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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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