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Bergamot polyphenol fraction prevents nonalcoholic fatty liver disease via stimulation of lipophagy in cafeteria diet-induced rat model of metabolic syndrome

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is the most common liver disease in industrialized countries. Defective autophagy of lipid droplets (LDs) in hepatocytes, also known as lipophagy, has recently been identified as a possible pathophysiological mechanism of NAFLD. Experimental and epidemiological evidence suggests that dietary polyphenols may prevent NAFLD. To address this hypothesis and analyze the underlying mechanisms, we supplemented bergamot polyphenol fraction (BPF) to cafeteria (CAF) diet-fed rats, a good model for pediatric metabolic syndrome and NAFLD. BPF treatment (50 mg/kg/day supplemented with drinking water, 3 months) potently counteracted the pathogenic increase of serum triglycerides and had moderate effects on blood glucose and obesity in this animal model. Importantly, BPF strongly reduced hepatic steatosis as documented by a significant decrease in total lipid content (−41.3%±12% S.E.M.), ultrasound examination and histological analysis of liver sections. The morphometric analysis of oil-red stained sections confirmed a dramatic reduction in LDs parameters such as total LD area (48.5%±15% S.E.M.) in hepatocytes from CAF+BPF rats. BPF-treated livers showed increased levels of LC3 and Beclin 1 and reduction of SQSTM1/p62, suggesting autophagy stimulation. Consistent with BPF stimulation of lipophagy, higher levels of LC3II were found in the LD subcellular fractions of BPF-expose livers. This study demonstrates that the liver and its lipid metabolism are the main targets of bergamot flavonoids, supporting the concept that supplementation of BPF is an effective strategy to prevent NAFLD.

Keywords: NAFLD; Lipid macroautophagy; Flavonoid; Hypercaloric diet; Lipid droplet fraction; Dyslipidemia

1. Introduction

Nonalcoholic fatty liver disease (NAFLD), the most common liver disease in industrialized countries, is associated with excessive fat and sugar consumption and poor life style behaviors and displays a genetic predisposition [1,2]. NAFLD is considered the hepatic manifestation of metabolic syndrome (MS), a highly prevalent clustering of common pathologies and cardiovascular risk factors affecting Western societies including abdominal obesity, hypertension, dyslipidemia and insulin resistance [3,4]. NAFLD is a chronic condition characterized by the accumulation of fatty droplets in hepatocytes (microvesicular and macrovesicular steatosis), ranging from benign simple steatosis (lipid accumulation in more than 5% of liver cells) [5], to more severe liver injury including lobular inflammation (with or without fibrosis), and hepatocyte swelling, as seen in nonalcoholic steatohepatitis (NASH) [6]. If untreated, NASH can progress over time to cirrhosis with the risk of developing liver cancer or liver failure [7]. Recent epidemiological studies show that NAFLD and MS are no longer confined to the adult population, as they are becoming alarmingly frequent among obese children. Increased snacking on palatable, but unhealthy foods rich in sugar and in highly saturated and oxidized fat, in concert with genetic factors, is considered the cause of this epidemic [1,2,8].

Combinations of fat- and carbohydrate-rich dietary components have been used in rodents to mimic MS. Among such diets, the "cafeteria" (CAF) diet (15% protein, 70% carbohydrates, 15% fat), closely resembles the Western-type snacks mostly consumed by children and teenagers. The CAF diet is considered to be the most appropriate regime to induce severe obesity with glucose intolerance and liver steatosis classifiable as NAFLD progressing to NASH [9]. In fact, 8 and 15 weeks of CAF diet feeding is more efficacious in inducing insulin resistance and high plasma triglyceride levels than a traditional lard-based high-fat diet used in rodent models [9,10].

The hallmark of NAFLD is triglyceride accumulation in the form of lipid droplets (LDs) in the cytoplasm of hepatocytes. This arises from

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an imbalance between lipid and fatty acid uptake, *de novo* lipogenesis and lipid removal, which can be mediated by lipolysis and mitochondrial fatty acid oxidation or lipid export as a component of very low-density lipoprotein (LDL) particles [11]. Neutral Lipids in the form of small LDs can be effectively removed by cytosolic lipases, but big LDs appear resistant to the action of cytoplasmic enzymes. Recently, it has been shown that the process of lipid specific autophagy (or "lipophagy") mediates sequestration of LDs and their delivery to lysosomes, where the lipid cargo is subjected to the action of acid hydrolases [12,13]. The machinery regulating lipophagy is largely overlapping with macroautophagy mechanisms, and proteins such as LC3, Atg5 and Atg7 serve the formation of autophagosomes, that is, double membrane vesicles used for both lipid and protein sequestration. Importantly, defective autophagy of LDs in hepatocytes has been recently proposed as a possible pathophysiological mechanism of NAFLD [12–16], just as a defective protein-specific autophagy is implicated in neurodegeneration [17,18].

To date, there are no specific medical treatments approved for NAFLD [2,7]. Insulin sensitizers and vitamin E can induce histological improvement in NASH, but their efficacy is limited by adverse side effects [2,7,19]. The only medical recommendation for patients diagnosed with NAFLD is low-fat, fruit and vegetables-based diet and regular physical activity [2,20]. Indeed, dietary polyphenols, a large and heterogeneous group of phytochemicals in plant-based foods, have been associated with lower risk of NAFLD. For example, resveratrol reduces steatosis in mice fed with a high calorie diet [21]. In a rat experimental model of NAFLD, the administration of epigallocatechingallate alleviated the severity of liver injury [22]. Moreover, *Citrus* polyphenols, such as naringenin and hesperitin, have been shown to ameliorate dyslipidemia and diabetes in different diet-induced and/or genetic animal models of metabolic diseases [23,24].

Among *Citrus* plants, bergamot (*Citrus bergamia* Risso et Poiteau) shows the highest content of flavonoids in juice and peels derived from its fruits [25,26]. The glutaryl derivatives of hesperidin and naringin (brutieridin and melitidin) have been implicated in statin-like activity of bergamot polyphenol fraction (BPF) and of bergamot juice in animal models of hypercholesterolemia [27–29]. Clinical studies have confirmed a strong reduction of cholesterol levels along with a minor hypoglycemic effect in patients with MS [29,30]. Importantly, the latter effect was associated with a very strong reduction in triglycerides levels in both MS patients and patients affected by simple dyslipidemia [29].

Considering that NAFLD is characterized by increased triglycerides levels, we have hypothesized that BPF could have beneficial effects on fatty liver. To address this hypothesis and analyze the underlying mechanisms, we administered BPF to CAF-diet-fed rats. BPF treatment potently counteracted the pathogenic increase in blood triglycerides and liver LDs accumulation. Increased expression of autophagy markers was found in liver homogenates and in LDs-enriched subcellular fractions of livers from BPF-treated animals, indicating lipophagy stimulation by BPF. These data support BPF supplementation as an effective strategy to prevent NAFLD and provide novel mechanistic insights into liver-specific action of polyphenols.

2. Materials and methods

2.1. Animal procedures

Twenty-six male 5-week-old Rcc:Han WIST rats (Harlan Laboratories, Indianapolis, IN, USA) were housed two rats/cage in the UMG animal house facility and maintained on a standard 12-h light/dark cycle (lights on at 7:00 a.m. and lights off at 7:00 p.m.), in a temperature-controlled environment (20±2 °C). The animals had access to water and were fed *ad libitum* with standard chow (SC) diet 2016 ("SC," Harlan Teklad) for 3 weeks before assignment to one of four experimental groups. All animal studies were approved by the Italian Health Ministry and by the local ethics committee.

2.2. Experimental design

At 8 weeks of age (day "−7"), the rats were weighed, marked with different colors on the tail for recognition, and randomly assigned to two basic experimental groups: CAF diet group (CAF, n=15 rats) or SC diet group (SC, n=11 rats). These were subsequently subdivided into two subgroups, of which one received BPF extract (~50 mg/kg body weight/day) as supplement in drinking water (SC+BPF, n=6; CAF+BPF, n=8) and the other received drinking water without BPF (SC, n=5; CAF, n=7). BPF dose, 50 mg/kg, was calculated as follows: the previously tested dose in humans 1000 mg/100 kg=10 mg/kg was multiplied 5× to account for higher metabolic rate in rodents [29,31]. After a week of adaptation to the new cage mate, the administration of the experimental diets started (day "0") and lasted 91–95 days until the day of sacrifice. Food consumption and body weight gain were monitored weekly for 13 weeks.

2.3. Diets and supplement

The CAF diet (similar to those used by Sampey et al. [9] to induce obesity in lean animals) included sweet or briny cookies, milk chocolate, cereals, potato chips, processed meats, condensed milk with sugar, high-fat cheese (parmesan or *provolone*), and so on, were provided in excess (see Supplementary data, Table S1). The CAF diet was offered every 2–3 days (75 kcal/rat/day) on Monday, Wednesday and Friday in addition to SC diet *ad libitum*. Each time a mix of salty and sweet snacks was offered to stimulate hyperphagy. Snack items were weighed once a week, before and after consumption (corrected for drying), to calculate the amounts ingested of each one in all cages. The energy intake was estimated by multiplying by the energy density of the singular item considered. According to the information on the product labels, SC diet provided an energy value of 3.0 kcal/g, against the mean 4.2±1.1 kcal/g of the items included in CAF diet (see Supplementary data, Table S1).

BPF, as previously prepared and characterized for polyphenol content [26,29], was kindly provided by Herbal and Antioxidant Derivatives srl. (Polistena, RC, Italy). BPF diluted in drinking water was provided daily or every 2 days in the SC+BPF and CAF+BPF groups. The amount of water and BPF consumption was monitored daily or every 2 days to calculate the daily intake of BPF. The concentration of BPF added to drinking water varied from 0.5 mg/ml up to 2 mg/ml and was progressively adjusted to the mean body mass in the cage to ensure a mean 50 mg/kg/rat/day dose over a 3-month period.

2.4. Rat liver ultrasonographic examination and US data analysis

Rat liver ultrasonographic (US) examination was performed with a high-resolution transducer (13 MHz and 24 MHz) using *Abdominal package* software settings on a dedicated ultrasound equipment for small animals (Vevo-LAZR 2100; VisualSonics, Toronto, Ontario, Canada). Before US examination, the rats were anesthetized by inhalation of 5% isoflurane (Merial, Toluse, France) by using an inhalation anesthesia system. Three percent isoflurane and 100% oxygen inhalation was maintained during the experiment by fixing a gas mask on the rat muzzle. The animals were positioned in dorsal recumbence on a rectangular table and the ventral abdomen was shaved by electric shaver. A sound-conducting gel was applied and the liver was assessed by placing the transducer just distal to the last right costal cartilages and angling its beam cranially, obtaining multiple transversal and longitudinal scans.

The liver echogenicity corresponding to the hepatic fat content was assessed with ImageJ software package (National Institute of Health, Bethesda, MD, USA). The method utilized was adapted from a method used to quantify stained liver tissue (see: http://rsbweb.nih.gov/ij/docs/examples/stained-sections/index.html and [32]). Briefly, US longitudinal high-magnification scans of the right lobe from five to six rats per group were imported into the software, and the mean brightness (the integrated pixel intensity of a 32-bit image/area) was quantified in hepatic parenchyma regions. Mean brightness levels were calculated for each experimental group, and the data were subjected to statistical analysis.

2.5. Blood and tissue collection

Food was removed for 4–5 h before sacrifice at week 14. Water or BPF solution (2 mg/ml) was removed for 2–3 h and then administrated for the subsequent 2 h. The animals were sacrificed under Zoletil (80 mg/kg) and Dormitor anesthesia for tissue collection. The blood was collected by cardiac puncture with heparinized 21G needle and divided for plasma (2 ml) and serum preparations (3–4 ml) in appropriate blood collection tubes. For blood tests on week 9 of the experiment, orbital sinus blood sampling was performed with Paster pipette by an experienced veterinarian after 5% isoflurane anesthesia (see below). Vacuette tubes (Greiner Bio-One GmbH, Kremsmunster, Austria), either EDTA- or Z serum clot activator-treated, were used for plasma or serum collection, respectively. Immediately after collection or after 1-h clotting, blood samples were centrifuged (1700×g; 10 min at room temperature) to collect plasma and serum, respectively, which was stored at −80°C until use. The liver accessory lobe and small part of the right liver lobe were collected first, rinsed in phosphate-buffered saline (PBS) and weighed. Next the animals were perfused with 150 mM NaCl solution to remove excess of blood and to collect the rest of the liver and other organs. All the organs were weighed, after removing excess liquid and subsequently divided for biochemical and histological processing (see below). Liver weight was calculated as the sum of perfused and nonperfused samples.

2.6. Blood analysis

The following parameters were determined in the serum: total cholesterol, LDL-cholesterol, triglycerides, glucose, blood urea nitrogen (BUN), creatinine, alanine