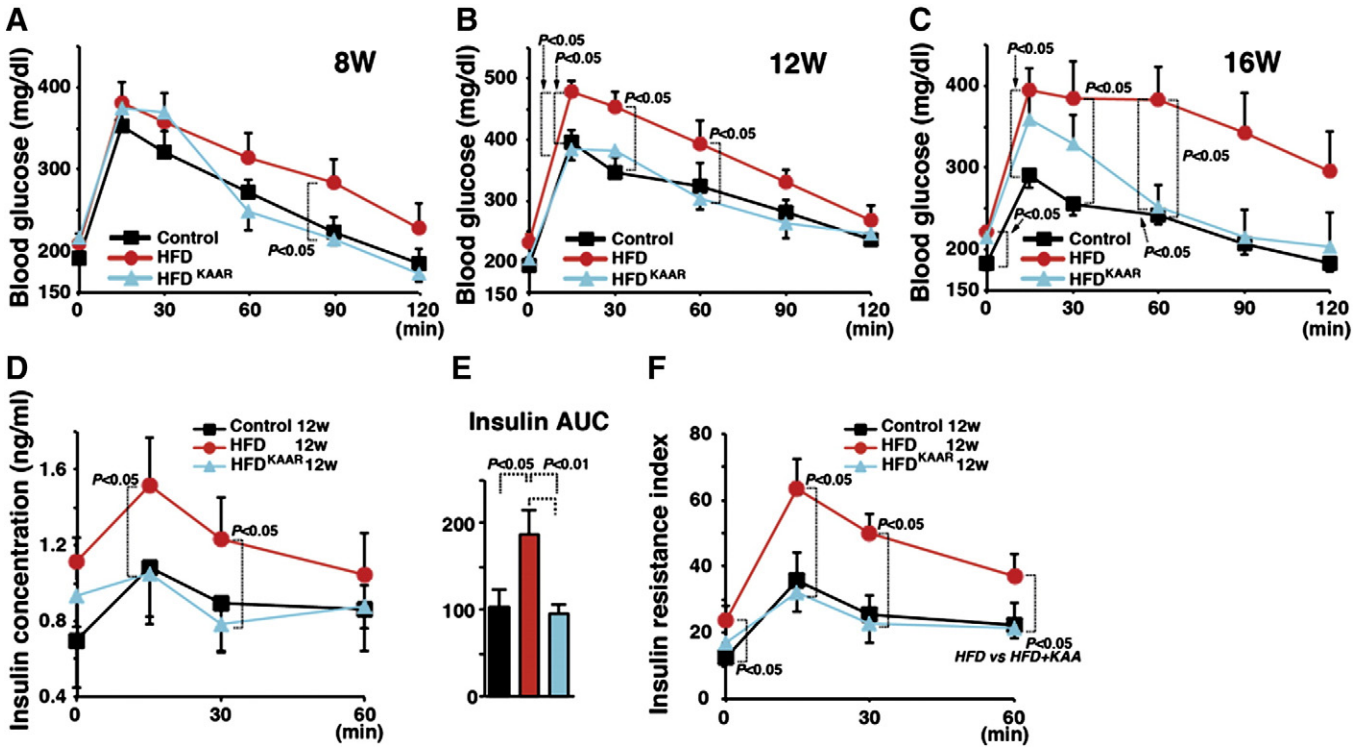

Fig. 2. Metabolic characteristic of mice. A–C. An intraperitoneal glucose tolerance test (IPGTT) at the indicated weeks after initiation of the experimental protocol. Mice were starved for 6 h starting at 8:30 AM, and subsequently, glucose (1 g/kg BW) was intraperitoneally injected. Blood glucose levels were monitored at the indicated times after glucose injection (n = 4 for each group). D. The insulin levels at the indicated time points during IPGTT. E. Area under the curve for insulin levels was analyzed in indicated groups. F. Insulin resistance indexes were evaluated as previously reported [24]. Data are expressed as the mean + s.e.m.

3.4. Liver autophagy induction by KAA replacement diet

A deficiency in autophagy has been implicated in metabolic defects in several organs, such as the liver, heart, kidney and muscles [4]. Upon analysis of GFP-LC3 mice, in which LC3-positive puncta formation can be visualized with fluorescence microscopy [25], the control diet-fed mice exhibited GFP-positive LC3-positive puncta formation in the liver (Fig. 4A) after 48 h of starvation. In contrast, the livers of HFD-fed mice displayed a significant suppression of LC3-positive puncta formation after 8 weeks of treatment with this diet (Fig. 4A, B), even with starvation. The HFD^{KAAR} diet-fed mice exhibited enhanced LC3-positive puncta formation compared to the HFD-fed mice (Fig. 4B, C). Similarly, the KAA replacement diet-fed mice exhibited increased LC3-positive puncta formation in the liver

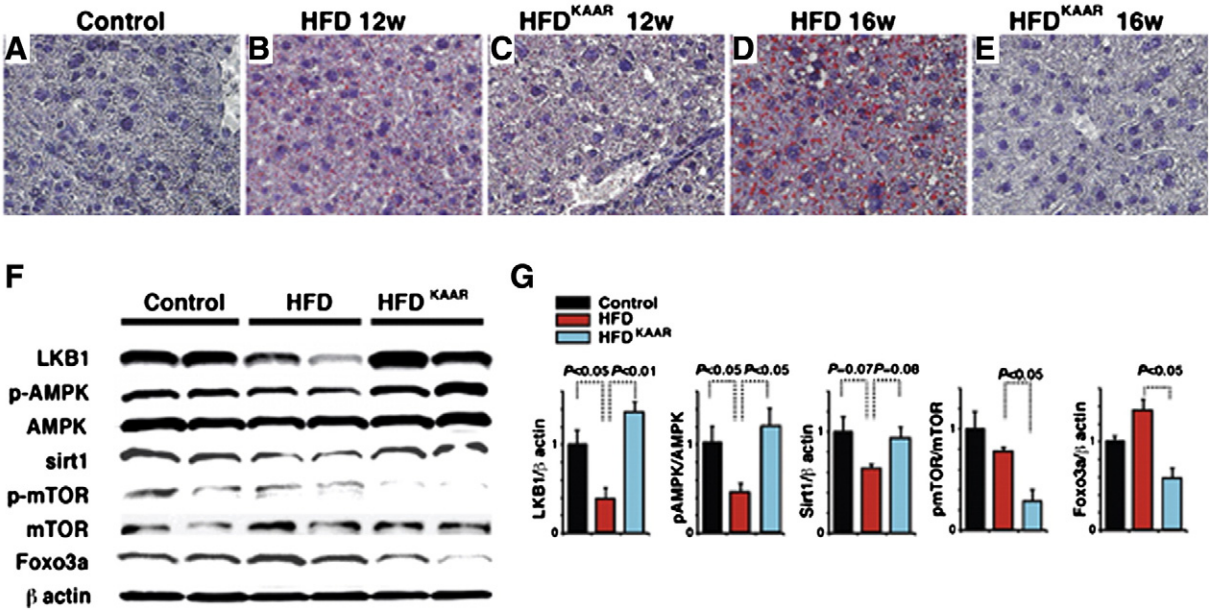

Fig. 3. Hepatic steatosis and nutrition-induced signal transduction in the liver. A–E. Oil Red O staining in the liver tissue. C57/Bl6 wild-type mice (8 weeks old) were fed a control diet, high-fat diet (HFD) or HFD with ketogenic amino acids (HFD^{KAAR}). Representative images (200×) are presented in the figure. At least four mice were analyzed in each group. F–G. The effects of the KAA diet on the nutrition-sensing pathway in the livers of the indicated groups of mice at 12 W (F) are shown. Representative data (n = 2) in each group are shown. G. Densitometric analysis of each protein expression normalized as indicated in figure. Data are shown as the mean ± s.e.m. in a graph (n = 4).

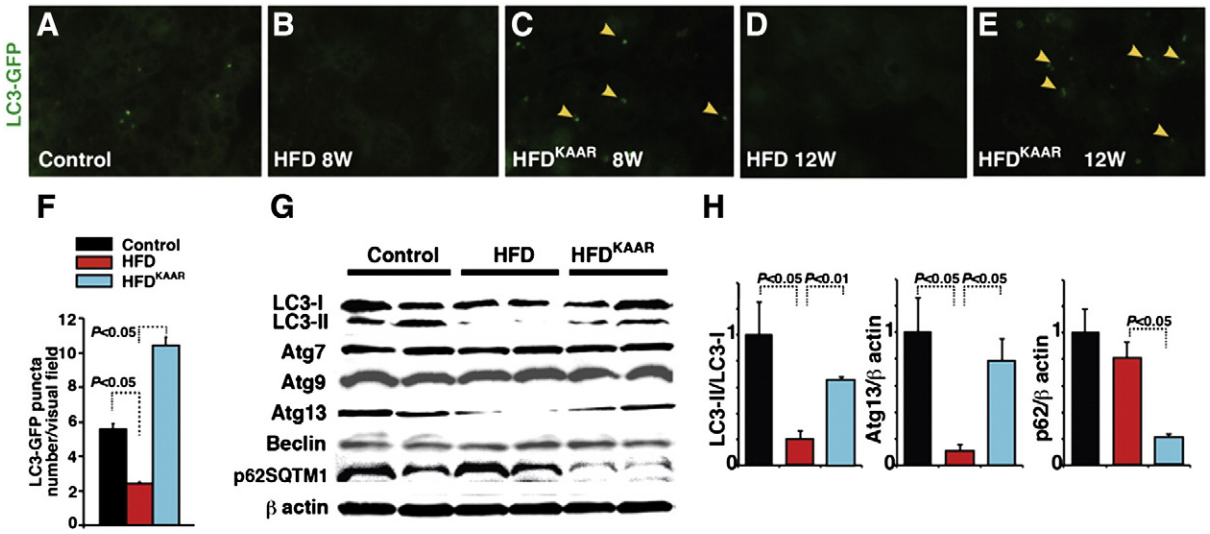

Fig. 4. KAA diet-induced autophagy in liver. A–E. LC3-GFP transgenic mice were fed a control diet, HFD or HFD^{KAAR}. Mice were euthanized at 8 or 12 weeks after the induction of the experimental protocol. After 48 h of starvation, the mice were perfused with fixative (4% paraformaldehyde in PBS) under general anesthesia, and the organs were harvested. After a 6-hour incubation in fixative, the fixed samples were transferred to a 30% sucrose solution in PBS for 24 h. Subsequently, the samples were placed in OCT mounting media, and frozen samples were sectioned at 5 μm. The sectioned samples were analyzed using fluorescence microscopy. LC-3 positive puncta formations were visualized by LC3-GFP-positive vacuoles (indicated by yellow arrowheads). F. Quantitative analysis of LC3-GFP-positive puncta formation in the liver. Liver sections (200×) were analyzed by fluorescence microscopy, and LC3-GFP puncta were counted in the visual field. In each group, 4 sections were analyzed. G. Autophagy-associated molecules analyzed by western blot analysis. C57/Bl6 wild type mice were used in this analysis. Representative data (n = 2) in each group are shown. H. Densitometric analysis of each protein expression normalized as indicated in figure. Data are shown as the mean ± s.e.m. in the graph (n = 4).

compared to the HFD-fed mice at 12 weeks after the initiation of the protocol (Fig. 4D, E, F). Livers of HFD-fed mice exhibited suppression of LC3-II and mTOR target protein Atg13, a component of an autophagy initiation factor, at 12 weeks (Fig. 4G, H). The ATG7, ATG9 and beclin protein levels exhibited no significant differences (Fig. 4G). Molecular defects associated with the autophagy system were also found in mice fed an HFD for 16 weeks (data not shown). The KAA diet-fed mice did not show the autophagy-related molecular defects, such as suppression of LC3-II and Atg13, observed in mice fed an HFD for 12 weeks (Fig. 4G, H). p62 levels were not altered the levels by HFD but KAA replacement diet decreased p62 levels in liver (Fig. 4G, H). These analyses suggested KAA-enhanced out-flux of autophagy.

3.5. KAA replacement diet inhibits HFD-induced macrophage accumulation in liver

Hepatic steatosis has been shown to associate with macrophage accumulation in the liver [26,27]. In our analysis, as expected, the HFD-fed mice exhibited enhanced macrophage accumulation in the liver compared to control diet-fed mice (Supplementary Fig. 1A, B, D). This macrophage accumulation was restored to normal levels in the KAA diet-fed mice (Supplementary Fig. 1B, C, D).

3.6. Intervention by KAA replacement diet cures established hepatosteatosis, defects in metabolism and autophagy, and macrophage accumulation in HFD-fed mice

Next, we investigated whether nutritional intervention with KAAs could reverse the metabolic abnormalities in established liver steatosis and glucose tolerance defects. After 8 weeks on an HFD diet, the mice exhibited liver steatosis (Fig. 5A). At 8 weeks after being fed the HFD, some of the mice were switched to an HFD^{KAAR} diet. A subsequent 8-week HFD^{KAAR} intervention (16 weeks total on HFD) resulted in mice that had a decreased body weight (Fig. 1A, B) and decreased liver and epididymal fat weights (Fig. 1C) compared to mice fed the HFD for 16 weeks. After 8 weeks of treatment with the KAA diet, hepatic steatosis was almost completely reversed compared to the established hepatic steatosis in mice fed an HFD for 8 weeks (Fig. 5A, B). Compared to the livers of mice on an HFD for 8 weeks, the KAA diet induced LKB1 and AMPK phosphorylation, which was associated with sirt1 protein induction and Foxo3a protein suppression, at both 4 and 8 weeks after treatment (Fig. 5C). These alterations in nutritional sensing pathway are associated with molecular defects of autophagy regulation, such as suppression of LC3-II, Atg13 and the accumulation of p62 (Fig. 5D). The KAA-replaced diet reversed the HFD-induced defects in nutritional-sensing and autophagy-regulated molecules (Fig. 5C, D). The amelioration of hepatic steatosis by a KAA-replacement diet was associated with some improvements in HFD-impaired glucose intolerance after 4 and 8 weeks of treatment with the KAA diet (Fig. 5E, F). Both insulin levels and IRI analysis exhibited suppressed trend in HFD^{KAAR} intervention group of mice (Fig. 5G, H) After 8 weeks, the HFD-fed mice exhibited enhanced macrophage accumulation in the liver (Supplementary Fig. 2A, D). This macrophage accumulation was decreased by the KAA-replacement diet after 8 weeks of treatment (Supplementary Fig. 2A, B, C, D).

3.7. Ketone bodies were not altered by KAA replacement diet

Ketone body levels in the plasma were similar in all groups' analysis (Supplementary Fig. 3A). When analyzed by LC–MS/MS analysis, ketone bodies such as β-hydroxybutylate (Supplementary Fig. 3B) and acetoacetate (Supplementary Fig. 3C), were not altered in KAA replacement diet, even though there was some trend of elevation in β-hydroxybutylate (Supplementary Fig. 3B).

4. Discussion

4.1. Obesity associated metabolic defects emerge important topic in medicine

The worldwide epidemic of obesity and associated metabolic defects reveals serious health problems. The medical costs for treating complications due to metabolic defects are a serious economic problem. Although modifications in lifestyle together with appropriate

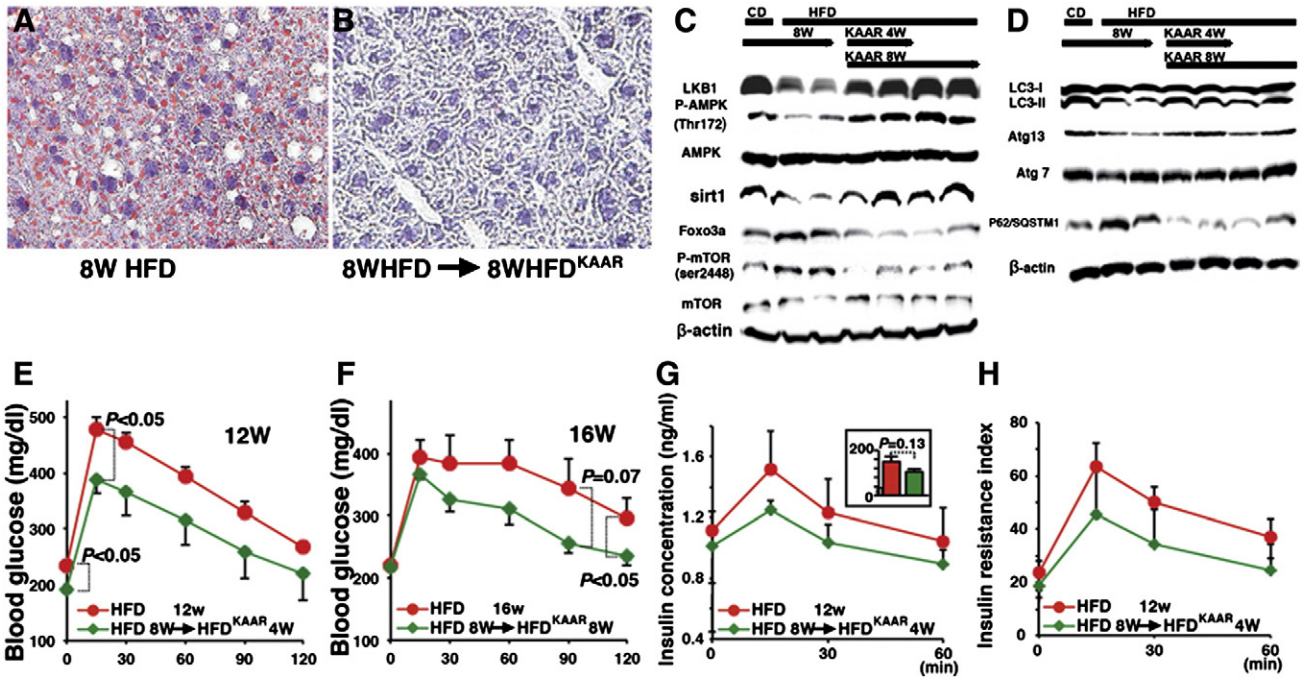

Fig. 5. The KAA diet reversed established hepatic steatosis and metabolic defects associated with enhanced autophagy. A, B. C57/Bl6 wild type mice were fed an HFD for 8 weeks. Mice were then switched to a, HFD^{KAAR} diet. Oil Red O staining indicated that an 8-week intervention with the KAA diet reverses the lipid accumulation in the liver. C. Nutritional-sensing pathways are ameliorated by a KAA diet. The KAA diet reversed the suppression of LKB1 and AMPK activity that was associated with sirt1 suppression and Foxo3a induction after treatment with an HFD for 8 weeks. The effect of the KAAs is most likely time-dependent. D. Autophagy-associated molecules. The KAA diet increased the LC3-II and Atg13 levels compared to mice treated with an HFD for 8 weeks. E, F. The effect of the KAA intervention on glucose tolerance. Mice treated with a high-fat diet for 8 weeks were divided into HFD or HFD^{KAAR} groups. At 4 or 8 weeks after the diet change to the KAA addition, IPGTT was performed again on the same mice. G. The insulin levels at the indicated time points during IPGTT. The inset in panel (G): area under the curve for insulin levels was analyzed in indicated groups. H. Insulin resistance indexes were evaluated as previously reported [24]. n = 4 or 5 in each group. Data are expressed as the mean + s.e.m.

exercise and a controlled diet could be essential therapies to address these metabolic defects, the strict control of these interventions coupled with an appropriate evaluation is challenging. Interventions for examining metabolic defects need to be easily performed and compliant for continuation. Therefore, a KAA replacement diet without alteration of total fat or calorie intake could be used to compare either the reduction in total food amount or fat consumption, suggesting the potential utility of KAA interventions for established metabolic diseases.

4.2. KAA diet is associated with amelioration of glucose tolerance in mice

A previous study revealed that branched-chain amino acids and their metabolic byproducts were linked to insulin resistance in humans and experimental animals [21]. In Newgard's seminal publication they compared the effect of HFD (kcal base: 45% fat, 35% carbohydrate, and 19% protein) vs. HFD + BCAA (kcal base: 43% fat, 34% carbohydrate, and 23% protein) in Wistar rats [21]; in our analysis we used identical calorie based recipe in either HFD or HFD^{KAAR} (kcal base: 53% fat, 29% carbohydrate, and 19% protein) in mice (Supplementary Table 2). We can find several important differences between Newgard's study and our current study. First of all, Newgard's study demonstrated that the HFD diet fed Wistar rats exhibited significantly heavier in body weight, whereas in our current study with C57/B6 mice we could not find any differences in body weight by HFD fed at least up to 12 weeks. These differences cannot be simply explained by the amount of food intake because animals ate about the similar calories between control and HFD fed in either Newgard's study or our current study. Another important point was that Newgard's study demonstrated the reduction of calorie intake by BCAA supplementation; therefore they performed the pair-fed study in which they again found BCAA-induced deterioration of glucose tolerance [21]. In our study basically we could not find any differences in food intake in all groups of prevention studies and the only tiny temporal reduction in food intake was found by HFD^{KAAR} in intervention study. Although the recipe of experimental food etc. looking similar, such differences found in the studies indicated that the experimental food of both studies did not induce the same metabolic alteration in the rats and mice studied, and that as a consequence of such differences, the biochemical and metabolic changes induced by the KAA supplementation may have been different from the finding by Newgard's work. It could be possible that the degree of caloric and/or HFD intake is a fundamental factor in determining the outcome of BCAA (or KAA) supplementation/replacement.

Regarding this finding, people with higher levels of branched-chain amino acids in their blood are much more likely to develop diabetes [20]. Gastric bypass surgery has been shown to decrease the levels of total amino acids and branched-chain amino acids in association with better glucose tolerance compared to dietary interventions in which the amino acid levels never changed [22]. Recently, however, Theytaz et al. reported that supplementation with essential amino acids blunts the deteriorated metabolic profiling during fructose overfeeding in healthy volunteers [28]. These reports are indeed contradictory to each other, although the causal role of branched-chain amino acids in the onset of insulin resistance remains elusive. Future research should be performed to answer such important discrepancy by appropriate diet feeding design.

4.3. Induction of autophagy associated with AMPK activation by KAA

Another important finding in our analysis was the amelioration of HFD-mediated autophagy defects by a KAA-replacement diet. The precise molecular mechanisms by which a KAA-replacement diet enhanced hepatic autophagy are not yet clear; however, it is possible that enhanced autophagy could induce degradation and a subsequent

rearrangement of cellular systems that are rich in damaged and old cellular components, thus potentially reducing harmful metabolic effects on the maintenance of cellular homeostasis [4,29]. Autophagy has been shown to be essential in liver protection against alcoholic or non-alcoholic liver injury [14,15]. In our analysis, a KAA-replaced diet increased the LKB1 levels and subsequent phosphorylation of AMPK, which is a master regulator and sensor of cellular energy [30], even with the HFD treatment. Enhanced AMPK activation and suppression of mTOR have been shown to be associated with an induction of autophagy [31,32] and organ protection [33–36]. Indeed Atg13, one of the autophagy initiation factor and target of mTOR, was induced in KAA diet, suggesting that a KAA diet can induce autophagy, normalization of hepatic histology and restoration of metabolic disease states.

5. Conclusion

We conclude that a KAA replacement diet could be a potential nutritional intervention for treatment in patients with metabolic defects, such as hepatic steatosis, glucose intolerance and metabolic syndrome, via a normalization of the nutritional signal responses and associated autophagy defects. The effect of a KAA-replacement diet can be confirmed in mice with established metabolism defects. Our analysis enables us to establish an evidence-based nutritional intervention for patients with devastating metabolic defects, which result in associated health problems worldwide.

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Authors' contributions

L.X. and M.K.: contributed equally to performing the experiments, analyzing the data and writing/editing the manuscript. J.H.: performed some experiments. M.K.: participated in discussions. K.N., H.J. and Y. N.: performed some experiments and provided intellectual input. H.M.: participated in discussions and provided intellectual input. K.K.: conceived the project, provided intellectual contribution, and mainly contributed to the manuscript writing and editing. D.K.: as principle investigator, conceptually designed the strategy for this study, participated in discussions, provided intellectual input, supervised the studies and performed the final edit on the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2013.05.003.

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