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Quercetin attenuates high fat diet-induced atherosclerosis in apolipoprotein E knockout mice: A critical role of NADPH oxidase

Lin Xiao [a, b, c], Liang Liu [d], Xiaoping Guo [a, b, c], Shanshan Zhang [a, b, c], Jing Wang [a, b, c], Feng Zhou [a, b, c], Liegang Liu [a, b, c], Yuhan Tang [a, b, c, *], Ping Yao [a, b, c, **]

[a] Department of Nutrition and Food Hygiene, School of Public Health, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China
[b] Hubei Key Laboratory of Food Nutrition and Safety, School of Public Health, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China
[c] Ministry of Education Key Laboratory of Environment, School of Public Health, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China
[d] College of Food Science and Engineering, Wuhan Polytechnic University, Wuhan, 430023, China

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ABSTRACT

Reactive oxygen species (ROS) have emerged as important molecules in cardiovascular function. Nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase is the major source of ROS in phagocytic and vascular cells. Several lines of evidence indicate that quercetin contributes to protecting against atherosclerosis. Herein, we investigated the effect of quercetin on alleviating atherosclerosis by regulating NADPH oxidase subunits expression in vivo, and explored the mechanism of quercetin suppressing the ROS overproduction stimulated by ox-LDL in mouse peritoneal macrophages (MPMs). Model ApoE KO mice were fed with either a normal chow diet or a high fat diet (HFD) supplemented with or without dosed quercetin for 24 weeks. Quercetin significantly reduced the atherosclerotic plaque area, alleviated the systemic oxidative stress, and suppressed aortic p47phox, p67phox expressions but partially reversed the NOX4 expression as compared to those in the HFD group. In vitro, quercetin effectively inhibited the ox-LDL induced ROS formation in MPMs, and blocked the vital step in activation of NADPH oxidase — membrane translocation of p47phox. Our findings suggest that regular consumption of dietary quercetin plays a role in preventing atherosclerosis giving its evident regulatory effect on subunits of NADPH oxidase.

1. Introduction

Quercetin is one of the most abundant flavonoids existed in human diet. Several existing epidemiologic studies suggest that supplementation of quercetin displays a protective effect on cardiac patients or populations at cardiovascular risk (Pfeuffer et al., 2013; Serban et al., 2016). At present, many properties have been proved to make quercetin a most promising 'nutriceutical' for cardiovascular disease prevention. Immuno-histochemical studies demonstrate that quercetin specifically accumulates in human atherosclerotic lesions, but not in the normal aorta (Kawai et al., 2008). In apolipoprotein E-knockout mice (ApoE KO mice) quercetin inhibits the atherogenesis by interfering with foam cell formation and reducing the prooxidant/proinflammatory responses associated to macrophages activation (Lara-Guzman et al., 2012). In addition, quercetin alleviates the inflammatory detrimental effects induced by hyper cholesterol diet in rabbits, thus suggesting an outstanding effect of quercetin on inhibiting inflammation in atherosclerotic progression in vivo (Bhaskar et al., 2013). Nevertheless, up to now, the exact mechanisms responsible for the protective effects reported for quercetin on atherosclerosis have not been fully elucidated.

It is increasingly evident that ROS initiate key intracellular signals that dictate cellular responses to a variety of stresses important in atherogenesis and its related cardiovascular events (Madamanchi et al., 2005). Unfortunately, most of the well-known

* Corresponding author. Department of Nutrition and Food Hygiene, School of Public Health, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China.
** Corresponding author. Department of Nutrition and Food Hygiene, School of Public Health, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China.
E-mail addresses: tyh043@126.com (Y. Tang), yaoping@mails.tjmu.edu.cn (P. Yao).

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Abbreviations

ApoE KO	apolipoprotein E-knockout
GSH	glutathione
H&E	hematoxylin and eosin
H2DCF-DA	2′,7′-Dichlorodihydrofluorescein diacetate
HFD	high fat diet
MDA	malonaldehyde
MPMs	mouse peritoneal macrophages
mRNA	messenger RNA
NADPH	nicotinamide-adenine dinucleotide phosphate
Ox-LDL	oxidized low-density lipoprotein
PBS	phosphate-buffered saline
ROS	reactive oxygen species

antioxidants such as vitamins C and E, and β-carotene do not show any clinical efficacy to prevent cardiovascular events (Fortmann et al., 2013), even though they can, at high concentrations, chemically quench ROS in vitro and in experimental animal models of cardiovascular disease. These unexpected results suggest that knowledge of the vital enzymes responsible for ROS generation in vascular disease is urgently required. It is more important to inhibit ROS generation directly by targeting these vital enzymes rather than to scavenge the highly reactive molecules when they have been formed. It has been well-documented that NADPH oxidase, especially of infiltrated macrophages into atherosclerotic plaque, is the major source of ROS in vasculature and is a key player in mediating redox signaling under the pathophysiological situations of cardiovascular disease. Previous studies have shown that NADPH oxidase activation is correlated with both severity of atherosclerosis (Sorescu et al., 2002) and features of plaque stability in human coronary arteries (Azumi et al., 2002). However, little research is focused on the regulatory effects of quercetin on NADPH oxidase subunits expression in vivo.

Recently, a few evidences from in vitro studies have demonstrated that quercetin suppresses ROS overproduction induced by oxidized low-density lipoprotein (ox-LDL) in human endothelial cells and bone marrow-derived macrophages (Hung et al., 2015; Lara-Guzman et al., 2012), and the suppression of NADPH oxidase, or blockage of vicious circle between LDL oxidation and inflammatory attack, may contribute to the protective effect. Moreover, p47phox is an essential subunit of NADPH oxidase, and its translocation from cytosol to membrane has been presumed as one of the critical early steps for NADPH oxidase activation. Nevertheless, whether interfering with p47phox membrane translocation is alternative potential mechanism of quercetin to suppressing the ROS generation induced by ox-LDL in macrophages infiltrated into atherosclerotic plaque, there is no data available to date.

Therefore, the objectives of our present study were to explore the effects of quercetin on atherosclerosis prevention and aortic NADPH oxidase subunits expression in ApoE KO mice, and to investigate the underlying mechanism of quercetin inhibiting the overproduction of ROS triggered by ox-LDL in mouse peritoneal macrophages.

2. Materials and methods

2.1. Chemicals and reagents

Quercetin (purity: 98%), 2-thiobarbituric acid (TBA), 5,5'-dithobis (2-nitrobenzoic acid) (DTNB), 1, 1, 3, 3-tetraethoxypropane (TTOP), glutathione (GSH) and ROS assay kit were purchased from Sigma-Aldrich (St Louis, Missouri, USA). Anti-GAPDH rabbit polyclonal antibody, anti-rabbit IgG (secondary antibody), anti-mouse IgG (secondary antibody), Alexa Fluor 594 fluorescence-conjugated anti-rabbit IgG (secondary antibody) and DAPI were obtained from Cell Signaling Technology. Anti-gp91phox, anti-p67phox and anti-NOX4 rabbit monoclonal antibodies were provided by Abcam Inc. Anti-p22phox, anti-p47phox rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology (USA). The kits of Glutathione (GSH) and Malonaldehyde (MDA) were provided by the Nanjing Jiancheng Corporation (Nanjing, China). The commercial kit of NADPH oxidase activity was purchased from Genmed Scientifics Inc., (Shanghai, China). Ox-LDL was obtained from Yiyuan Biotechnologies (Guangzhou, China). Reagents for cell culture were bought from Gibco (Grand Island, USA). All other chemicals and organic solvents were of reagent grade and purchased from a local reagent supplier.

2.2. Animal and treatment

Six-week-old male ApoE KO mice weighing 18–20 g with the genetic background of C57BL/6 J (n = 75), were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. After one week of acclimatization with chow diet, the animals (n = 15 per group) were randomly assigned to one of groups with following diets: 1) normal diet (ND), 2) high fat diet (HFD), 3) high fat + low dose of quercetin (HFD + LQR; HF + 25 mg/kg bw quercetin), 4)high fat + middle dose of quercetin (HFD + MQR; HF + 50 mg/kg bw quercetin), 5) high fat + high dose of quercetin (HFD + HQR; HF + 100 mg/kg bw quercetin). High fat diet was comprised of 21% fat and 1.25% cholesterol. Quercetin was added to the HFD + QR diet by cold processing and diets were stored at 4 °C in light-protective, airtight containers. The doses of quercetin (25, 50, 100 mg/kg bw) to mice were largely equivalent to 125, 250, and 500 mg/d for human adults according to the ratio of body surface between mice and human (Reagan-Shaw et al., 2008), approximating to average daily intake of flavonoids in adult population with quercetin as one of the most common one (Ivey et al., 2015; Jun et al., 2016; Lajous et al., 2016).

Animals were treated in compliance with the Guiding Principles in the Care and Use of Laboratory Animals published by the US National Institutes of Health, and all animal procedures were approved by the Tongji Medical College Council on Animal Care Committee. All mice were housed in shoebox cages with corncob bedding in a temperature-controlled room (25 °C ± 2 °C) under 12-h light/12-h dark cycles and were given water and diet *ad libitum* for 24 weeks. Food consumption was calculated weekly through weighing food before and after a 48 h period and body weight was measured weekly.

2.3. Atherosclerosis lesion assessment

After 24 weeks, three animals were randomly selected from each group for en face aorta Oil-Red O staining. As previously described (Liu et al., 2016), after perfusion with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde via the left ventricle, the heart and aorta were removed, and then whole aortas were dissected from the heart, opened longitudinally and stained with Oil Red O for en face morphometric analysis of the atherosclerotic lesions. Images were captured with Canon IXUS 220 HS camera. The lesion size of the aorta was analyzed by Image Pro Plus 6.0.

After feed-deprived for 8 h, the remaining animals were sacrificed by cervical dislocation. Serum was collected from blood by centrifuge at 3500 g for 10 min. Aortas were snap-frozen in liquid nitrogen and stored at −80 °C until use.