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Quercetin-3-*O*-glucuronide induces ABCA1 expression by LXRα activation in murine macrophages

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ABSTRACT

Reverse cholesterol transport (RCT) removes excess cholesterol from macrophages to prevent atherosclerosis. ATP-binding cassette, subfamily A, member 1 (ABCA1) is a crucial cholesterol transporter involved in RCT to produce high density lipoprotein-cholesterol (HDLC), and is transcriptionally regulated by liver X receptor alpha (LXRα), a nuclear receptor. Quercetin is a widely distributed flavonoid in edible plants which prevented atherosclerosis in an animal model. We found that quercetin-3-*O*-glucuronide (Q3GA), a major quercetin metabolite after absorption from the digestive tract, enhanced ABCA1 expression, *in vitro*, via LXRα in macrophages. In addition, leaf extracts of a traditional Asian edible plant, *Nelumbo nucifera* (NNE), which contained abundant amounts of quercetin glycosides, significantly elevated plasma HDLC in mice. We are the first to present experimental evidence that Q3GA induced ABCA1 in macrophages, and to provide an alternative explanation to previous studies on arteriosclerosis prevention by quercetin.

1. Introduction

Excess cholesterol accumulation is associated with multiple diseases, especially atherosclerotic cardiovascular disease [1]. During atherosclerosis development, macrophage-derived foam cells accumulate excess cholesterol in a plaque, which is a characteristic feature of atherosclerosis [2]. To prevent atherosclerosis progression, reverse cholesterol transport (RCT) activation by high density lipoprotein (HDL) is important in transporting excess cholesterol from peripheral tissues to the liver, where it is subsequently converted to bile acids and excreted. During this process, ABCA1 plays a central role in removing excess cholesterol from the cells by elevating HDLC levels through the efflux of free cholesterol to lipid-poor apoA-I, a major apolipoprotein of HDL [3]. Mutations in ABCA1 causes Tangier disease, where patients cannot produce HDLC, resulting in cholesterol deposition in tissue macrophages and prevalent atherosclerosis [4]. Similarly, ABCA1 knockout mice virtually lack HDLC [5]. In contrast, increased ABCA1 activity elevates plasma HDLC levels by increased efflux of cholesterol from macrophages and protects against atherosclerosis in mice [6]. ABCA1 is widely expressed and is abundant in macrophages [7]. ABCA1 expression is transcriptionally regulated by a nuclear receptor known as liver X receptor alpha (LXRα), whose endogenous ligands include a variety of oxidized cholesterol derivatives referred to as oxysterols [8]. LXRα is thought to be a sterol sensor, protecting the cells from cholesterol overload by stimulating RCT [9]. LXRα performs a dominant role in limiting atherosclerosis *in vivo*, whereas another isoform, LXRβ which is not thought to limit atherosclerosis, is ubiquitously expressed [10]. To prevent atherosclerosis, synthetic LXRα ligands have been developed: oral administration of synthetic LXRα ligands increased plasma HDLC levels in mice [11]. However, a representative LXRα ligand had a profound side effect: it caused hypertriglyceridemia and hepatic steatosis by activating *sterol regulatory element binding transcription factor 1c* (*SREBP1c*), and *fatty acid synthase* (*FAS*) in the liver [12].

Quercetin is a flavonoid, reported to prevent atherosclerosis in an animal model [13], and it is widely distributed as glycosides in edible plants. During absorption from the digestive tract, quercetin glycosides and quercetin aglycon are quickly converted

Abbreviations: ABCA1, ATP-binding cassette, subfamily A, member 1; HDL, high density lipoprotein; HDLC, high density lipoprotein-cholesterol; LXRα, liver X receptor alpha; NNE, *Nelumbo nucifera* leaf extracts; Q3GA, quercetin-3-*O*-glucuronide; RCT, reverse cholesterol transport.

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to Q3GA [14,15]. Recently, Q3GA was thought to not only be a detoxified metabolite but also a hydrophilic anti-oxidant and precursor of hydrophobic quercetin aglycon [16]. Significant increases in HDLC by oral quercetin aglycon consumption [17], and Q3GA accumulation in macrophages of atherosclerotic arteries were reported in humans. These data imply there is a relationship between anti-atherosclerosis effects and Q3GA in humans [18]. To understand the mechanisms underlying the anti-atherosclerosis effects of quercetin *in vivo*, Q3GA activation of the RCT mechanism in macrophages should be studied.

Nelumbo nucifera has been utilized as a herbal medicine, and dried leaves have been traditionally used for tea in Japan. The leaves contain quercetin glycosides [19,20]. In this study, we investigated the effects of *N. nucifera* leaf extract (NNE) feeding on murine RCT *in vivo*, and the *in vitro* contribution of Q3GA to RCT in macrophages.

2. Materials and methods

2.1. Materials

Quercetin and quercetin-3-*O*-rutinoside were purchased from Sigma–Aldrich (MO, USA), quercetin-3-*O*-glucoside and quercetin-3-*O*-galactoside were provided by EXTRASYNTHESE (Genay, France). Geranylgeraniol was purchased from Sigma–Aldrich.

2.2. Preparation of NNE

Dried *N. nucifera* leaves were purchased from a local market. Extraction was performed according to Ono et al. [21] with slight modifications. Briefly, the dried leaves (200 g) were extracted with 4 L of 15% aqueous ethanol at room temperature for 1 h. The extract was filtered, and then concentrated under reduced pressure. The concentrate was lyophilized to give 41.0 g (yield 20.5%) of dark brown extract.

2.3. HPLC analysis of NNE

NNE was dissolved in 50% ethanol (10 μg/μl, w/v), and 20 μl (NNE 200 μg) was subjected to HPLC. The HPLC system used a Waters 2996 Photodiode Array Detector and Waters 2690 Separations Module: column, CAPCELL-PAC AQ C18, 4.6 × 250 mm, 5 μm (Shiseido, Japan); solvent system, acetonitrile/water linear gradient solvent system containing 0.1% (v/v) formic acid from 5% to 27.5% (v/v) acetonitrile in 50 min; flow rate, 1.0 mL/min. Column oven was kept at 40 °C.

2.4. Structural analysis of isolated compounds

Quercetin-3-*O*-arabinopyranosyl-(1 → 2)-galactopyranoside and quercetin-3-*O*-glucuronide were isolated using semi-preparative HPLC from NNE: system controller, SCL-10Avp (Shimadzu); column oven, L-7300 (Hitachi); UV–Vis detector, SPD-10A (Shimadzu); pump, LC-10ADvp (Shimadzu); Degasser, DGU-14A (Shimadzu); column, CAPCELL-PAC AQ C18, 10 × 250 mm, 5 μm (Shiseido); flow rate, 4.7 ml/min; solvent system; acetonitrile/water linear gradient solvent system containing 0.1% (v/v) formic acid from 5% to 27.5% (v/v) acetonitrile in 50 min. ^{1}H and ^{13}C-NMR data of isolated compounds (TMS as internal standard) were recorded in CD_3OD on a Bruker AM-400 spectrometer at 400 MHz.

2.5. Cell culture

The mouse macrophage-like cell line, RAW264.7, was maintained in RPMI1640 medium supplemented with 10% FCS (v/v) and 1% penicillin/streptomycin (v/v). The cells were inoculated at 0.5×10^6 cells in 35 mm dishes and incubated for two days, the medium was then changed with fresh medium containing the appropriate concentration of test sample.

2.6. Animals and diets

Male BALB/c mice (5 weeks old) were obtained from Charles River Japan (Tokyo, Japan). The mice were housed under a 12 h light/12 h dark cycle in a temperature- and humidity-controlled room. They were assimilated for one week and fed *ad libitum* with the CE-2 diet (CLEA Japan, Tokyo, Japan). The mice were divided into two groups matched for body weight (control group, $n = 8$; NNE group, $n = 7$), and fed AIN-93G [22] with or without 5% NNE for two weeks. Food intake and body weights were measured once a week. The study was conducted in accordance with the guidelines for animal care, handling, and termination from Kirin Company, which are in line with international and Japanese guidelines of animal care and welfare.

2.7. Measurements of liver and plasma lipids contents

To measure the liver lipids content, 0.2 g tissue was homogenized and extracted with chloroform:methanol mixture (2:1 v/v), as previously described [23]. The amounts of extracted liver lipids and plasma lipids were quantified using Triglyceride G Test Wako, Phospholipid G Test Wako and Total-Cholesterol G Test Wako (Wako Pure Chemicals, Osaka, Japan), respectively. Lipoprotein fractionation (Chylomicron; very low-density lipoprotein, VLDL; low-density lipoprotein, LDL; and HDL) and HDL subfractionation of plasma were performed using the HPLC column method (LipoSEARCH, Skylight-biotech, Tokyo, Japan) [24].

2.8. RNA preparation and quantitative RT-PCR from cultured cells and tissues

Total RNA was isolated from RAW264.7 using Qiashredder and RNeasy Mini kits (Qiagen, Hilden, Germany). Trizol (Invitrogen, Carlsbad, CA) and RNeasy Mini kits were used according to manufacturer's instructions. Three micrograms of total RNA were utilized for reverse transcription using oligo (dT) primers with the ThermoScript RT-PCR system (Invitrogen). The resulting products were subjected to quantitative RT-PCR using a LightCycler PCR system and FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Basel, Switzerland). The relative expression levels of genes were normalized using *ribosomal protein large P0* (*36B4*) for RAW264.7 and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) for liver. The primers used for PCR are described in Supplementary material 1.

2.9. Immuno-blotting

Total proteins were prepared from RAW264.7 according to a previously described method [25]. Briefly, cells were washed with PBS, and lysed in 10 mM Tris–HCl (pH 7.5), 1 mM $MgCl_2$, 0.25% SDS, and 1% Triton X-100 in the presence of protease inhibitors (Complete Protease Inhibitor Cocktail; Roche Applied Science). Cellular extracts were centrifuged to remove debris. For ABCA1 immuno-blotting, 20 μg of total protein were reduced using loading buffer (without boiling), and separated using 3–8% NuPAGE™ Tris acetate gels (Invitrogen), and transferred to PVDF membranes (Hybond P; GE Healthcare, Waukesha, WI, USA). Rabbit polyclonal antibody to mouse ABCA1 (Novus Biologicals, Littleton, CO, USA), and anti-rabbit Ig horseradish peroxidase linked whole antibody from donkey (Amersham Life science, Amersham, UK) were used for immuno-blotting. Signals were detected using chemiluminescence