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Cyanidin-3-O-β-glucoside regulates the activation and the secretion of adipokines from brown adipose tissue and alleviates diet induced fatty liver

Lei Pei[a,c], Ting Wan[a,c], Sufan Wang[a,c], Mingtong Ye[a,c], Yun Qiu[a,c], Rui Jiang[a,c], Nengzhi Pang[a,c], Yuanling Huang[a,c], Yujia Zhou[a,c], Xuye Jiang[a,c], Wenhua Ling[a], Zhenfeng Zhang[b], Lili Yang[a,c,*]

[a] Department of Nutrition, School of Public Health, Sun Yat-sen University, Guangzhou, Guangdong Province, 510080, PR China
[b] Department of Radiology, The Second Affiliated Hospital of Guangzhou Medical University, Guangzhou, Guangdong Province, 510260, PR China
[c] Guangdong Provincial Key Laboratory of Food, Nutrition and Health, Guangzhou, Guangdong Province, 510080, PR China

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ABSTRACT

Aim: Cyanidin-3-O-β-glucoside (Cy-3-G) the most abundant monomer of anthocyanins has multiple protective effects on many diseases. To date, whether Cy-3-G could regulate the function of brown adipose tissue (BAT) is still unclear and whether this regulation could influence the secretion of adipokines from BAT to prevent non-alcoholic fatty liver disease (NAFLD) indirectly remains to be explored. In this study we investigated the effect of Cy-3-G on BAT and the potential role of Cy-3-G to prevent fatty liver through regulating the secretion of BAT.
Methods: Male C57BL/6 J mice were fed with a high fat high cholesterol (HFC) diet with or without 200 mg/kg B.W Cy-3-G for 8 weeks. In *in vitro* experiments, the differentiated brown adipocytes (BAC) and C3H10T1/2 clone8 cells were treated with 0.2 mM palmitate with or without Cy-3-G for 72 or 96 h. Then the culture media of C3H10T1/2 clone8 cells were collected for measuring the adipokines secretion by immunoblot assay and were applied to culture HepG2 cells or LO2 cells for 24 h. Lipid accumulation in HepG2 cells or LO2 cells were evaluated by oil red O staining.
Results: Here we found that Cy-3-G regulated the activation of BAT and the expression of adipokines in BAT which were disrupted by HFC diet and alleviated diet induced fatty liver in mice. In *in vitro* experiments, Cy-3-G inhibited the release of adipokines including extracellular nicotinamide phosphoribosyltransferase (eNAMPT) and fibroblast growth factor 21 (FGF21) from differentiated C3H10T1/2 clone8 cells induced by palmitate, which was accompanied by a reduction of lipid accumulation in HepG2 cells and LO2 cells cultured by the corresponding collected media of C3H10T1/2 clone8 cells.
Conclusions: These results indicate that Cy-3-G can regulate the thermogenic and secretory functions of BAT. Furthermore, our data suggest that the protective effect of Cy-3-G on hepatic lipid accumulation is probably via regulating the secretion of adipokines from BAT.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease characterized by excessive fat deposition in liver cells due to causes other than excessive alcohol consumption. NAFLD is a syndrome ranging from simple steatosis, which is relatively benign, to non-alcoholic steatohepatitis (NASH), which can progress to cirrhosis. Central obesity, insulin resistance, type 2 diabetes mellitus and dyslipidemia are the most important risk factors. In the past several years, it has been shown that the clinical burden of NAFLD is not only confined to liver-related morbidity and mortality, but there is now growing evidence that NAFLD is a multisystem disease, affecting several extra-hepatic organs and regulatory pathways [1,2].

Brown adipose tissue (BAT) is the main site of adaptive thermogenesis in mammals mainly existing at the shoulder blades and abdominal large vessels through uncoupling of the oxidative

Abbreviations: Cy-3-G, cyanidin-3-O-β-glucoside; BAT, brown adipose tissue; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; UCP1, uncoupling protein 1; NGF, nerve growth factor; VEGFA, vascular endothelial growth factor A; PET, positron emission tomography; NRG4, neuregulin 4; NAMPT, nicotinamide phosphoribosyltransferase; iNAMPT, intracellular NAMPT; eNAMPT, extracellular NAMPT; HFC, high fat high cholesterol; T_3, 3,3'5-Triiodo-L-thyronine; IBMX, xanthine; DMEM, dulbecco's modified eagle's medium; FBS, fetal bovine serum; ALT, alanine aminotransferases; AST, aspartate aminotransferases; TG, triglyceride; TC, total cholesterol; BAC, brown adipocytes; BMPs, bone morphogenetic proteins; IGF1, insulinlike growth factor 1; HPLC, high-performance liquid chromatography

* Corresponding author at: Department of Nutrition, School of Public Health, Sun Yat-sen University, No. 74 Zhongshan Road 2, Yuexiu District, Guangzhou, Guangdong Province, 510080, PR China.
E-mail address: yangll7@mail.sysu.edu.cn (L. Yang).

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phosphorylation respiratory chain in the mitochondrion, with the generation of heat instead of ATP, an effect mediated by uncoupling protein 1 (UCP1) in inner membrane [3,4]. Mature brown adipose tissue contains abundant mitochondria and expresses high level of UCP1, thus activating its thermogenic capacity or transplanting of embryonic BAT would raise the utilization of glucose and lipids which brings several biological effects including promoting energy expenditure, reducing obesity, lowering plasma lipids and protecting from some chronic metabolic alterations such as hyperglycemia [5] and insulin resistance [6–9]. In addition, recent studies using positron emission tomography (PET) demonstrated that there was more metabolically active BAT present in some adults than expected as classical or beige adipocytes. Besides, BAT with UCP1 deficiency had a modest effect on diet-induced obesity in mice when housed at ambient room temperature [10–12]. Therefore, it suggests that BAT contributes to the whole-body energy homeostasis through other mechanisms beyond thermogenesis.

Experimental evidence indicates that BAT also synthesizes diverse regulatory molecules (the so-called brown adipokines) that alter metabolic physiology via autocrine, paracrine and endocrine mechanisms. For example, nerve growth factor (NGF), fibroblast growth factor 2 and vascular endothelial growth factor A (VEGFA) are thought to serve as autocrine and/or paracrine factors to increase the sympathetic innervation, the number of preadipocytes and the vascularity in BAT [13–15]. While the secreted factors exerting metabolic effects on other tissues via endocrine are still necessary to be investigated further arises our interest. Neuregulin 4 (NRG4) is identified as a BAT-enriched secreted factor that can attenuate hepatic lipogenic signaling and preserve glucose and lipid homeostasis in obesity [16,17]. Nicotinamide phosphoribosyltransferase (NAMPT), the rate-limiting enzyme that initiates NAD^+ biosynthesis from nicotinamide in mammals, has two distinct forms: intracellular and extracellular NAMPT (iNAMPT and eNAMPT, respectively). iNAMPT which mainly reflects enzymatic activity is present in cytoplasm and nucleus [18,19]. eNAMPT is secreted by multiple cell types, including adipocytes [20] and immune cells and it is fully activated as an NAD^+ biosynthetic enzyme capable of catalyzing the generation of NMN, whereas it has also been reported to have a role as an inflammatory cytokine in the pathogenesis of diet-induced diabetes [21,22] and other pathophysiological functions [23] that are independent of catalytic activity. Consequently, the exploration of BAT-secreted regulatory molecules might reveal targets for novel drugs to treat or prevent chronic metabolic diseases such as NAFLD.

Cy-3-G is the most abundant monomer of polyphenolic compounds (anthocyanins) which are widely distributed in fruits, vegetables and pigmented cereals. Numerous studies have demonstrated that Cy-3-G can exhibit significant pharmacological activities including anti-oxidative [24], anti-inflammatory [25], anti-thrombotic effects [26], attenuating body weight gain [27] and hepatic lipid biosynthesis [28]. Cy-3-G extracted from black soybean seed coat had hypoglycemic and hypolipidemic actions and could protect against liver, kidney and pancreas damages in diabetic mice [29]. Mulberry ethanol extracts improved glucose homeostasis and ameliorated hepatic steatosis in high-fat diet fed mice [30]. Cy-3-G combined with its metabolite protocatechuic acid explained the inhibiting effect on hepatic stellate cells activation and liver fibrosis [31]. Our team's previous research has found that Cy-3-G protected against alcohol induced liver injury through regulating energy homeostasis [32]. However, whether Cy-3-G can regulate the activation of BAT and its secretion of adipokines is unclear. Furthermore, whether the regulation of the secretion of adipokines from BAT by Cy-3-G contributes to the prevention of fatty liver is unknown. In this study, we will explore how the activity of BAT is regulated by Cy-3-G, and how this process affects the formation of fatty liver induced by HFC diet.

2. Materials and methods

2.1. Reagents and antibodies

Anti-UCP1 (Cat.D9D6X) were purchased from Cell Signaling Technology (Danvers, MA). Anti-UCP1 (Cat.ab23841) and Anti-Visfatin (Cat.ab45890) were purchased from Abcam (Cambridge, UK). Anti-FGF21 (Cat.H-105) and all secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), and antibiotic mixtures (penicillin-streptomycin) were from Gibco BRL (Grand Island, NY). 3,3',5-Triiodo-L-thyronine (T_3) (T2877), xanthine (IBMX) (X0626), dexamethasone (D4920) and indomethacin (I7378) were purchased from Sigma-Aldrich (MO, USA). Insulin from bovine pancreas (I8040) was from Solarbio (Sci-Tech, Beijing, China). All other chemicals used in these experiments were purchased from commercial sources.

2.2. Cy-3-G extraction

The ripe fruits of mulberry were bought from a local market. The samples were freeze-dried with a lyophilizer (Labconco, UK). Mulberry pigment fraction was extracted with 65% methanol (0.1% trifluoroacetic acid) at room temperature. Then the extraction solution was passed through a macroporous adsorption resin column and the adsorbed anthocyanins were eluted by 80% ethanol. The Cy-3-G was obtained from the anthocyanins evaporated eluent by medium-performance liquid chromatography. The detailed extraction procedure of Cy-3-G from mulberry was performed as described in the literatures [33–35]. The purity of the extracted Cy-3-G measured by high-performance liquid chromatography (HPLC) was > 96% (Fig.S2; Table S1,2) [32].

2.3. Animal and experimental protocols

All animal experiments were approved by the Sun Yat-sen University Animal Ethics Committees (2012–0080). Male C57BL/6 J mice (6 weeks old) were purchased from the Sun Yat-sen University animal center (Guangzhou, China). The mice were maintained at 25 ℃ with a 12 h light/dark cycle and had ad libitum access to water and standard rodent chow diet (5% fat w/w; Guangdong Medical Laboratory Animal Center). The HFC diet (contained 17% fat [50% lard and 50% cacao-butter], supplemented with 1.25% cholesterol and 0.5% cholate) as described [32,36]. After 2-week adaptation, mice were randomly allocated into the following four experimental groups (n = 6–10): Control (chow diet), Cy-3-G (chow diet + 200 mg/kg B.W Cy-3-G), HFC (HFC diet), HFC + Cy-3-G (HFC diet + 200 mg/kg B.W Cy-3-G). Mice in the Cy-3-G and HFC + Cy-3-G group were orally gavaged with 200 mg/kg B.W Cy-3-G solution per day for 8 weeks. Mice in the Control and HFC group were orally gavaged with an equivalent volume of saline. After 8-week treatment, the animals were fasted for 16 h, and then were sacrificed. Tissue samples and serum were collected, either fixed in 10% formalin or rapidly frozen in liquid nitrogen and stored at −80℃.

2.4. Adipocyte differentiation and treatments

Brown preadipocytes were isolated from the interscapular region of newborn male C57BL/6 J mice (3 days old) purchased from the Sun Yat-sen University animal center (Guangzhou, China). Brown preadipocytes isolation and differentiation were performed as previously described [37,38]. Brown preadipocytes were cultured in dulbecco's modified eagle's medium (DMEM) with 10% fetal bovine serum (FBS) (Gibco, USA) for 2 days after reaching confluence (denoted as day 0 of differentiation). Brown adipocytes (BAC) differentiation was induced by adding a cocktail containing 0.5 mM xanthine (IBMX), 125μM