Food and Chemical Toxicology 94 (2016) 203–212

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Hepatotoxicity and endothelial dysfunction induced by high choline diet and the protective effects of phloretin in mice

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ARTICLE INFO

Article history:
Received 6 January 2016
Received in revised form
6 April 2016
Accepted 6 June 2016
Available online 15 June 2016

Keywords:
Phloretin
Choline
Atherosclerosis
Vascular endothelial dysfunction
Liver injury

ABSTRACT

The involvement of choline and its metabolite trimethylamine-*N*-oxide (TMAO) in endothelial dysfunction and atherosclerosis has been repeatedly confirmed. Phloretin, a dihydrochalcone flavonoid usually present in apples, possesses a variety of biological activities including vascular nutrition. This study was designed to investigate whether phloretin could alleviate or prevent high choline-induced vascular endothelial dysfunction and liver injury in mice. Mice were provided with 3% high choline water and given phloretin orally daily for 10 weeks. The high choline-treated mice showed the significant dyslipidemia and hyperglycemia with the impaired liver and vascular endothelium ($p < 0.01$). Administration of phloretin at 200 and 400 mg/kg bw significantly reduced the choline-induced elevation of serum TC, TG, LDL-C, AST, ALT, ET-1 and TXA2 ($p < 0.01$), and markedly antagonized the choline-induced decrease of serum PGI2, HDL-C and NO levels. Furthermore, phloretin elevated hepatic SOD and GSH-Px activities and decreased hepatic MDA levels of the mice exposed to high choline water. Moreover, histopathological test with the H&E and Oil Red O staining of liver sections confirmed the high choline diet-caused liver steatosis and the hepatoprotective effect of phloretin. These findings suggest that high choline causes oxidative damage, and phloretin alleviate vascular endothelial dysfunction and liver injury.

1. Introduction

Choline, L-carnitine and lecithin (phosphatidyl choline) as methyl-donor foods are the essential nutrients in humans, and remain a very active topic of research (Wang et al., 2011; Tang et al., 2013; Koeth et al., 2013; Bennett et al., 2013). However, it was recently found that dietary choline, L-carnitine or lecithin was metabolized by gut microbes to trimethylamine (TMA), which in turn was metabolized by liver flavin monoxygenases (especially FMO3) to form harmful trimethylamine-*N*-oxide (TMAO), which was linked to cardiovascular diseases (CVD) (Wang et al., 2011; Tang et al., 2013; Koeth et al., 2013; Bennett et al., 2013). As a result, plasma TMAO has emerged as an important biomarker for vascular diseases, and the concern has arisen from the realization that methyl-donor foods can promote vascular endothelial damage. Clearly, exploring the potential adverse action of choline will be important in future studies, and this striking discovery also provides opportunities for the development of new diagnostic technology and therapeutic approaches of incident major adverse cardiovascular events.

Extensive epidemiological studies have demonstrated that a diet rich in fruits and vegetables has protective effects on various chronic diseases including CVD, and polyphenolic compounds may be largely responsible for the cardioprotective effects of colored fruits, mainly through antioxidant and anti-inflammatory actions (Ho and Wang, 2013). It is also well known that endothelial dysfunction is a driving force in CVD development, and growing evidence has indicated that reactive oxygen species (ROS) generation and nitric oxide (NO) reduction under pathophysiological conditions are related to the development of endothelial damage or atherosclerosis (George et al., 2012). Despite the established antioxidative and anti-inflammatory properties of flavonoids, no data exist concerning the role of natural dietary flavonoids in the regulation of high choline-induced CVD, including vascular endothelial dysfunction.

Phloretin and its glucoside phloridzin are abundantly presented

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http://dx.doi.org/10.1016/j.fct.2016.06.004
0278-6915/

in various species of apples, especially in the peels, and glycosylated derivatives of phloretin are nearly entirely converted into phloretin by hydrolytic enzymes in the small intestine (Vasantha-Rupasinghe and Yasmin, 2010). Phloretin has been shown to have wide biological activities, including antioxidant, antitumor, and anti-inflammatory properties, and suppress the stimulated expression of endothelial adhesion molecules and reduce activation of human platelets (Ho and Wang, 2013; Vasantha-Rupasinghe and Yasmin, 2010; Gaucher et al., 2013; Stangl et al., 2005). More recently, we have found that polyphenols can inhibit CCl_4-induced hepatic injury in mice by mediating antioxidative activity (Tian et al., 2012; Yang et al., 2010; Cheng et al., 2013). However, no scientific studies have reported the inhibitory effect of phloretin against high choline-mediated vascular endothelial dysfunction. The present study was therefore to evaluate whether besides vascular injury, long-term dietary intake of 3% high-choline water also led to liver damage in mice. Furthermore, we also determined whether the treatment with phloretin could attenuate high choline-induced vascular endothelial dysfunction and liver injury in mice. As far as we know, no result of such an investigation was published.

2. Materials and methods

2.1. Materials and chemicals

Phloretin (purity > 97%, food grade) was purchased from National Institutes for Food and Drug Control (Beijing, China). Food grade choline was obtained from Ya-xixi Co., Ltd. (Beijing, China). Food grade carboxymethylcellulose sodium (CMCNa) was purchased from China National pharmaceutical Co., Ltd. (Beijing, China). Hemataxylin and eosin (H&E) and Oil Red O were from Shanghai Lanji Technological Development Co., Ltd. (Shanghai, China). Assay kits of serum high density lipoprotein-cholesterol (HDL-C, No. 2400066), low density lipoprotein-cholesterol (LDL-C, No. 2400074), total cholesterol (TC, No. 2400065), total triglyceride (TG, No. 2400059), alanine aminotransferase (ALT, No.2400043), and aspartate aminotransferse (AST, No.2400051) were purchased from Changchun Huili Biotechnology Co. Ltd. (Changchun, China). Detection kits for nitric oxide (NO, No. A013-1), superoxide dismutase (SOD, No. A001-1), glutathione peroxidase (GSH-Px, No. A005), malonaldehyde (MDA, No. A003-1) and non-esterified fatty acid (NEFA, No. A042) were the products of Nanjing Jiancheng Bioengineering Institute (Nanjing, China). ELISA kits of thromboxane B2, 6-keto-PFF1a and endothelium-1 (ET-1) were also obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Deionized water was prepared using a Millipore Milli Q-Plus system (Millipore, Bedford, MA, USA).

2.2. Animals and experimental design

A total of 40 healthy male Kunming mice aged 4 weeks with a body weight of 15–18 g were purchased from the Experimental Animal Center of the Fourth Military Medical University (Xi'an, China). Mice were housed under a controlled room temperature at 22 ± 2 °C and maintained with a 12/12 h light-dark cycle and a humidity of 60 ± 5%. The mice were held for one week prior to experiments and free access to tap water and rodent chow (1% coarse meal, 1% vitamin complex, 2% mineral, 10% bran, 10% fish meal, 10% bean cake, 26% wheat flour and 40% corn flour, Qianmin Feed Factory). All the experiments were approved by the Fourth Military Medical University Committee on Animal Care and Use.

After laboratory environmental adaptation for one week, the mice with a body weight of 20–25 g were randomly divided into five groups (n = 8). The phloretin was dissolved with the 10% carboxymethylcellulose sodium (CMCNa). In normal control group, the mice received only tap water and were administrated intragastrically (ig., 0.4 mL) with 10% CMCNa aqueous vehicle once daily for 10 weeks. In high-choline (HC) control group (model group), the mice received 3% HC water (w/v) and administered intragastrically with 10% CMCNa vehicle (ig., 0.4 mL) once daily during the experimental period. In phloretin-treated groups, the mice received 3% HC water and administrated with phloretin at 100, 200, and 400 mg/kg bw (ig., 0.4 mL, 10% CMCNa aqueous solution) as low-, middle- and high-doses of treatments for 10 consecutive weeks, respectively. The mice were allowed free access to tap water or 3% HC water, and the 3% HC water was renewed every other day and the body weight of all the tested mice was measured once a week (Table 1). The dosages of phloretin were selected on the basis of the results of a previous experiment in mice and considered as the appropriate dosages (Crespy et al., 2001). All the experiments were approved by the Fourth Military Medical University Committee on Animal Care and Use (approval number, SYXK-007-2007).

2.3. Biological sample preparation in mice

At the end of the experimental period, all the mice were kept fasted strictly two hours after the last administration, but allowed free access to water as usual for 8 h before bleeding. All of the animals were anaesthetized with intraperitoneal injection of pentobarbital sodium (50 mg/kg), and then the animals were sacrificed by cervical dislocation to obtain blood, livers and blood vessels. The blood of mice was drawn using the 1.0 mL microcontainers, and the mouse liver was immediately removed and washed by ice-cold physiologic saline. The blood samples were centrifuged at 3000*g* for 10 min, and stored at 4 °C until use. During the preparation, 1.0 g of each liver tissue was homogenized in 9-times ice-cold physiologic saline in volume by automatic homogenate machine (F6/10-10G, FLUKO Equipment Shanghai Co. Ltd., Shanghai, China), and the resulting homogenate was centrifuged at 3000*g* for 10 min to obtain postnuclear supernatant and was refrigerated at −80 °C for further analysis.

2.4. Assay for serum ET-1, NO, TXA2, and PGI2 levels

Serum ET-1 and NO levels were used to evaluate the vascular endothelial dysfunction of mice and were measured using competitive inhibition method of enzyme linked immunosorbent ELISA kit according to the kit manufacturer's instructions. The results were expressed in pg/mL and μmol/L, respectively. The exudation of TXA2 and PGI2 was estimated by using the enzyme immunoassay of thromboxane B2 (TXB2) and 6-keto-PGF1a, where TXB2 is a metabolite of non-enzymatic hydrolysis of TXA2, and 6-keto-PGF1a is also a metabolite of non-enzymatic hydrolysis of PGI2, respectively (Davi and Patrono, 2007; Yamagata et al., 2015). The OD values at 450 nm were read by ELISA reader. All samples were tested in triplicate, and the absorbance values were averaged and the results were expressed as pg/mL and ng/mL, respectively.

2.5. Determination of lipid profile and liver function

Serum was assayed for the contents of fasting HDL-C, LDL-C, TC, TG, AST and ALT, which were conventional physical parameters of dyslipidaemia in clinical diagnosis (Lee et al., 2010). The levels of serum HDL-C, LDL-C, TC and TG were determined by visible spectrometer using commercially available diagnostic kits according to instructions, respectively. All the results were expressed in mmol/L. The activities of serum AST and ALT were determined by 2,4-dinitrophenylhydrazine method according to the assay kits, respectively, and the results of AST and ALT were expressed as U/L.