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Research Paper

Branched Chain Amino Acids Cause Liver Injury in Obese/Diabetic Mice by Promoting Adipocyte Lipolysis and Inhibiting Hepatic Autophagy

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ABSTRACT

The Western meat-rich diet is both high in protein and fat. Although the hazardous effect of a high fat diet (HFD) upon liver structure and function is well recognized, whether the co-presence of high protein intake contributes to, or protects against, HF-induced hepatic injury remains unclear. Increased intake of branched chain amino acids (BCAA, essential amino acids compromising 20% of total protein intake) reduces body weight. However, elevated circulating BCAA is associated with non-alcoholic fatty liver disease and injury. The mechanisms responsible for this quandary remain unknown; the role of BCAA in HF-induced liver injury is unclear. Utilizing HFD or HFD + BCAA models, we demonstrated BCAA supplementation attenuated HFD-induced weight gain, decreased fat mass, activated mammalian target of rapamycin (mTOR), inhibited hepatic lipogenic enzymes, and reduced hepatic triglyceride content. However, BCAA caused significant hepatic damage in HFD mice, evidenced by exacerbated hepatic oxidative stress, increased hepatic apoptosis, and elevated circulation hepatic enzymes. Compared to solely HFD-fed animals, plasma levels of free fatty acids (FFA) in the HFD + BCAA group are significantly further increased, due largely to AMPKα2-mediated adipocyte lipolysis. Lipolysis inhibition normalized plasma FFA levels, and improved insulin sensitivity. Surprisingly, blocking lipolysis failed to abolish BCAA-induced liver injury. Mechanistically, hepatic mTOR activation by BCAA inhibited lipid-induced hepatic autophagy, increased hepatic apoptosis, blocked hepatic FFA/triglyceride conversion, and increased hepatocyte susceptibility to FFA-mediated lipotoxicity. These data demonstrated that BCAA reduces HFD-induced body weight, at the expense of abnormal lipolysis and hyperlipidemia, causing hepatic lipotoxicity. Furthermore, BCAA directly exacerbate hepatic lipotoxicity by reducing lipogenesis and inhibiting autophagy in the hepatocyte.

Abbreviations: β-AR, β-adrenergic receptor; 4-HNE, 4-hydroxynonenal; ACC, acetyl-coA carboxylase; ALT, alanine aminotransferase; AMPK, adenosine monophosphate-activated protein kinase; ANOVA, analysis of variance; AST, aspartate transaminase; ATGL, adipose triglyceride lipase; BCAA, branched chain amino acids; BCKA, branched chain α-ketoacids; BCKD, branched-chain α-ketoacid dehydrogenase; BDK, branched-chain α-ketoacid dehydrogenase kinase; BSA, bovine serum albumin; cAMP, cyclic adenosine monophosphate; DG, diacylglycerol; DGAT1, diacylglycerol acyltransferase-1; ELOVL6, elongation of very long chain fatty acids protein-6; FASN, fatty acid synthase; FFA, free fatty acids; GFP-LC3, green fluorescent protein-light chain-3; GTT, glucose tolerance test; HE, hematoxylin-eosin; HFD, high fat diet; HOMA-IR, homeostasis model assessment of insulin resistance; HPLC, high performance liquid chromatography; HSL, hormone sensitive lipase; IL-1β, interleukin-1β; IL-6, interleukin-6; IP, intraperitoneal injection; IRS1, insulin receptor substrate-1; ISO, isoprenaline; ITT, insulin tolerance test; IU, international unit; MCP-1, monocyte chemotactic protein-1; MDA, malondialdehyde; mTOR, mammalian target of rapamycin; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; ND, normal diet; OA, oleic acid; PKA, protein kinase A; PP2Cm, protein phosphatase-2Cm; ROS, reactive oxygen species; SCD1, stearoyl-CoA desaturase-1; SEM, standard error of the mean; siRNA, small interfering RNA; SOD, superoxide dismutase; SREBP-1c, sterol regulatory element binding protein-1c; TG, triglyceride; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; TUNEL, terminal deoxynucleotidyl transferased UTP nick end labeling.

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1. Introduction

With the prevalence of obesity, non-alcoholic fatty liver disease (NAFLD) has become the most common chronic liver disease in the world (Angulo, 2002). Although most patients are asymptomatic, NAFLD may progress to non-alcoholic steatohepatitis (NASH), a predisposing condition of liver cirrhosis, end-stage liver disease, and hepatocellular carcinoma (Angulo, 2002). Unfortunately, as there are no specific and effective therapies for NASH, both better understanding of the molecular mechanisms underlying NAFLD and efficacious therapies are in great need.

Excessive carbohydrate intake is causatively linked to obesity/diabetes. Low-carbohydrate, high-fat/high protein diets like Atkins diet are often recommended to the obesity patients to promote weight loss (Astrup et al., 2004). More importantly, typical western meat-rich foods contain both high protein and high fat (Heidemann et al., 2008; Cordain et al., 2005). Although the hazardous effect of high fat upon hepatic structure/function is well-recognized, the impact of concomitant high protein intake upon HF-induced liver injury remains unclear (de Wit et al., 2012). Branched chain amino acids (BCAA, including leucine, isoleucine, and valine) are a group of essential amino acids. Relatively abundant in food, they account for 20% of total protein intake (Harris et al., 2005). Part of the high-protein diet often recommended for obese patients, BCAA intake reduces body weight (Hutson et al., 2005). However, recent studies demonstrate elevated circulating BCAA are strongly associated with NAFLD-related metabolic disorders, such as obesity, metabolic syndrome, and type 2 diabetes mellitus (Lynch and Adams, 2014). Moreover, as opposed to patients with simple fatty liver, hepatic BCAA accumulation is a signature metabolic finding in patients with steatohepatitis (Lake et al., 2015). Finally, downregulated expression of hepatic BCAA-degrading enzymes is also a hallmark of non-alcoholic fatty liver (Lake et al., 2015; Mardinoglu et al., 2014). Together, these clinical studies strongly suggest BCAA intake may have negative impact upon liver structure/function, particularly in obesity. Mechanisms responsible for this quandary (how BCAA induces weight-loss but damages the liver) remain unknown. Moreover, whether elevated circulatory BCAA plays a causative role in the liver injury observed in NAFLD patients has not been determined.

Emerging evidence demonstrates free fatty acids (FFA) and their metabolites play a critical role in the pathogenesis of NASH (Neuschwander-Tetri, 2010). Excess hepatic FFA accumulation results in increased oxidative stress, lipid peroxidation, and hepatocellular apoptosis, leading to NASH development (Fuchs and Sanyal, 2012). These FFA-mediated hepatotoxic effects are termed hepatic lipotoxicity. Uptake from adipocyte-released FFA in circulation and de novo synthesis by hepatocytes are the two most significant sources of hepatic FFA (Fuchs and Sanyal, 2012). As such, adipocyte lipolysis inhibition and hepatic lipogenic suppression are effective interventions attenuating hepatic FFA accumulation and resultant liver injury. The effect of BCAA supplementation upon adipocyte lipolysis, hepatic lipogenesis, and ultimately hepatic FFA accumulation (particularly during obesity or diabetes) remains unknown.

Utilizing high-fat-diet (HFD) and HFD + BCAA models, the current study has three specific aims: 1) to determine whether BCAA contributes to or protects against HF-induced liver injury; 2) to determine whether BCAA may have direct negative impact upon liver structure/function in HFD-induced obese animals; and 3) if so, clarify the responsible underlying molecular mechanisms.

2. Methods and Materials

2.1. Animals, Diets, and Treatments

All study protocols were approved by the Animal Care and Use Committee of the Fourth Military Medical University, and strictly followed guidelines regarding the humane use and care of laboratory animals for biomedical research published by the National Institutes of Health (No. 85-23, revised 1996). Adult male wild-type C57BL6J mice (aged from 8 to 10 weeks) and green fluorescent protein-light chain-3 (GFP-LC3) transgenic C57BL6J mice (aged from 8 to 10 weeks) were purchased from the Vital River Company, China. There was no statistically difference in the animal age between respective groups. During the study duration, animals were maintained in a temperature-controlled barrier facility at 26 °C with a 12 hour-light/dark cycle. Both wild-type and GFP-LC3 transgenic mice were randomly divided into four groups: normal diet (ND) group, ND + BCAA group, high fat diet (HFD) group, and the HFD + BCAA group. The HFD (60% of kcal fat, D12492) and ND (10% of kcal fat, D12450) were purchased from Research Diets Inc. (USA). The BCAA mixture (L-leucine 25 g, L-isoleucine 12.5 g, and L-valine 12.5 g) was added into 1 L drinking water. All the mice had unrestricted access to food and water. These diets were fed for 12 weeks. During this period, food and water intake were monitored bi-weekly. Acipimox (10 mg/kg body weight, Selleck Inc., China) was administered daily by intraperitoneal (IP) injection. Rapamycin (1 mg/kg body weight, Selleck Inc., China) was given IP every other day. After overnight fasting (from 9 pm to 9 am next morning), animals were anesthetized with 2% isoflurane inhalation and sacrificed by cutting the carotid artery. After animal sacrifice, the blood, epididymal white fat, liver, and gastrocnemius muscle were collected and used for the following biological analyses.

2.2. In Vitro and In Vivo Lipolysis Assay

In cultured 3T3-L1 cell-derived adipocytes, the lipolysis assay was performed as previously described (Wu et al., 2015). Briefly, adipocytes were incubated and were treated with or without insulin (10 ng/ml) for 10 min, followed by 90 min of stimulation with 10 μM isoproterenol (ISO). Subsequently, 1 ml of the incubation medium of adipocytes was removed and acidified with 100 μl 30% trichloroacetic acid. The mixture was carefully vortexed and was centrifuged at 3000*g* for 10 min at 4 °C. 100 μl supernatant was collected and was neutralized with 10 μl 10% KOH, and tested for glycerol concentration (nM glycerol released per mg protein) via commercial glycerol assay kit (Biovision, USA). For in vivo lipolysis, mice were fasted for 3 h and subjected to specific β3-adrenergic receptor agonist CL-316243 (0.1 mg/kg body weight, Sigma, US) IP 5 min after insulin (0.75 IU/kg body weight, Novo Nordisk, Sweden) or saline injection. Blood samples were obtained via tail vein 0, 30, 60, and 120 min after CL-316243 injection to determine FFA and glycerol levels.

2.3. GFP-LC3 Droplets Detection

After 12 week-diet intervention, male GFP-LC3 transgenic mice were sacrificed. Livers were harvested. Frozen hepatic sections were prepared, and subjected to confocal microscopy (Olympus, Japan) at 588 nm by argon laser at <2% of full power, described previously in detail (Kim and Lemasters, 2011). All images were captured and were analyzed via Image-Plus software.

2.4. Leucine Tolerance Test

As described by Lu et al., briefly, overnight fasted mice were subjected to IP leucine solution (150 mM, dosed 15 μl/g body weight) (Lu et al., 2009). Blood samples were obtained via tail vein 0, 1, 2, and 4 h after leucine administration. Plasma BCAA levels were measured.

2.5. Lean and Fat Mass Calculation

The body fat mass was calculated as previously described (D'Antona et al., 2010). Briefly, the total epididymal, perirenal, mesentery, and subcutaneous fat pads were carefully dissected, collected, and weighted. The lean body mass equals body weight minus body fat mass.

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2.6. BCKD Complex Activity Assay

Branched-chain α-ketoacid dehydrogenase (BCKD) complex actual activity was measured by spectrophotometric assay, as described in detail previously (Lian et al., 2015). Briefly, BCKD complex was isolated from tissue extracts using 9% polyethylene glycol. BCKD enzyme activity was determined spectrophotometrically by measuring the rate of NADH production resulting from the conversion of α-keto-isovalerate into isobutyryl-CoA. A unit of enzyme activity was defined as 1 μmol NADH formation per minute at 37 °C.

2.7. Statistical Analyses

All data are presented as mean + standard error of the mean (SEM). The student's *t*-test with a two-tail distribution tested statistical significance for two groups. For more than two groups, two-way ANOVA followed by post-hoc analysis was performed for analyzing two parameters. GraphPad Prism software was utilized. P values < 0.05 were considered statistically significant.

A further description of employed materials and methods is provided in "Supplementary Materials".

3. Results

3.1. BCAA Supplementation in HFD-Induced Obesity/Diabetic Mice Reduced Fat Mass and Body Weight, and Suppressed Hepatic De Novo Lipogenesis

The catabolism of BCAA is tightly regulated in vivo by the BCKD complex (Harris et al., 2005). In normal diet (ND) mice, plasma levels of BCAA and its downstream metabolite BCKA were low, as dietary BCAA was rapidly degraded as determined by leucine tolerance test (Fig. 1A–C). However, in high-fat diet (HFD)-induced obese mice, plasma BCAA and BCKA levels were significantly elevated (Fig. 1A and B). The leucine tolerance test consistently demonstrated that BCAA clearance capacity was significantly impaired in HFD-fed mice (Fig. 1B) which might be a main contributor to increased BCAA accumulation in HFD-induced obesity/diabetic mice. BCAA administration significantly increased daily consumptions of leucine, isoleucine, and valine (Supplementary Table 3). BCAA supplementation had no significant effect upon plasma BCAA in ND mice. However, supplementation of BCAA atop HFD significantly further increased plasma BCAA levels, including leucine/isoleucine and valine (Fig. 1C and Supplementary Fig. 3). Mechanistically, adipose and liver BCKD complex activity (the rate-limiting enzyme catalyzing BCAA catabolism) is significantly decreased (see Supplementary Fig. 1A and B) due to increased phosphorylation of the BCKD E1α subunit at serine^{293} (Lu et al., 2009) in HFD animals (see Supplementary Fig. 1D and E). However, HFD had no significant effect upon BCKD phosphorylation or activity in skeletal muscle (see Supplementary Fig. 1C and F). Taken together, these data indicated that HFD inhibited BCAA catabolism in the liver and adipose tissue, increased circulating BCAA abundance, and further exacerbated BCAA accumulation when BCAA was supplemented atop HFD.

Because BCAA supplementation alone (ND + BCAA) had no significant effect upon plasma BCAA levels (Fig. 1C) and, more importantly, long-term BCAA supplementation alone have no significant effects on metabolic parameters (such as body weight, glucose homeostasis, and blood lipids) and liver function and structure in ND group (see Supplementary Fig. 2), all subsequent experimental results were thus

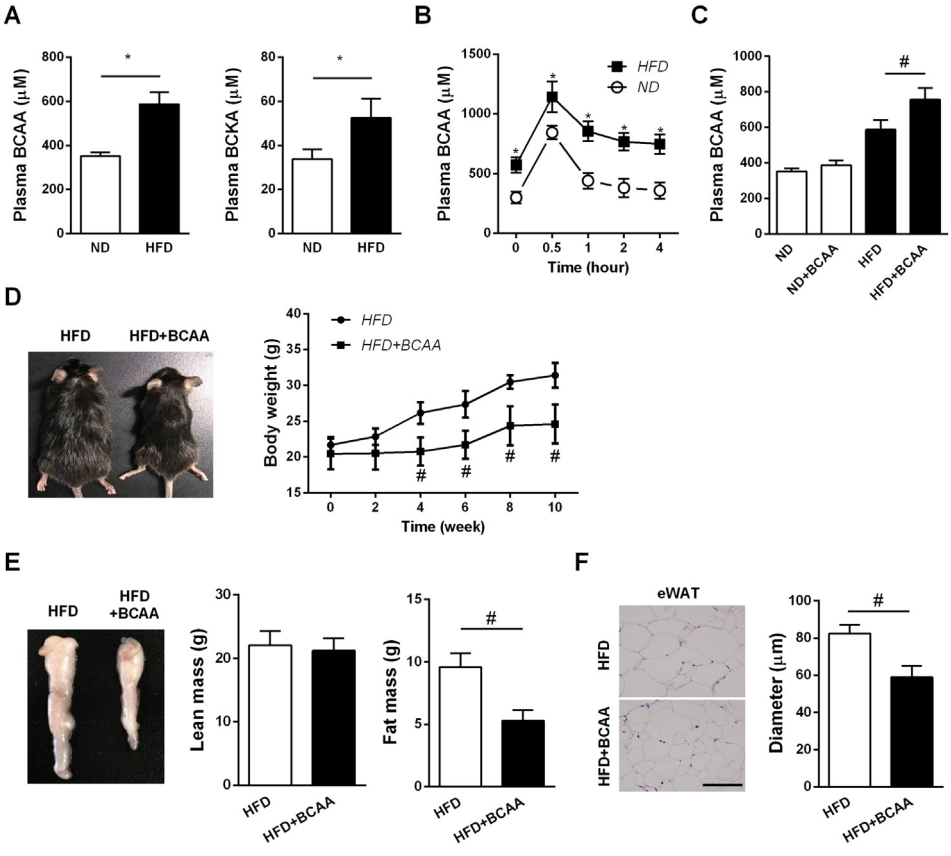

Fig. 1. BCAA supplementation reduced fat mass and body weight gain in HFD-induced obese/diabetic mice. (A–B) Adult male C57BL6J mice were fed 12 weeks of normal diet (ND) or high-fat diet (HFD). (A) Plasma BCAA was determined via commercial kit and plasma BCKA was determined via high performance liquid chromatography (HPLC). (B) Leucine tolerance test results in ND and HFD mice. (C to F) ND and HFD-fed mice were then orally administered BCAA (ND + BCAA, HFD + BCAA) or vehicle (ND, HFD) for 12 weeks. (C) Quantification of plasma BCAA. (D) Body weight was determined every two weeks. (E) Lean and fat mass, and (F) adipocyte size were determined. n = 8–10 per group. *P < 0.05 versus ND; #P < 0.05 versus HFD. Data presented as mean + SEM.

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presented in HFD and HFD + BCAA animals to determine the effects of BCAA supplementation during HFD-induced obesity/diabetic conditions. To clarify whether increased circulating BCAA contributes to or protects against HF-induced metabolic disorders, the effects of BCAA supplementation upon body weight, fat mass, and liver lipogenesis were determined. Compared to the HFD group, the HFD + BCAA group consumed equal water, but moderately decreased food quantity (see Supplementary Fig. 4A and B). Moreover, BCAA supplementation attenuated HFD-induced weight gain (Fig. 1D), decreased fat mass (Fig. 1E), and reduced adipocyte cell diameter (Fig. 1F).

Increased hepatic de novo lipogenesis and resultant ectopic lipid deposition contributes to liver injury in obese individuals (Donnelly et al., 2005). Suppression of hepatic lipogenesis improves metabolic profile and insulin sensitivity (Postic and Girard, 2008). HFD caused significant hepatic lipid accumulation as evidenced by large lipid droplets and elevated liver triglyceride (TG) content (Fig. 2A). Interestingly, BCAA supplementation atop HFD significantly reduced abundance of large hepatic lipid droplets and liver TG content (Fig. 2A). Because sterol regulator element binding protein-1c (SREBP-1c) is the master regulator of hepatic lipogenic program (Neuschwander-Tetri, 2010), we next determined the effect of BACC upon HFD-induced SREBP-1c expression. As expected, hepatic expression level of SREBP-1c [full-length SREBP-1c: SREBP-1c (fl); partial SREBP-1c: SREBP-1c (p)] was significantly elevated in HFD mice (Fig. 2B). Consistent with prior observations (reduced large lipid droplets and reduced hepatic TG content), BCAA supplementation significantly inhibited SREBP-1c expression (Fig. 2B). Consequently, the mRNA expression levels of SREBP-1c targeted genes related to de novo lipogenesis, including acetyl-coA carboxylase-1 (ACC1), fatty acid synthesis (FASN), stearoyl-CoA desaturase-1 (SCD1), elongation of very long chain fatty acids protein-6 (ELOVL6), and diacylglycerol acyltransferase-1 (DGAT1) were all suppressed by BCAA supplementation (Fig. 2C). Taken together, these data demonstrated BCAA blocked hepatic lipogenic responses during HFD conditions.

Recent studies have demonstrated that the mammalian target of rapamycin (mTOR) activation plays a critical role in BCAA biological functions (Lynch and Adams, 2014). Moreover, persistent activation of hepatic mTOR signaling reduces AKT activity, subsequently inhibiting SREBP-1c expression and lipogenic responses under HFD conditions (Porstmann et al., 2008; Yecies et al., 2011). To determine whether BCAA inhibition of SREBP-1c expression and lipogenic responses in HFD conditions is mediated by mTOR/AKT signaling, additional in vivo and in vitro experiments were performed. In vivo BCAA supplementation significantly increased mTOR phosphorylation and inhibited AKT phosphorylation (Fig. 2B). Co-treatment with rapamycin (RAPA) inhibited BCAA-induced mTOR phosphorylation, increased AKT phosphorylation, stimulated SREBP-1c expression (Fig. 2B), enhanced lipogenic enzyme expression (Fig. 2C), and increased both large lipid droplet abundance and hepatic TG content (Fig. 2A). To further determine whether the lipogenic inhibitory effect of in vivo BCAA and its involved signaling mechanisms occur directly in hepatic cells, in vitro experiments were performed in cultured primary hepatocytes isolated from C57BL6J mice. BCAA administration significantly blocked oleic acid (OA)-mediated AKT activation and subsequent SREBP-1c expression, all of which were restored by rapamycin co-treatment (Fig. 2D). Consistently, BCAA suppressed OA-induced TG accumulation, an effect reversed by RAPA and mimicked by AKT inhibition (Fig. 2E). Collectively, the experimental results presented in Figs. 1 and 2 demonstrated BCAA reduced body weight and inhibited hepatic de novo lipogenesis in HFD-induced obese animals, suggesting a beneficial metabolic impact.

3.2. BCAA Supplementation Caused Significant Liver Damage in HFD Mice

Having demonstrated the systemic and hepatic metabolic effects of BCAA supplementation, we next determined the effects of BCAA upon HFD-induced liver injury. Compared with plasma levels of ALT (~50 IU/L) and AST (~100 IU/L) in ND group (Supplementary Fig. 2),

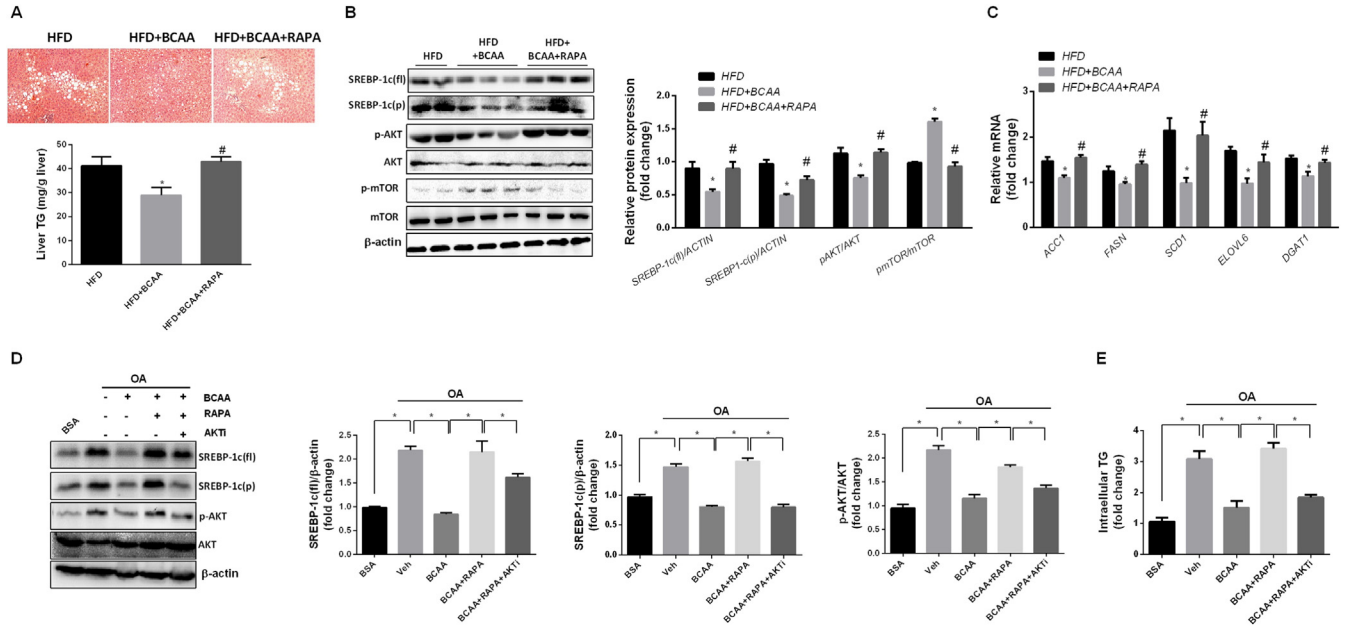

Fig. 2. BCAA supplementation suppressed hepatic de novo lipogenesis in HFD-induced obese/diabetic mice via mTOR signaling. (A–C) Adult C57BL6J mice were fed on high-fat diet (HFD), high-fat plus BCAA diet (HFD + BCAA), or rapamycin (RAPA, 1 mg/kg body weight intraperitoneal injection, every other day) atop HFD + BCAA (HFD + BCAA + RAPA) for 12 weeks. (A) Upper: HE staining in liver sections (n = 10 per group). Lower: hepatic TG content (n = 10 per group). (B) Expression of hepatic SREBP-1c (full length, fl), SREBP-1c (partial, p), p-AKT, AKT, p-mTOR, and mTOR by Western blot (n = 6 per group). (C) Expression of hepatic ACC1, FASN, SCD1, ELOVL6, and DGAT1 mRNA by real-time PCR (n = 6 per group). For (A, B, and C): *P < 0.05 versus HFD and #P < 0.05 versus HFD + BCAA. Data presented as mean + SEM. (D–E) Cultured primary hepatocytes were treated with bovine serum albumin (BSA) or oleic acid (OA, 1 mM) for 12 h. Effects of BCAA (5 mM), rapamycin (RAPA, 1 μM), and AKT inhibitor (AKTi, 10 μM) co-treatment upon hepatocyte lipogenesis were determined. (D) Expression of SREBP-1c (fl), SREBP-1c (p), and p-AKT/AKT ratio by Western blot. Left panel: representative blots; right three panels: quantification data. (E) Intracellular TG content, as measured via commercial kit. For (D and E): n = 4 per group. *P < 0.05. Data presented as mean + SEM.

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long-term HFD caused a mild elevation of plasma ALT (~100 IU/L) and AST (~150 IU/L), suggesting that HFD induced lipotoxic liver damage in mice (Fig. 3A). Somewhat to our surprise, plasma AST and ALT were significantly elevated when BCAA was concomitantly given atop HFD (Fig. 3A). Hepatic inflammation is a hallmark of HFD-induced fatty liver damage (Angulo, 2002). As shown in Fig. 3B, compared with HFD group, BCAA supplementation significantly upregulated the expression of proinflammatory cytokines (TNF-α, IL-6, IL-1β, and MCP-1), strongly demonstrating that BCAA exacerbated NAFLD-related hepatic inflammation. Taken together, these data suggest BCAA supplementation exacerbated HFD-induced liver structure/function injury, divergent from the apparent beneficial metabolic effects presented in Figs. 1 and 2.

Additional studies were conducted to identify the underlying mechanisms responsible for this contradictory effect. Given that FFA-mediated lipotoxicity is a common cause of liver damage in HFD conditions, it was plausible the hepatic pathological changes observed in BCAA supplemented mice were due to hepatic FFA accumulation and resultant lipotoxicity. Indeed, our results demonstrated BCAA supplementation significantly increased hepatic FFA content (Fig. 3C). To further determine how increased FFA may cause liver damage, we determined the effect of BCAA supplementation upon lipid peroxidation and hepatic apoptotic cell death, two well-defined pathways mediating FFA lipotoxicity (Neuschwander-Tetri, 2010). BCAA supplementation significantly increased lipid peroxidation, evidenced by increased malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) (Fig. 3D). Moreover, compared to the HFD group, the HFD + BACC group exhibited significantly increased hepatic apoptosis, evidenced by TUNEL staining (Fig. 3E) and cleaved caspase-3 (Fig. 3F). Taken together, these data demonstrated BCAA supplementation atop HFD increased hepatic FFA accumulation and lipid peroxidation, damaging liver structure and function.

3.3. BCAA Supplementation Aggravated HFD-Induced Hyperlipidemia Due to AMPKα2-Mediated Adipocyte Lipolysis

The above results demonstrated that, despite suppression of de novo hepatic lipogenesis by BCAA supplementation atop HFD, hepatic FFA accumulation increases with consequent liver injury. Fig. 1 demonstrated BCAA supplementation significantly reduced fat mass, the major source of circulating lipids. Together, these results suggest increased hepatic FFA accumulation is likely a consequence of increased adipose lipolysis with resultant hyperlipidemia. To obtain direct evidence supporting this notion, in vivo and in vitro experiments were performed. First, BCAA supplementation significantly aggravated HFD-induced hyperlipidemia, indicated by increased circulating triglycerides (TG) and FFA levels (Fig. 4A). Second, although BCAA did not influence lipolysis induced by CL-316243 (β3-adrenergic receptor agonist), the inhibitory effects of insulin upon CL-316243-induced lipolysis were significantly impaired in the BCAA group, as evidenced by increased FFA and glycerol release (Fig. 4B + insulin). Third, isoprenaline (ISO) caused significant lipolysis in cultured adipocytes, in both the presence and absence of BCAA (Fig. 4C, middle two bars). Insulin significantly attenuated ISO-induced lipolysis in control adipocytes. However, this inhibitory effect was significantly blunted by BCAA (Fig. 4C, last two bars). Finally, BCAA supplementation resulted in significant whole body insulin resistance, as evidenced by increased plasma insulin levels during the glucose tolerance test (GTT, Fig. 4D), attenuated the glucose-countering effect of insulin during the insulin tolerance test (ITT, Fig. 4E), and increased the HOMA-IR index (Fig. 4F). Taken together, these in vivo and in vitro data suggest that BCAA reduced adiposity, increased FFA release, and exacerbated hyperlipidemia, potentially through blocking the inhibitory effects of insulin on adipocyte lipolysis.

As observed in Fig. 4B/C, BCAA itself failed to affect β-AR-induced adipocyte lipolysis in vivo and in vitro; however, BCAA significantly promoted lipolysis through reducing insulin-mediated anti-lipolytic effects in the adipocyte. Next, we determined the molecular mechanisms to explain how BCAA influenced insulin-mediated anti-lipolytic effects in cultured adipocytes. As we have demonstrated BCAA inhibited hepatic lipogenesis via mTOR activation (Fig. 2), we first determined the role of mTOR in BCAA-induced adipocyte lipolysis. BCAA significantly activated adipocyte mTOR signaling, an effect abolished by rapamycin (Fig. 5A). However, because rapamycin failed to inhibit BCAA-enhanced lipolysis upon insulin stimulation (Fig. 5A), BCAA likely promotes adipocyte insulin resistant-lipolysis in an mTOR-independent fashion. Recently, it was demonstrated nicotine promotes adipocyte lipolysis via reactive oxygen species (ROS)-mediated AMP-activated protein kinase-α2 (AMPKα2) activation (Wu et al., 2015). As we have demonstrated BCAA supplementation caused significant superoxide

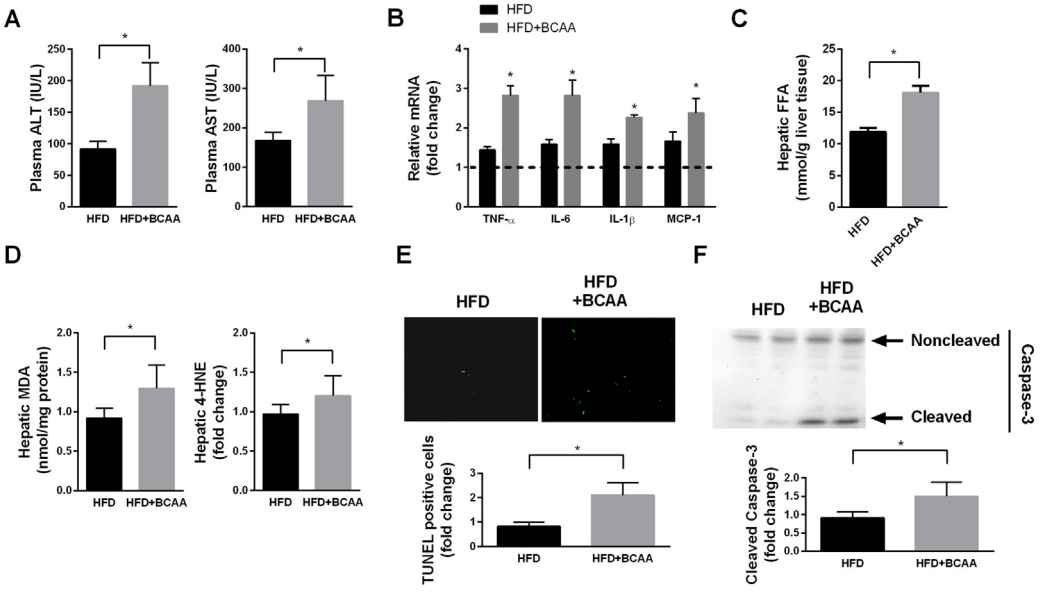

Fig. 3. BCAA caused significant liver damage in HFD mice. (A) Plasma ALT and AST were measured in HFD and HFD + BCAA group (n = 10 per group). (B) Hepatic proinflammatory cytokines mRNA levels were determined, which was compared to normal diet (dotted line), n = 10 per group. (C) Hepatic FFA content in HFD and HFD + BCAA group (n = 6 per group). (D) Hepatic MDA and 4-HNE (two common indices of lipid peroxidation, n = 8 per group). (E) Hepatic apoptosis determined by TUNEL staining (n = 8 per group). (F) Hepatic cleaved and non-cleaved caspase-3 expression levels determined by Western blot (n = 6 per group). *P < 0.05 versus HFD. Data presented as mean + SEM.

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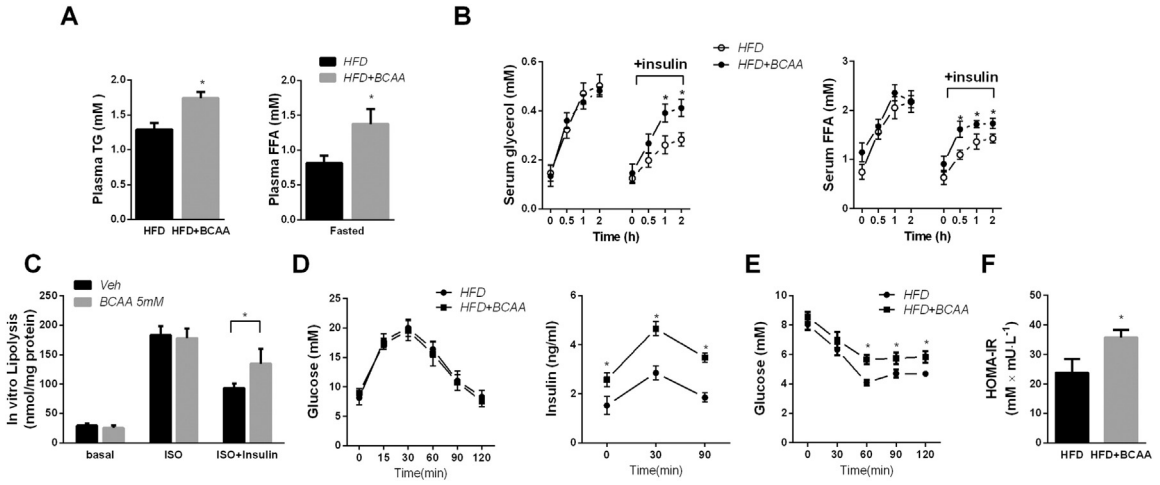

Fig. 4. BCAA exacerbated HFD-induced hyperlipidemia and insulin resistance, due to aberrant lipolysis. HFD-fed mice were given BCAA daily for 12 weeks. (A) After 6 hour-fasting, plasma TG and FFA levels were determined. (B) In vivo lipolysis was determined in HFD and HFD + BCAA group. n = 6 per group. (C) In vitro lipolysis was performed in cultured adipocytes with or without BCAA (5 mM) co-treatment for 6 h per Methods. n = 6 per group. (D to F) After 6 hour-fasting, glucose homeostasis was determined in HFD and HFD + BCAA mice. (D) Glucose tolerance test (GTT) and insulin release during GTT. (E) Insulin tolerance test (ITT). (F) HOMA-IR indexes, calculated after GTT and ITT. n = 6 per group. *P < 0.05. Data presented as mean + SEM.

accumulation (Supplementary Fig. 4C), superoxide dismutase (SOD) inactivation (Supplementary Fig. 4D), and lipid peroxidation (Fig. 3D), we hypothesized BCAA-enhanced adipocyte lipolysis may also involve AMPKα2 activation. Several in vivo and in vitro lines of evidence strongly support this notion. First, in vivo BCAA supplementation atop HFD significantly increased phosphorylation levels of AMPK and acetyl-coA

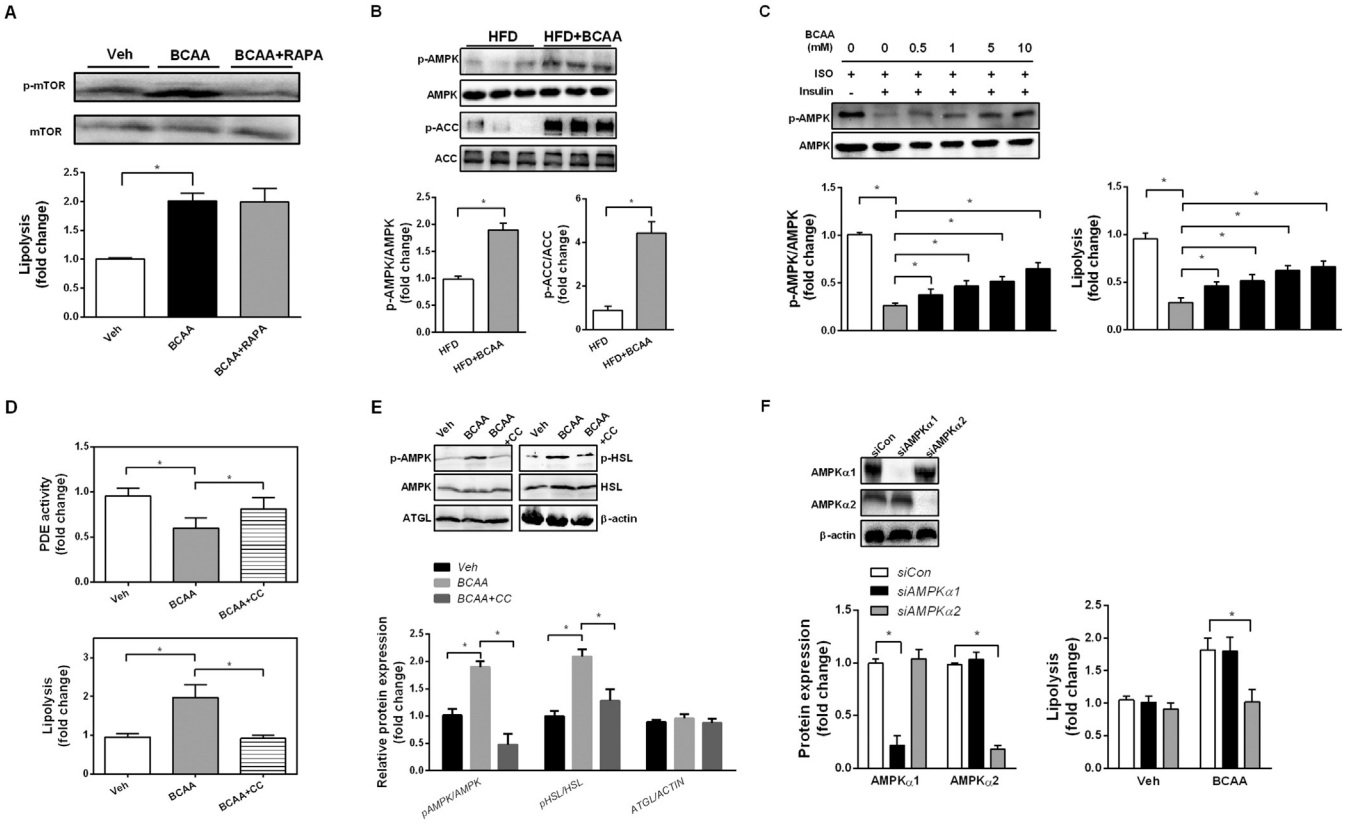

Fig. 5. AMPKα2, but not mTOR, signaling mediates BCAA-enhanced lipolysis in adipocytes. (A) Cultured adipocytes were treated with vehicle (Veh), BCAA (5 mM), or BCAA + rapamycin (RAPA, 1 μM) for 6 h. Insulin (10 ng/ml) was added to culture medium for 10 min, followed by 90 minute-stimulation by isoprenaline (ISO, 10 μM). Expression of p-mTOR and mTOR in adipocytes by Western blot (upper panel); lipolytic rates were measured by glycerol release into supernatant (lower panel). n = 6 per group. (B) Expression of p-AMPK, AMPK, p-ACC, and ACC in white adipose tissue isolated from HFD and HFD + BCAA group mice (n = 6 per group). (C to E) Cultured adipocytes were treated with vehicle (Veh), BCAA, or BCAA + Compound-C (CC, 10 μM) for 6 h. Insulin and ISO were added to culture medium as described in Fig. 5A. (C) Effects of different doses of BCAA upon expression of p-AMPK and AMPK in adipocytes (left panel) and glycerol release in supernatant (right panel). (D) PDE activity in adipocytes (upper panel) and glycerol release in supernatant (lower panel). (E) p-AMPK, AMPK, p-HSL, HSL, and ATGL expression in adipocytes by Western blot (n = 6 per group). (F) On Day 5 after differentiation, adipocytes treated by control siRNA (siCon), AMPKα1 siRNA (siAMPKα1), or AMPKα2 siRNA (siAMPKα2). 48 h after siRNA transfection, expression of AMPKα1 and AMPKα2 was determined by Western blot (left two panels). Effects of BCAA upon lipolysis were determined in siRNA-transfected adipocytes (right panel). n = 5 per group. *P < 0.05. All data presented as mean + SEM.

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carboxylase (ACC) in adipose tissue (Fig. 5B). Second, in cultured adipocytes, insulin significantly decreased ISO-induced AMPK activation. BCAA addition blunted the inhibitory effects of insulin and increased AMPK phosphorylation in a concentration-dependent manner (Fig. 5C, representative blot and summary data in left two panels). Consequently, insulin's inhibitory effect upon ISO-induced lipolysis was reversed by BCAA in a concentration-dependent fashion (Fig. 5C, right panel). Third, BCAA significantly suppressed the activity of phosphodiesterases (PDE), a key anti-lipolytic molecule (Fig. 5D, top panel) (Chaves et al., 2011), and significantly increased lipolysis in cultured adipocytes (Fig. 5D, bottom panel). Inhibition of AMPK by Compound-C (CC, specific AMPK inhibitor) significantly attenuated the inhibitory effects of BCAA upon PDE, and reduced BCAA-enhanced lipolysis (Fig. 5D). Fourth, to further investigate which lipase is involved in BCAA-enhanced lipolysis, we determined the expression levels of two dominant lipases in the adipocyte, hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) (Duncan et al., 2007). BCAA significantly increased HSL phosphorylation at serine^{660} without altering ATGL expression level (Fig. 5E). Inhibition of AMPK by Compound-C abolished BCAA-enhanced HSL phosphorylation (Fig. 5E). Finally, to determine the specific AMPK subunits responsible for BCAA-induced lipolysis, cultured adipocytes were transfected with AMPKα1 or AMPKα2 specific siRNA (Fig. 5F, left two panels). As summarized in Fig. 5F (right panel), BCAA-enhanced lipolysis was blocked by AMPKα2, but not AMPKα1, knockdown. Collectively, these results demonstrated BCAA blocks insulin-mediated anti-lipolytic effects and subsequently enhances lipolysis via AMPKα2-mediated PDE suppression and HSL activation, resulting in increased adipocyte FFA release.

3.4. Inhibition of Lipolysis Abolished BCAA-Enhanced Hyperglycemia and Weight Loss, Improved Insulin Sensitivity, but Failed to Normalize BCAA-Induced Liver Injury

To determine whether increased adipocyte lipolysis and subsequent FFA release are causatively related to BCAA-induced weight loss, hyperlipidemia, insulin resistance, and liver damage, HFD + BCAA mice were treated with acipimox, a potent lipolysis inhibitor (Fuccella et al., 1980). As summarized in Fig. 6, acipimox abolished BCAA-induced body weight loss (A, left) and adipose reduction (A, middle), reversed adipocyte size (A, right), reduced plasma TG and FFA levels to slightly below control (B), and improved whole body insulin sensitivity (C). These data support lipolysis is responsible for body weight loss, adipose reduction, hyperlipidemia, and insulin resistance in HFD + BCAA animals. However, BCAA-induced liver damage was not completely blocked by acipimox. Plasma AST/ALT levels (Fig. 6D), hepatic MDA and 4-HNE content (Fig. 6E), and hepatic apoptotic cell death (Fig. 6F) were all significantly greater in acipimox-treated animals than HFD control animals. These data suggest BCAA may have direct deleterious effect upon hepatic structure/function in HFD-fed mice.

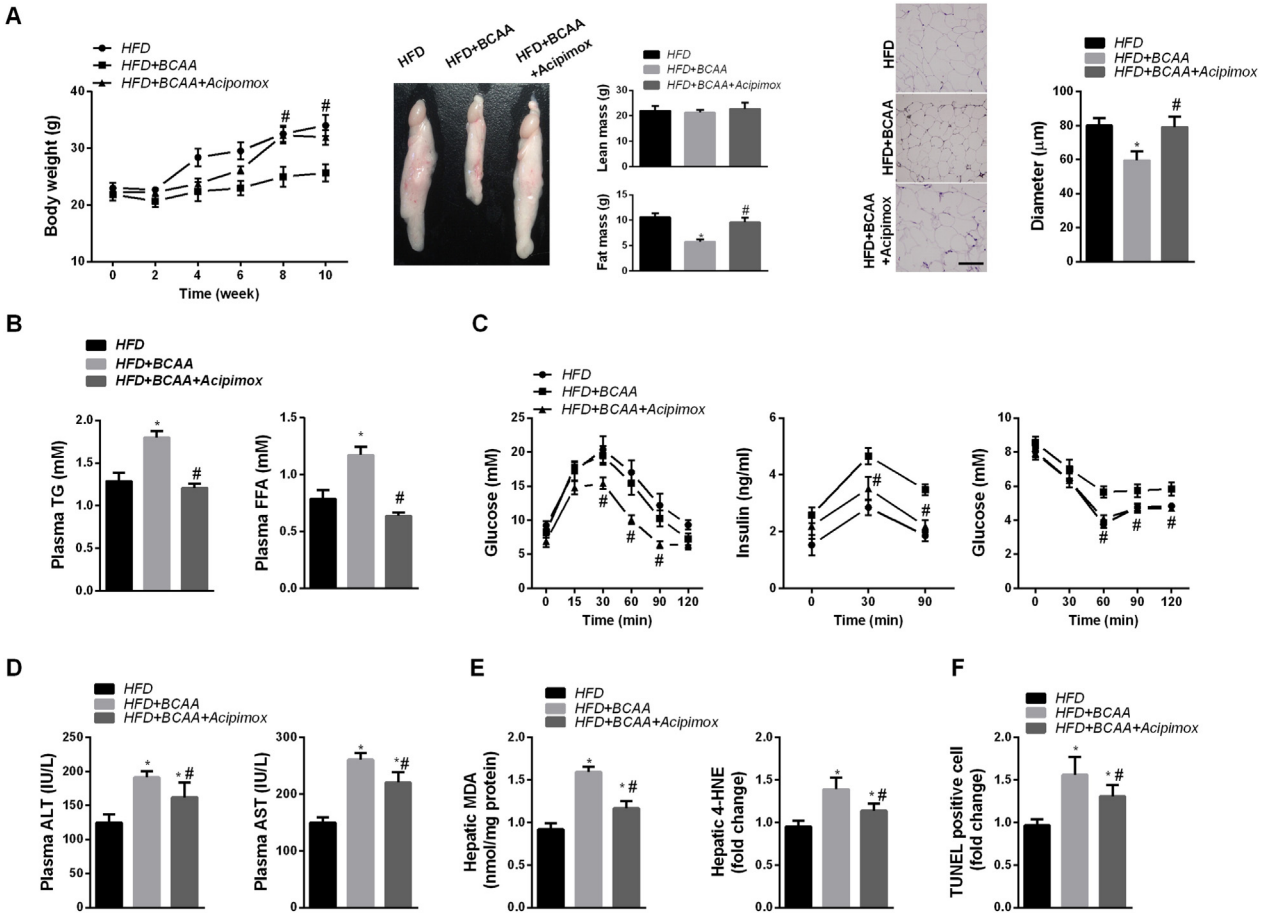

Fig. 6. Inhibition of lipolysis abolished BCAA-enhanced hyperglycemia and weight loss, improved insulin sensitivity, but failed to normalize BCAA-induced liver injury. Adult C57BL6J mice were treated with HFD, HFD + BCAA, or HFD + BCAA + acipimox (10 mg/kg body weight, daily IP injection) for 12 weeks. (A) Left panel, body weight was monitored every two weeks (n = 10 per group). Lean and fat mass (middle panel), and adipocyte size (right panel) were determined (n = 10 per group). (B) Quantification of plasma TG and FFA (n = 8 per group). (C) Glucose homeostasis (GTT, insulin release during GTT, and ITT) was evaluated (n = 6 per group). (D) Quantification of plasma ALT and AST (n = 8 per group). (E) Determination of hepatic MDA and 4-HNE (n = 8 per group). (F) Hepatic apoptosis determined by TUNEL staining (n = 8 per group). *P < 0.05 versus HFD and #P < 0.5 versus HFD + BCAA. All data presented as mean + SEM.

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3.5. BCAA Inhibited Hepatic Autophagy in Response to Lipid Exposure by mTOR Signaling Activation

Hepatic autophagy is a compensatory protective process against FFA-induced lipotoxicity (Lin et al., 2013; González-Rodríguez et al., 2014). Insufficient autophagy results in apoptotic death (Codogno and Meijer, 2005). We have demonstrated BCAA inhibition of hepatic lipogenesis is mediated by mTOR activation (Fig. 2), the master regulatory molecule in autophagosome formation. Next, we determined if BCAA may inhibit hepatic autophagy, contributing to hepatic apoptosis (Fig. 3E/F) and liver injury. BCAA supplementation indeed blocked HFD-induced autophagy activation, as evidenced by reduced light chain 3-II (LC3-II) expression (Fig. 7A: GFP-LC3 droplets formation; Fig. 7B: LC3-II western analysis) and increased hepatic p62 expression (Fig. 7B). Rapamycin treatment blocked the inhibitory effect of BCAA upon autophagy, evidenced by increased LC3-II and reduced p62 expression (Fig. 7C). Moreover, BCAA inhibited palmitate-induced autophagy in cultured hepatic cells in a dose-dependent manner (Fig. 7D), an effect reversed by rapamycin (Fig. 7E). These data demonstrate BCAA activates mTOR signaling, and subsequently inhibits hepatic autophagy in response to lipid exposure.

3.6. In Vivo mTOR Inhibition Had No Significant Effect Upon BCAA-Induced Plasma FFA Elevation but Significantly Attenuated Liver Injury

The aforementioned in vitro and in vivo experimental results demonstrated two separate signaling systems are activated in adipocytes and hepatic cells, which are respectively responsible for lipolysis (AMPKα2) and liver injury (mTOR). In a final attempt to provide direct evidence that inhibition of hepatic lipogenesis and autophagy by mTOR activation contributes to BCAA-enhanced hepatic lipotoxicity and liver injury, the effect of mTOR inhibition upon plasma FFA and liver injury was determined. Rapamycin does not block BCAA-induced plasma FFA elevation (Fig. 8A left panel). This result is consistent with our in vitro results (Fig. 4) demonstrating adipocyte lipolysis is mediated by AMPKα2 but not mTOR. However, rapamycin treatment significantly attenuated BCAA-induced liver damage, as evidenced by normalized plasma AST/ALT levels (Fig. 8B), reduced BCAA-enhanced lipid peroxidation (Fig. 8C), attenuated hepatic apoptosis (Fig. 8D), and decreased mRNA expression levels of proinflammatory cytokines in the liver (Fig. 8E). In cultured hepatocytes, BCAA obviously increased FFA-induced apoptosis, which was reversed by rapamycin or carbamazepine co-treatment (Supplementary Fig. 6). Carbamazepine is an autophagy inducing drug (Hidvegi et al., 2010; Lin et al., 2013). These in vitro data revealed that mTOR-mediated autophagy inhibition plays a crucial role in hepatic lipotoxicity regulated by BCAA. Interestingly, although rapamycin failed to normalize plasma FFA concentration, hepatic FFA accumulation was completely normalized by rapamycin (Fig. 8A, right panel).

4. Discussion

We have made several important observations in the current study. Firstly, we demonstrated BCAA supplementation attenuated HFD-induced weight gain, decreased fat mass, inhibited expression of hepatic lipogenic enzymes, and reduced hepatic triglyceride contents. However, BCAA reduced HFD-induced body weight at the expense of abnormal lipolysis, resulting in hyperlipidemia, whole body insulin resistance, and hepatic lipotoxicity. BCAA are abundant ingredients in the human diet accounting for nearly 20% of the total protein intake (Harris et al., 2005). It has been suggested that BCAA intake reduces body weight gain and inhibits appetite by acting upon the central nervous system (Hutson et al., 2005). However, our experimental results suggest increased adipose lipolysis, not reduced food intake, is primarily responsible for BCAA supplementation-induced weight loss. Although BCAA moderately reduced food intake, inhibition of lipolysis completely restored fat mass and body weight in BCAA supplemented HFD mice. Taken together, these results provide a likely explanation for the

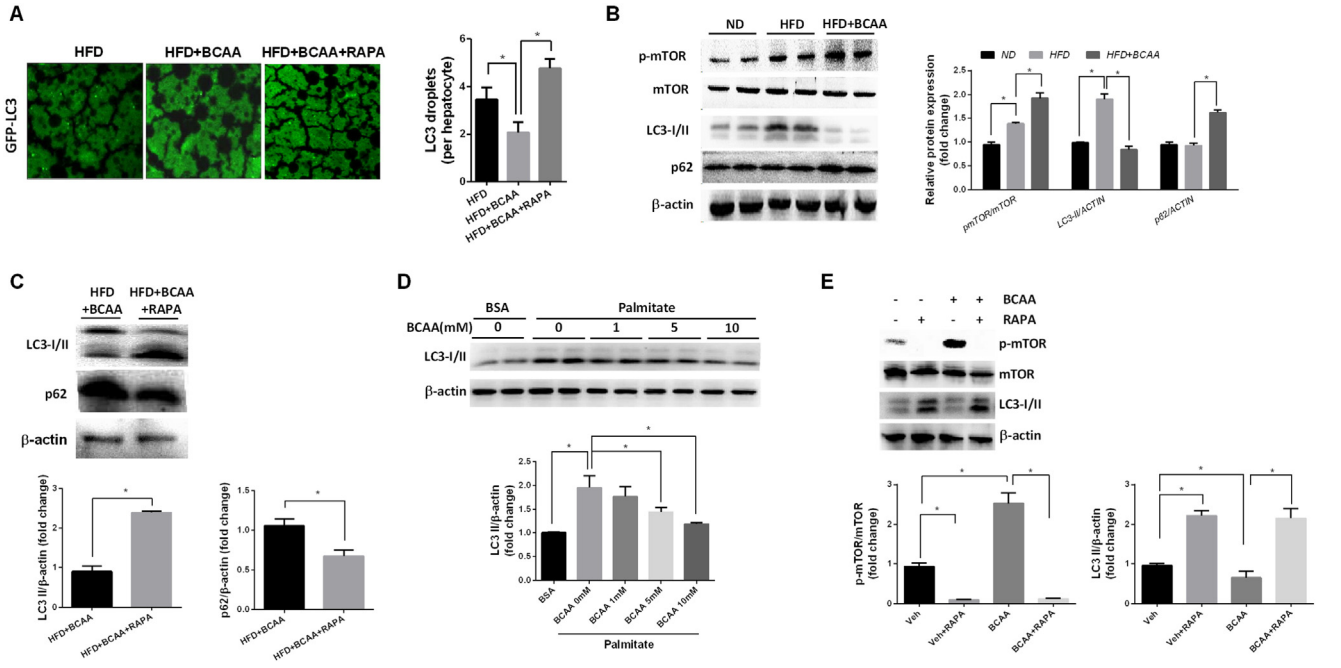

Fig. 7. BCAA inhibited hepatic autophagy in response to lipid exposure by activating mTOR signaling. GFP-LC3 transgenic mice were treated with ND, HFD, HFD + BCAA, or HFD + BCAA + RAPA (rapamycin, 1 mg/kg body weight, IP injection every other day) for 12 weeks. (A) GFP-LC3 droplets were analyzed in the liver (n = 10 per group). (B) Hepatic p-mTOR, mTOR, LC3-I/II, and p62 protein expression were determined by Western blot from ND, HFD, and HFD + BCAA groups (n = 4 per group). (C) Hepatic LC3-I/II and p62 expression were determined by Western blot from HFD + BCAA and HFD + BCAA + RAPA groups (n = 4 per group). (D) Cultured hepatocytes were treated with different doses of BCAA (0, 1, 5, 10 mM) in the absence or presence of palmitate (500 μM) for 24 h. LC3-I/II protein expression was evaluated by Western blot and normalized against β-actin expression (n = 3 per group). (E) Cultured hepatocytes were treated with or without BCAA (5 mM) or rapamycin (1 μM) after 24 h of palmitate (500 μM) exposure. Expression of p-mTOR, mTOR, and LC3-I/II were determined by Western blot (n = 3 per group). *P < 0.05. All data presented as mean + SEM.

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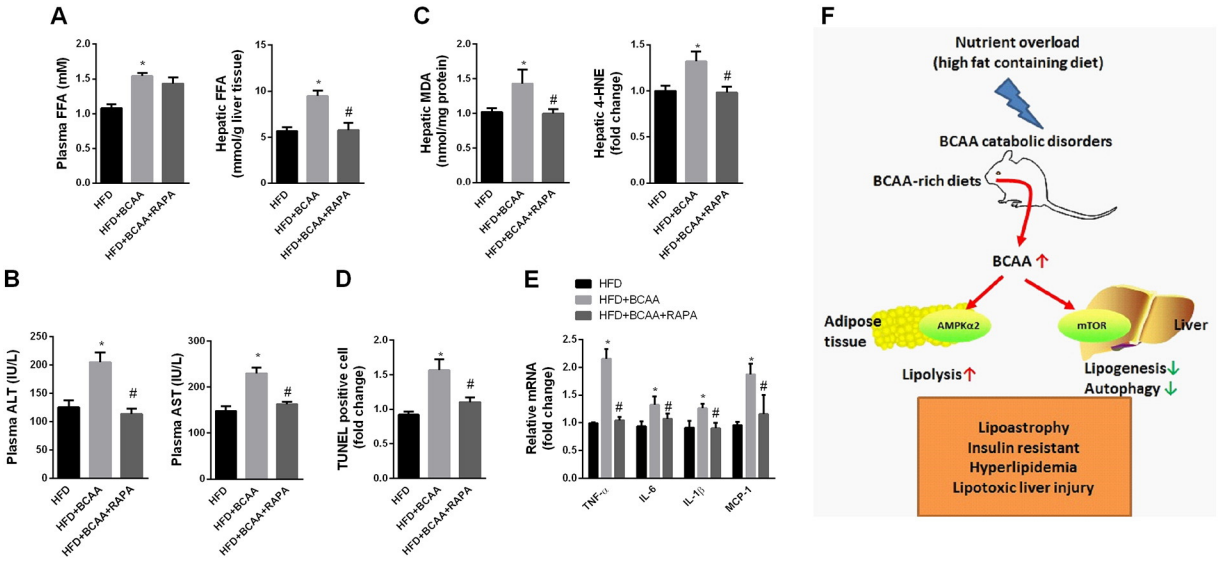

Fig. 8. In vivo mTOR inhibition had no significant effect upon BCAA-induced plasma FFA elevation, but significantly attenuated liver injury. Adult C57BL6J mice were treated with HFD, HFD + BCAA, or HFD + BCAA + RAPA (rapamycin, 1 mg/kg body weight, IP injection every other day) for 12 weeks. (A) Plasma and hepatic FFA were determined. (B) Plasma ALT and AST were determined. (C) Quantification of hepatic MDA and 4-HNE. (D) TUNEL staining on liver sections. (E) Hepatic mRNA expression levels of inflammatory cytokines were determined by real time-PCR. n = 10 per group. *P < 0.05 versus HFD group and #P < 0.05 versus HFD + BCAA group. Data presented as mean + SEM. (F) Schematic reviewing proposed mechanism underlying BCAA-aggravated hepatic lipotoxicity. HFD induces BCAA catabolic dysfunction in mice. Under HFD conditions, increased BCAA consumption increases circulating BCAA. BCAA-induced ROS promotes AMPK phosphorylation and AMPKα2 triggers lipolysis. BCAA-enhanced lipolysis induces hyperlipidemia. Elevated circulating FFA results in insulin resistance and lipotoxic liver injury. Meanwhile, BCAA activate hepatic mTOR signaling, inhibit lipogenesis and autophagy, therefore increasing hepatic susceptibility to FFA-mediated lipotoxicity. Overall, increased BCAA consumption aggravates HFD-induced hepatic lipotoxicity, providing mechanistic insight regarding diet-induced steatohepatitis pathogenesis.

contradictory clinical observations that BCAA supplementation attenuates weight gain, whereas increased plasma BCAA is associated with NAFLD and liver injury.

Secondly, we have demonstrated that BCAA increased adipocyte lipolysis and FFA release via AMPKα2-dependent HSL activation. Lipolysis rates are precisely regulated through hormonal and biochemical signals. Initiation of TG hydrolysis in adipose tissue is controlled by two enzymes, HSL and ATGL (also known as desnutrin). HSL hydrolyses triglycerides, diglycerides, and cholesteryl esters, although with much greater specificity for diacylglycerol (DG). Lipolytic agents (such as β-adrenergic agonists) acutely regulate HSL by increasing cellular cyclic adenosine monophosphate (cAMP) levels, thus activating cAMP-dependent protein kinase (protein kinase A, PKA). PKA phosphorylates HSL at serine^{563}, serine^{659}, and serine^{660}, thereby increasing its intrinsic activity. PKA also promotes HSL translocation from cytosol to the lipid droplet (Yeaman, 2004). Activation of phosphodiesterase (PDE) reduces cAMP levels, with consequent reduced PKA activity, inhibiting HSL phosphorylation and translocation. In contrast to HSL, the activity of ATGL is specific for TG and has limited activity against DG. Our current study demonstrated BCAA supplementation had no significant effect on ATGL expression. However, BCAA supplementation significantly reduced PDE activity and increased HSL phosphorylation without altering its expression. These results suggest BCAA increases adipocyte lipolysis likely via attenuating PDE-inhibition of HSL.

AMPK is a crucial sensor of redox status and energy balance. Its role in lipolysis is controversial. Initial evidence of a regulatory role for AMPK in adipocyte lipolysis originated from the in vitro observation that HSL is phosphorylated by AMPK at serine^{565}. Phosphorylation at serine^{565} blocks the phosphorylation of HSL by PKA at serine$^{563,\ 659,\ 660}$ (Yeaman, 2004). Based upon these in vitro studies, it was proposed AMPK activation would exert an anti-lipolytic effect in adipocytes. However, several recent studies have demonstrated AMPK activation promotes lipolysis (Yin et al., 2003; Koh et al., 2007), supported by evidence acute and chronic exercise increase catecholamine release, lipolysis, and AMPK activation in the adipose tissue (Koh et al., 2007). An anti-lipolytic role for AMPK appears counterintuitive because during exercise, circulating levels of FFA are increased significantly. Such contradictory results may be explained by the opposite regulatory effects exerted by different AMPK isoforms. Specifically, adipocytes from AMPKα1-knockout mice exhibit increased lipolysis, indicating an anti-lipolytic role of this enzyme (Daval et al., 2005). In contrast, mice lacking the AMPKα2 subunit manifest increased adiposity and weight gain (Villena et al., 2004), suggesting a pro-lipolytic role of this subunit. More importantly, a recent study demonstrated nicotine-induced lipolysis is lost in AMPKα2, but not AMPKα1, knockout adipocytes, further supporting the role of AMPKα2 in promoting lipolysis (Wu et al., 2015). To this end, our current study demonstrated BCAA significantly increased adipocyte lipolysis, an effect blocked by AMPKα2, not AMPKα1, knockdown. However, the detailed molecular signaling by which BCAA inhibits PDE activity via AMPKα2 remains unidentified. Moreover, whether BCAA-induced HSL phosphorylation is a direct result of AMPKα2, or an indirect effect via PDE inhibition, was not directly addressed in this study. These important questions warrant additional ongoing investigation, underway in our laboratory.

Thirdly, we have provided the direct evidence that BCAA supplementation atop HFD caused significant liver damage via two mTOR-activated signaling pathways: inhibition of hepatic lipogenesis and blockage of autophagy. De novo hepatic lipogenesis is regulated by a group of genes controlled by SREBP-1c, the master regulator of the hepatic lipogenic program, regarded as a therapeutic target against hepatic lipogenesis and its resultant metabolic disorders (Postic and Girard, 2008). Our current study demonstrated BCAA supplementation significantly inhibited hepatic SREBP-1c expression and reduced lipogenesis enzyme expression in an mTOR-dependent manner. However, mTOR blockade with rapamycin significantly attenuated, not exacerbated, BCAA-induced liver injury. These results suggest the beneficial effect of mTOR-mediated global inhibition of hepatic lipogenesis by BCAA is overwhelmed by stronger deleterious signaling pathways concurrently activated by mTOR.

Although de novo hepatic lipogenesis contribute to liver FFA abundance, circulating FFA represent greater pools for hepatic FFA accumulation. Under physiologic conditions, intrahepatic FFA can be re-esterified

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and stored as TG in lipid droplets (Neuschwander-Tetri, 2010). Recent studies demonstrated converting FFA to TG is critical in protecting the liver from FFA-induced lipotoxicity. Clinical observations reveal patients with advanced steatohepatitis express significantly reduced key hepatic lipogenic enzymes, and have increased hepatic FFA and significantly reduced hepatic TG (Nagaya et al., 2010; van der Poorten et al., 2013). Specifically, genetic interruption of hepatic TG synthesis leads to increased hepatic FFA accumulation, inflammation, fibrosis, and exacerbated liver damage (Yamaguchi et al., 2007). These results suggest that although global inhibition of hepatic de novo lipogenesis is metabolically beneficial, inhibition of hepatic FFA-TG conversion under hyperlipidemic conditions will be injurious to the liver. Our current study demonstrated that BCAA not only globally inhibited hepatic lipogenesis, but also stimulated adipocyte lipolysis, increasing circulating FFA. Unfortunately, FFA accumulated in hepatic cells cannot be re-esterified and stored as TG, because DGAT1 (a key enzyme for TG synthesis from FFA) is inhibited by BCAA (Fig. 2C). FFA-mediated hepatic lipotoxicity is thus intensified. Our results support this conclusion: although in vivo inhibition of mTOR failed to reduce BCAA-induced hyperlipidemia, BCAA-induced liver injury was attenuated.

Autophagy is a pivotal self-repair mechanism maintaining intracellular homeostasis via degradation of injured organelles and misfolded proteins in response to stress. Insufficient autophagy activates apoptosis signaling, resulting in cell death (Codogno and Meijer, 2005). In nonalcoholic fatty liver, hepatic autophagy is impaired due to mTOR activation. Restoring autophagy by rapamycin or carbamazepine treatment ameliorates disease severity (Lin et al., 2013). Furthermore, autophagy activation protects against FFA-mediated lipotoxicity both in vivo and in vitro (Lin et al., 2013; González-Rodríguez et al., 2014). In this study, we demonstrated BCAA activated mTOR and significantly inhibited hepatic autophagy in response to lipid exposure, resulting in hepatic apoptosis. Blockade of mTOR signaling by rapamycin restored hepatic autophagy response, attenuated FFA-induced lipotoxicity, and reduced hepatic apoptosis.

In summary, our results demonstrated BCAA causes hepatic injury via complex mechanisms involving both adipocytes and hepatic cells. In adipocytes, BCAA activates AMPKα2 and stimulates lipolysis, increasing plasma FFA, which in turn results in hepatic FFA accumulation. In the liver, BCAA activates mTOR, which inhibits FFA to TG conversion and autophagy. Inhibition of hepatic FFA to TG conversion blocks the hepatic FFA outflow route, intensifying FFA lipotoxicity. Blockade of autophagy impedes the self-repairing mechanism protective against lipotoxicity, increasing apoptotic cell death. There exist many similarities between BCAA-induced (as demonstrated in this study) and nicotine-induced (recently reported by Zou and colleagues) metabolic alterations (Wu et al., 2015). However, BCAA-induced liver injury is far worse than that caused by nicotine. Lipolysis inhibition completely normalized the liver structure and function in nicotine-treated, but not BCAA-treated, animals due to the directly hepatocyte toxic effects of BCAA. As BCAA are abundant in protein, our results call for caution regarding the ingestion of high protein diets in obesity and diabetic individuals, unless their BCAA metabolic pathways are determined normal. Moreover, our results call to attention that high fat is not the only harmful factor in the western meat-rich diet. The combination of high fat and high protein consumption may generate further dangerous metabolic disorders and organ injury.

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Disclosure

None.

Author Contributions

Study concept and design: L. Tao, F. Zhang, and W. Yan. Acquisition of data: F. Zhang, S. Zhao, Y. Xia, W. Wang, C. Peng, J. Zhang, and W. Yan. Analysis and interpretation of data: S. Zhao, H. Zhao, K. Lian, C. Gao, F. Zhang. Drafting of the manuscript: F. Zhang. Critical revision of the manuscript for important intellectual content: X. Ma, W. Lau, L. Tao. Statistical analysis: F. Zhang, F. Yan, L. Zhang, Y. Lee. Obtained fundings: L. Tao.

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Appendix A. Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2016.10.013.

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