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Research Paper

Microbiome Remodeling *via* the Montmorillonite Adsorption-Excretion Axis Prevents Obesity-related Metabolic Disorders

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ABSTRACT

Obesity and its related metabolic disorders are closely correlated with gut dysbiosis. Montmorillonite is a common medicine used to treat diarrhea. We have previously found that dietary lipid adsorbent-montmorillonite (DLA-M) has an unexpected role in preventing obesity. The aim of this study was to further investigate whether DLA-M regulates intestinal absorption and gut microbiota to prevent obesity-related metabolic disorders. Here, we show that DLA-M absorbs free fatty acids (FFA) and endotoxins *in vitro* and *in vivo*. Moreover, the combination of fluorescent tracer technique and polarized light microscopy showed that DLA-M crystals immobilized BODIPY® FL C16 and FITC-LPS, respectively, in the digestive tract *in situ*. HFD-fed mice treated with DLA-M showed mild changes in the composition of the gut microbiota, particularly increases in short-chain fatty acids (SCFA)-producing *Blautia* bacteria and decreases in endotoxin-producing *Desulfovibrio* bacteria, these changes were positively correlated with obesity and inflammation. Our results indicated that DLA-M immobilizes FFA and endotoxins in the digestive tract *via* the adsorption-excretion axis and DLA-M may potentially be used as a prebiotic to prevent intestinal dysbiosis and obesity-associated metabolic disorders in obese individuals.

1. Introduction

Obesity is a metabolic disturbance that has high prevalence worldwide and has increased over the past four decades. It is associated with several chronic diseases, including type 2 diabetes (T2D), nonalcoholic fatty liver disease (NAFLD), dyslipidemia, cardiovascular diseases and cancer, which are the main causes of mortality (Wormser et al., 2011, Bhaskaran et al., 2014, Arnold et al., 2015). Obesity results from a complex interaction between genetic background and environmental factors, such as a fatty diet and low physical activity (Everard and Cani, 2013). When fat synthesis continually exceeds lipolysis, the surplus lipids are stored in white adipose tissue (WAT), thus leading to the development of obesity (Hoffmann et al., 2015). The overweight and obesity epidemic poses a considerable global public health threat. The treatment and prevention of obesity and its complications are a major challenge at present.

The connection between the intestinal microbiota and energy homeostasis and the pathogenesis of obesity-associated metabolic disorders is increasingly being recognized (Musso et al., 2010, Hildebrandt et al., 2009). Accumulating evidence suggests that changes in the gut microbiota composition are associated with glucose metabolism, lipid metabolism, energy balance and immune homeostasis, thereby influencing whole-body metabolism (Cani, 2014). Approximately 1000 different bacterial species exist in the human gut, and these bacteria have 150 times more genes than humans do (Qin et al., 2010). Hosts and their gut microbiomes have co-evolved, and the gut microbiome thus regulates the host metabolism and extraction of energy from ingested food (Shen et al., 2013, Shin et al., 2014). The intestinal microbiota exchange metabolites with the host and perform a crosstalk with host signaling pathways, thereby modulating both host gene expression and host metabolism.

Abbreviations: DLA-M, dietary lipid adsorbent-montmorillonite; Epi-WAT, epididymal white adipose tissue; FFA, free fatty acid; HFD, high fat diet; HOMA-IR, homeostasis model index of insulin resistance; IBS, irritable bowel syndrome; IGTT, intraperitoneal glucose tolerance test; ITT, insulin tolerance test; LPS, lipopolysaccharide; Mes-WAT, mesenteric white adipose tissue; NAFLD, nonalcoholic fatty liver disease; NCD, normal chow diet; NEFA, non-esterified fatty acids; OA, oleic acid; OGTT, oral glucose tolerance test; OUT, operational taxonomic unit; PA, palmitic acid; Per-WAT, perirenal white adipose tissue; QUICKI, quantitative insulin check index; PCoA, principal coordinates analysis; RDA, redundancy analysis; SCFA, short-chain fatty acid; T2D, type 2 diabetes; TC, total cholesterol; TG, triglyceride; VFA, volatile fatty acid; WAT, white adipose tissue.

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Increasing clinical and experimental data indicate that bacteria lipopolysaccharide (LPS), or endotoxin, is closely related to the development of obesity-associated low-grade inflammation (Cani et al., 2008, 2009). High plasma endotoxemia may result from the increase in Gram-negative bacteria in the gut microbiota and intestinal permeability that result from a high fat diet (HFD) (Serino et al., 2012). Moreover, LPS treatment causes mild obesity and NAFLD in mice and humans (Chen et al., 2011, Thuy et al., 2008, Harte et al., 2010). These observations indicate that decreasing the amount of endotoxin in the bloodstream may protect animals against obesity induced inflammation.

We have previously shown that dietary lipid adsorbent-montmorillonite (DLA-M) can adsorb dietary lipids (triglycerides and cholesterol) and increase fecal lipid excretion, thus preventing obesity in HFD-fed rats (Xu et al., 2016). The adsorption-excretion axis decreases the energy intake from the digestive tract. In the present study, we further found that DLA-M also adsorbs free fatty acids (FFA) and endotoxin and affects the composition of the gut microbiota, thereby preventing obesity, improving insulin sensitivity and ameliorating hepatic steatosis in mouse model of obesity. Our results thus demonstrate that DLA-M is an excellent adsorbent agent that may potentially contribute to the prevention of obesity and its associated metabolic disorders.

2. Materials & Methods

2.1. Materials

DLA-M was prepared in our laboratory, as previously described (Xu et al., 2016). BODIPY (D3922) and BODIPY® FL C16 (D3821) were obtained from Thermo Fisher Scientific. Lipopolysaccharides (LPS), FITC-LPS (F3665), oleic acid (OA) and palmitic acid (PA) were purchased from Sigma-Aldrich.

2.2. Animal Experiments

Animal experiments were approved and performed in accordance with the guidelines of Ethics and Animal Welfare Committee of Beijing Normal University (Approval No. CLS-EAW-2013-014 and CLS-EAW-2015-013). C57BL/6J and CD-1 (ICR) mice were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China) and housed in a light and climate controlled room at 12 h light - dark cycle and 25 ± 2 °C, 3–4 mice/cage, with free access to water and different diets. Both the normal chow diet (NCD) and HFD (containing 60% and 45% fat by energy, as shown in Supplementary Table 1) were obtained from Beijing HFK Bioscience Co. Ltd. (China). Ten-week-old male C57BL/6J mice were randomly allocated into three groups containing six to fifteen animals each, and they were fed the NCD or HFD (60% kcal fat) for ten weeks. Ten-week-old male CD-1 mice were randomly distributed into three groups of 10 animals each, and they were fed NCD or HFD (45% kcal fat) for five weeks (Supplementary Figs. 1a–f and 2a–d). The treatment groups (HFD + DLA-M) were fed an HFD with DLA-M at 1 g/kg body weight by intragastric gavage daily, whereas the NCD and HFD groups, which were used as controls, were orally administered normal saline. The body weight and food intake were measured weekly. Fresh stool samples were collected during the final five days and immediately stored at −80 °C for further analysis. During the final two weeks of the experiment, six animals from each group were subjected to the oral glucose tolerance test (OGTT), intraperitoneal glucose tolerance test (IGTT) and insulin tolerance test (ITT) according to previously described methods (Gao et al., 2009). At the end of the experimental period, all mice were starved for 12 h and then sacrificed. Blood was collected from the eye venous plexus. The epididymal-WAT (Epi-WAT), perirenal-WAT (Per-WAT), mesenteric-WAT (Mes-WAT), liver and gastrointestinal system (with or without chyme) were immediately excised, weighed, measured and frozen in liquid nitrogen after sacrifice.

Ten-week-old male RFP (Rosa26-mTmG) mice ($n = 3$) were treated with DLA-M (1 g/kg) and then treated with BODIPY® FL C16 (5 mg/kg) or FITC-LPS (5 mg/kg) separately by gavage for 4 h, and the mice were observed with a whole-body imaging scope (Lumazone FA1300, USA).

2.3. Cell Culture and Steatotic Hepatocyte Model Construction

L-02 cells were obtained from the Cell Resource Center, Peking Union Medical College (Beijing, China), and they were cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin at 37 °C with 5% CO_2. Steatotic hepatocyte model construction was performed as previously described (Gomez-Lechon et al., 2007, Zhou et al., 2014). L-02 cells at 75% confluency were exposed to a 1 mmol/l FFA mixture (OA:PA ratio, 2:1). After incubation for another 48 h, the cells were used for further analysis.

2.4. Quantitative Real-time PCR

Total RNA was isolated using an RNAprep Pure tissue kit or RNAprep Pure cell kit (Tiangen, China). Equal amounts of total RNA (2 μg) were then reverse transcribed to cDNA using M-MLV transcriptase (Promega, USA). Quantitative real-time PCR was performed in triplicate using the SYBR Green qPCR SuperMix (Transgen Biotech, China) on an ABI 7500 instrument (Applied Biosystems, USA) according to the manufacturer's instructions. Gene expression was normalized against the housekeeping gene: murine or human glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers used are listed in Supplementary Table 2.

2.5. Western Blot Analyses

An equal amount of cell or tissue protein lysates (10 μg) was subjected to 10% SDS-PAGE and electro-blotted onto Immobilon®-P transfer membranes (Millipore, USA). Then western blot analysis was performed using standard procedures. The band intensity was quantified using ImageJ software. The following antibodies were used: antibodies against FABP (sc-18661), TLR-4 (sc-293072), NF-κB (sc-372) and IκB-α (sc-847) from Santa Cruz, and antibodies against β-actin from Sigma.

2.6. Biochemical Analyses

ELISA kits were used to evaluate insulin, tumor necrosis factor α (TNFα), and interleukin-6 (IL-6), which were purchased from Neobioscience (China). Serum endotoxin and LPS content quantification were performed using a quantitative chromogenic tachypleus amebocyte lysate kit, which was purchased from the Chinese Horseshoe Crab Reagent Manufactory, Co., Ltd. Triglyceride (TG), total cholesterol (TC), and FFA concentrations in serum, tissue or cell were measured using commercially available kits from Applygen Technologies Inc. (China).

2.7. Histological Analyses

Tissues were fixed in 4% formaldehyde, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. The sizes of the adipocytes and the proximal jejunum villi length were analyzed using Image-pro plus software. To observe the lipid droplets, frozen liver sections and steatotic L-02 cells were staining by using Oil Red O or BODIPY.

2.8. DLA-M Absorption Analyses

For the *in vivo* experiment, RFP mice were treated as described above, and the gastrointestinal contents (stomach, duodenum, jejunum, ileum, cecum and colon) were evaluated by using smears. BODIPY® FL C16 and FITC-LPS were visualized as green fluorescence at an absorbance wavelength of 488 nm using a fluorescence microscope (ZEISS Imager M1, Germany), and DLA-M crystals were detected using a polarized brightfield *in situ*. For the *in vitro* experiment, the intestinal chyme was diluted with saline or certain concentrations of OA, PA and LPS and then