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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

(ECL Plus; GE Healthcare) according to the manufacturer's protocol. Anti-actin for broad range species detection (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a loading control. Protein concentration was determined according to the method of Bradford with bovine serum albumin as a standard [26].

2.10. In vitro LXRα activation measurement

In vitro LXRα activation was measured using the LXRα RCAS kit (FUJIKURA KASEI, Tsukuba, Japan) according to manufacturer's instructions. All tested samples were dissolved in dimethyl sulfoxide.

2.11. Statistical analyses

All values are presented as the mean ± s.d. The Mann–Whitney's U test, and Student's *t*-test were used in the statistical evaluation of the results. Significance was assumed if the *p* value was <0.05.

3. Results

3.1. NNE feeding elevated HDLC in mice

Dietary supplementation of 5% NNE increased plasma cholesterol in mice (Fig. 1A). In these mice, only HDLC was significantly increased; non-HDLC was not increased (Fig. 1B). Cholesterol content in fractionated HDL revealed that NNE feeding increased subfractions 3 and 4, namely large and medium HDL cholesterol (Fig. 1C). These results clearly showed that NNE could increase the content of plasma HDLC. While NNE feeding did not significantly alter plasma triglyceride and glucose levels, plasma triglyceride suppression was observed (Supplementary material 2). No significant differences in body weight and epididymal white adipose tissue weight were observed between the two groups (Supplementary material 3), except for liver weight (control, 0.90 ± 0.05 g; NNE, 0.97 ± 0.07 g: $p < 0.05$ using Mann–Whitney's U test). Liver triglyceride, cholesterol and phospholipid content were not significantly different between the two groups (Table 1).

3.2. Flavonoid profile and effects on ABCA1 expression in macrophages treated with NNE

HPLC analysis of NNE at 360 nm showed five detectable peaks, namely compounds 1–5, within the expected retention time ranges of flavonoid glycosides (Fig. 2A). Compounds 2, 3, and 4 were identified as quercetin-3-*O*-rutinoside, quercetin-3-*O*-galactoside, and quercetin-3-*O*-glucoside, respectively, using co-chromatography with authentic standards. Purified compounds 1 and 5 were subjected to 1H- and 13C-NMR analyses, and identified as quercetin-3-*O*-arabinopyranosyl-(1 → 2)-galactosylpyranoside and Q3GA, respectively, through comparison with previously reported flavonoid glycosides [14,20]. As shown in Fig. 2A, Q3GA is the most abundant flavonoid glycoside. The concentration of each compound in NNE is shown in Fig. 2A, and was calculated using a quercetin-3-*O*-rutinoside standard curve at 360 nm. NNE contains a total of 11.7% (w/w) quercetin glycosides.

To evaluate the involvement of macrophages in HDLC elevation of NNE fed mice, we investigated the effects on *ABCA1* mRNA expression in a mouse macrophage cell line, RAW267.4. As shown in Fig. 2B, NNE was fed for 24 h, dose dependently, resulting in an increase in *ABCA1* mRNA, which showed biphasic increments with a transient peak at 4 h. The biphasic increment was also observed in immuno-blotting (Fig. 2C), suggesting that NNE contained multiple active ingredients to up-regulate ABCA1 protein expression. To elucidate the effect of quercetin glycoside, the quercetin glycoside fraction, containing compounds 1–5, was separated from non-quercetin fractions using semi-separative HPLC. A total of 310.3 mg NNE was subject to separation: 49.8 mg quercetin glycosides fraction and 230.2 mg non-quercetin glycosides fraction were recovered. Both fractions induced *ABCA1* mRNA expression levels, but the induction pattern differed. Consecutive *ABCA1* mRNA up-regulation was observed using the quercetin glycosides fraction,

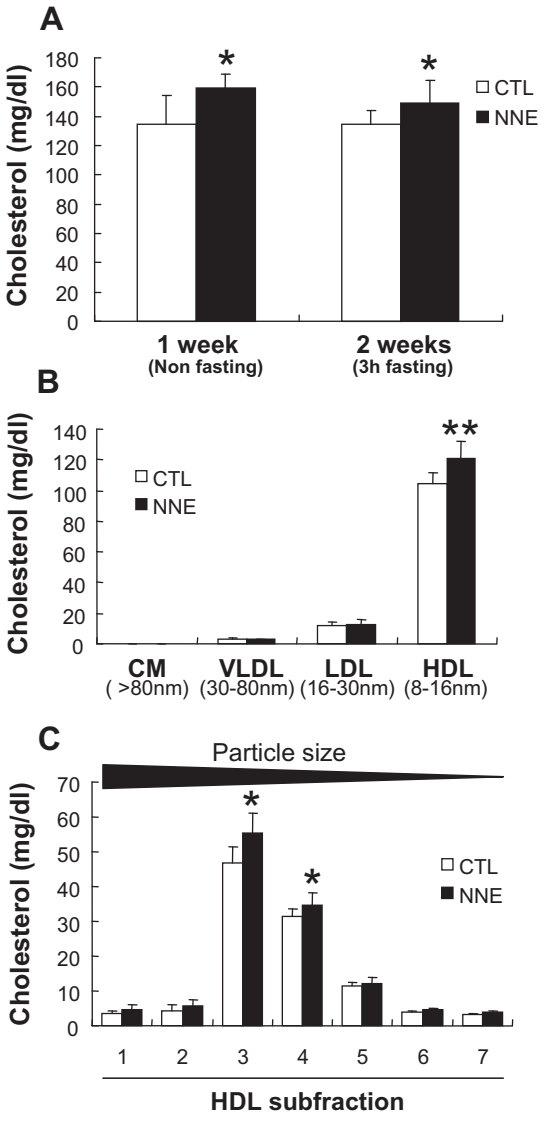

Fig. 1. NNE increased HDLC in mice. (A) Cholesterol content of mouse plasma, one or two weeks after the start of NNE feeding. (B) Cholesterol content of fractionated lipoproteins. (C) Cholesterol content of fractionated HDL. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. the control. Mann–Whitney's U test was used for statistical analyses. CTL, control mice; NNE, *N. nucifera* leaf extract fed mice.

Table 1
Liver lipids of NNE fed mice.

	Control	NNE
Cholesterol	10.2 ± 1.0	10.2 ± 0.8
Phospholipids	22.6 ± 2.4	23.2 ± 1.2
Triglycerides	49.0 ± 8.3	55.8 ± 9.7

All values are presented as the mean of μg per mg protein ± s.d. Statistical evaluation using the Mann–Whitney's U test showed no significant difference between the two groups. NNE, NNE fed mice.

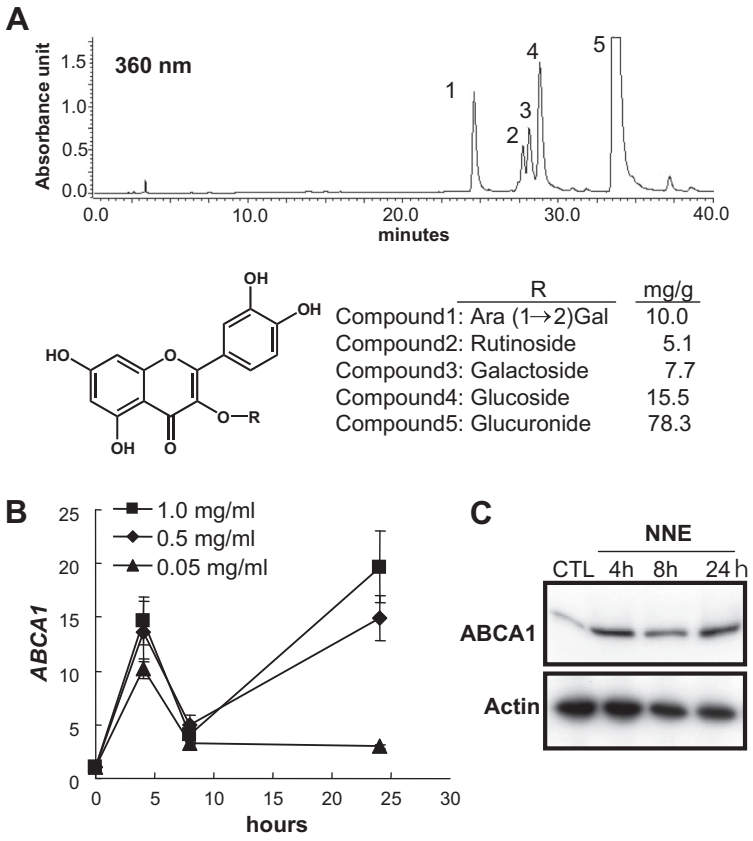

Fig. 2. Flavonoid profile of NNE and its effects on ABCA1 expression in macrophages. (A) HPLC chromatogram and quercetin glycosides detected in NNE. Compound 1, quercetin-3-*O*-arabinopyranosyl-(1 → 2)-galactopyranoside; 2, quercetin-3-*O*-rutinoside; 3, quercetin-3-*O*-galactoside; 4, quercetin-3-*O*-glucoside; 5, quercetin-3-*O*-glucuronide (Q3GA). (B, C) Effects of NNE on ABCA1 expression were estimated by quantitative RT-PCR (B), and immuno-blot analyses (C) in RAW264.7. Expression levels of mRNA were normalized by *36B4*. Actin was used as the load control for the immuno-blot. In immuno-blotting, the cells were treated with 0.5 mg/ml NNE.

while the non-quercetin glycosides fraction transiently induced *ABCA1* mRNA up-regulation at 4 h (Supplementary material 4).

3.3. Q3GA induces ABCA1 expression in RAW267.4

We subsequently focused on the quercetin glycosides fraction, as it produced consecutive *ABCA1* mRNA enhancement and we could technically identify and utilize each compound of this fraction. Among the quercetin glycosides in NNE, RT-PCR showed Q3GA (Compound 5) was the most effective in enhancing *ABCA1* mRNA expression; quercetin-3-*O*-arabinopyranosyl-(1 → 2)-galactopyranoside (Compound 1) and quercetin aglycon also significantly up-regulated *ABCA1* mRNA expression. Three other quercetin glycosides did not enhance *ABCA1* mRNA expression (Fig. 3A).

Immuno-blotting was performed to investigate protein expression level (Fig. 3B). Among the quercetin glycosides in NNE, only Q3GA (Compound 5) was effective in increasing ABCA1 at the protein level. Quercetin-3-*O*-arabinopyranosyl-(1 → 2)-galactopyranoside (Compound 1) and quercetin aglycon did not increase ABCA1 at the protein level, but did increase it at the mRNA level. These results indicated Q3GA was the most active ingredient in enhancing ABCA1 in macrophages among the quercetin glycosides detected in NNE.

3.4. Q3GA is a LXRα ligand

ABCA1 mRNA expression is transcriptionally regulated by nuclear receptor LXRα, which is activated, in turn, by chemical ligands. To reveal whether LXRα was involved, LXRα antagonist (geranylgeraniol) effects on *ABCA1* mRNA expression was investigated in macrophages [27]. RT-PCR showed *ABCA1* mRNA enhancement by 50 μM Q3GA was 34.4% suppressed by 50 μM geranylgeraniol treatment ($p = 0.08$, Student's *t*-test). In addition, the effects of NNE and quercetin aglycon were suppressed by 79.0% ($p < 0.01$, Student's *t*-test) and 43.5% ($p < 0.05$, Student's *t*-test), respectively, by geranylgeraniol treatment. A synthetic LXRα ligand T0901317 (3 μM) was also repressed by 44.8% by geranylgeraniol treatment ($p < 0.01$, Student's *t*-test). The data implied that LXRα ligands were contained in NNE, and Q3GA and quercetin aglycon were LXRα ligands.

To confirm involvement of LXRα, affinity levels between recombinant LXRα and the LXXLL motif peptide of co-activator steroid receptor coactivator-1 were investigated *in vitro*. The binding level of LXRα with the co-activator has been used previously to estimate LXRα activation [28]. Our results indicated that NNE, Q3GA, and quercetin aglycon could significantly enhance the binding level LXRα and the co-activator (Fig. 4), suggesting that Q3GA and quercetin aglycon are LXRα ligands.

4. Discussion

In this study, we first showed that NNE elevated plasma HDLC levels, indicative that RCT was activated by NNE. Medium and large size HDLC were increased (Fig. 1C), suggesting that ABCA1 mainly contributed to increasing HDLC because it could transport cholesterol into lipid-depleted HDL apolipoproteins, producing medium or large HDL [3]. The induction level of *ABCG1*, another cholesterol transporter, was ca. 10-times lower than *ABCA1* in macrophages (Supplementary material 5) and it could not transport cholesterol into lipid-depleted HDL apolipoprotein [29,30], suggesting that

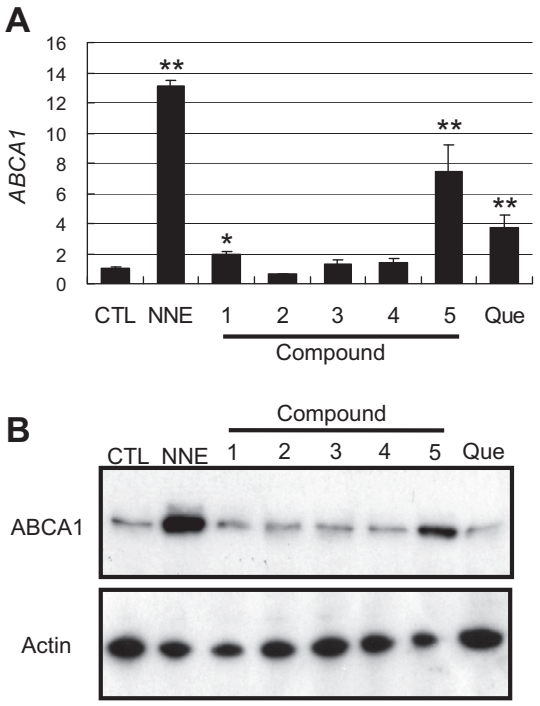

Fig. 3. Q3GA enhanced ABCA1 expression in macrophages. ABCA1 mRNA and protein expression levels were measured by quantitative RT-PCR (A) and immunoblot (B), respectively. The cells were treated with 50 μM of each compound or 0.5 mg/ml NNE for 24 h. Expression levels of mRNA were normalized using *36B4*. Actin was used as the load control for immuno-blotting. Compound 1, quercetin-3-*O*-arabinopyranosyl-(1 → 2)-galactopyranoside; 2, quercetin-3-*O*-rutinoside; 3, quercetin-3-*O*-galactoside; 4, quercetin-3-*O*-glucoside; 5, quercetin-3-*O*-glucuronide (Q3GA). $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. the control. All *p* values were determined using the Student's *t*-test. CTL, control; NNE, *N. nucifera* leaf extract; Que, quercetin.

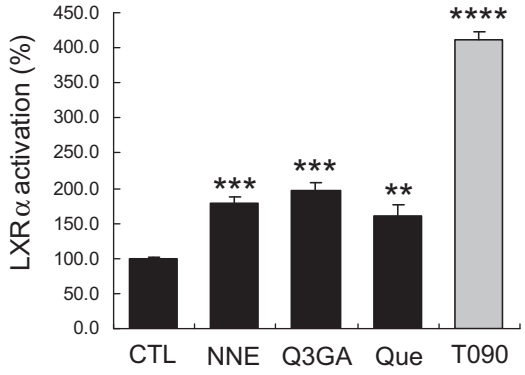

Fig. 4. Q3GA activated LXR. The effects of each sample on affinity level between LXRα and peptide containing the LXXLL motif of the co-activator steroid receptor coactivator-1, which is used to estimate LXRα activation. NNE, 1.0 mg/ml; Q3GA and quercetin, 50 μM; T090 (positive control), 25 nM. $^{**}p < 0.01$; $^{***}p < 0.001$, $^{****}p < 0.0001$ vs. the negative control. All *p* values were determined using Student's *t*-test. CTL, control; NNE, *N. nucifera* leaf extract, Q3GA; quercetin-3-*O*-glucuronide; Que, quercetin; T090, T0901317 synthetic LXRα ligand.

ABCG1 contribution may be limited to elevating HDLC by NNE feeding.

Quercetin usually exists as a sugar-bound form in edible plants: NNE contains quercetin as a glycoside (Fig. 2A). Fortunately, the major quercetin glycoside in NNE was Q3GA, enabling us to purify and utilize it for an *in vitro* study to investigate the effects of the major circulating quercetin metabolite in macrophages. Q3GA could enhance ABCA1 expression in macrophages, partly by LXRα activation. Our data suggested that NNE feeding supplied Q3GA to the plasma and macrophages where Q3GA may then induce ABCA1 expression in macrophages, via LXRα activation, producing an elevation in HDLC.

NNE contained an abundant amount of quercetin glycosides as the source of Q3GA, a LXRα ligand. A synthetic LXRα agonist caused hypertriglyceridemia and hepatic steatosis by inducing *SREBP1c* and *FAS* mRNA in the liver [12]. However, NNE did not significantly elevate liver triglycerides (Table 1). In addition, NNE did not induce liver *SREBP1c*, *FAS* or *ABCA1* mRNA levels in the liver (Supplementary material 6). These data indicated that NNE feeding did not activate LXRα in hepatocytes.

A previous report showed that Q3GA was taken up and deconjugated to quercetin aglycon in macrophages [18]. Our results showed 50 μM quercetin aglycon induced ABCA1 mRNA, even when 50 μM Q3GA was applied to macrophages *in vitro*, suggesting that quercetin aglycon may be involved in ABCA1 mRNA induction. During the preparation of this manuscript, Chang et al. reported quercetin aglycon enhanced *ABCA1* expression in macrophages: 100 μM quercetin aglycon significantly induced Sp1 and LXRα binding to the *ABCA1* promoter via a p38 dependent pathway, which subsequently enhanced ABCA1 protein expression *in vitro* [31]. They also showed knockdown of p38 significantly attenuated the enhancement of Sp1 and LXRα binding to the *ABCA1* promoter, however, only LXRα binding enhancement was partially detected, whereas Sp1 binding was completely lost [31]. Our data showed that quercetin aglycon and Q3GA could directly activate LXRα (Fig. 4), suggesting there is an alternative route for quercetin to enhance ABCA1 expression in macrophages: one route is the p38 dependent Sp1 and LXRα activation, as reported by Chang et al., and the other is direct LXRα activation, as presented in this report. Interestingly, Q3GA was more effective in inducing ABCA1 than the same *in vitro* concentration of quercetin aglycon in our experiments (Fig. 3). The data suggests that Q3GA may affect other mechanisms to enhance ABCA1 besides the p38 dependent pathway and direct activation of LXRα, and further studies are required to reveal the difference between Q3GA and quercetin aglycon in ABCA1 expression.

Our findings provide the first experimental evidence of enhancement of ABCA1 expression through LXRα activation by Q3GA in macrophages. Because Q3GA is a major circulating metabolite of dietary quercetin, our results could be important in explaining the HDLC elevation after quercetin oral administration observed in a recent human study [17] and a previous animal model experiment in which quercetin prevented arteriosclerosis [13]. Quercetin is a natural product which can be added to functional foods and beverages to produce potential hypolipidemic activity by enhancing cholesterol removal from macrophages to prevent arteriosclerosis.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.168.

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