Effect of quercetin and its metabolite on caveolin-1 expression induced by oxidized LDL and lysophosphatidylcholine in endothelial cells

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Oxidized low-density lipoprotein contributes to atherosclerotic plaque formation, and quercetin is expected to exert anti-atherosclerotic effects. We previously reported accumulation of conjugated quercetin metabolites in the aorta of rabbits fed high-cholesterol diets with quercetin glucosides, resulting in attenuation of lipid peroxidation and inhibition of lipid accumulation. Caveolin-1, a major structural protein of caveolae in vascular endothelial cells, plays a role in atherosclerosis development. Here we investigated effects of oxidized low-density lipoprotein, quercetin and its metabolite, quercetin 3-O-β-glucuronide, on caveolin-1 expression. Oxidized low-density lipoprotein significantly upregulated caveolin-1 mRNA expression. An oxidized low-density lipoprotein component, lysophosphatidylcholine, also induced expression of both caveolin-1 mRNA and protein. However, lysophosphatidylcholine did not affect the location of caveolin-1 proteins within caveolae structures. Co-treatment with quercetin or quercetin 3-O-β-glucuronide inhibited lysophosphatidylcholine-induced caveolin-1 expression. Quercetin and quercetin 3-O-β-glucuronide also suppressed expression of adhesion molecules induced by oxidized low-density lipoprotein and lysophosphatidylcholine. These results strongly suggest lysophosphatidylcholine derived from oxidized low-density lipoprotein contributes to atherosclerotic events by upregulating caveolin-1 expression, resulting in induction of adhesion molecules. Quercetin metabolites are likely to exert an anti-atherosclerotic effect by attenuating caveolin-1 expression in endothelial cells.

Key Words: caveolin-1, adhesion molecule, quercetin, quercetin metabolite, vascular endothelial cell

Quercetin (3,3',4',5,7-pentahydroxyflavone; Q), a typical flavonol-type flavonoid, frequently presents as its glycoside form in fruits and vegetables. Epidemiological studies indicate dietary intake of flavonoids, including Q, is inversely associated with risk of cardiovascular diseases such as coronary heart disease2,3 and stroke.3 A Japanese study reported that intake of flavonoids, in particular Q, inversely correlated with plasma total cholesterol and low-density lipoprotein (LDL) concentrations.4 In addition to reducing oxidative stress, dietary Q appears to exert effects on vascular function by elevating both endothelial nitric oxide synthase expression and blood glutathione redox ratio.5 Dietary Q is converted to glucurononate and/or sulfate derivatives, or their O-methyl derivatives, during absorption into the body, and presents exclusively as these conjugated metabolites in circulating blood.6 In a previous study utilizing high-cholesterol fed rabbits, we reported that dietary intake of Q glucoside resulted in accumulation of its conjugated metabolites within the aorta, leading to inhibition of aortic lipid peroxidation and accumulation.(7) We also demonstrated that Q 3-O-β-glucuronide (Q3GA), one of Q’s major conjugated metabolites, can return to its aglycone form to exert physiological functions in activated macrophages, an event expected to be involved in early atherosclerosis development.(8,9) Although oxidative damage to endothelial cells may profoundly affect vascular functions, effects of Q metabolites on vascular endothelial cells remain obscure.

Circulating LDL is oxidized in intima and resulting oxidized LDL (ox-LDL) plays diverse roles in atherosclerosis pathogenesis and progression.10 In the early stages of atherosclerotic plaque formation, ox-LDL activates endothelial cells by inducing cell surface adhesion molecules, especially intracellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), which mediate rolling and adhesion of monocytes.11 Caveolae, a lipid raft subtype, are 50–100 nm flask-shaped invaginations of plasma membrane, which are abundant in endothelial cells. Caveolin-1 (CAV-1) is a major structural protein of caveolae.12 Endothelial-specific over-expression of CAV-1 accelerates progression of atherosclerosis by reducing endothelial cell proliferation, migration and nitric oxide (NO) production leading to VCAM-1 expression.13 Caveolae and CAV-1 mediate LDL uptake/transcytosis and regulate LDL entry into arterial walls.14-16 Furthermore, it has been suggested that CAV-1 directly regulates expression of these adhesion molecules.15 These facts suggest CAV-1 plays a critical role in regulation of endothelial cell activation. Therefore, we aimed to clarify effects of Q metabolites on ox-LDL-induced CAV-1 expression and adhesion molecule expression in human umbilical vein endothelial cells (HUVEC).

Materials and Methods

Reagents. Quercetin dehydrate, α-tocopherol, L-α-lysophosphatidylcholine (lysoPC) and rabbit polyclonal antibody to CAV-1 were purchased from Sigma-Aldrich (St. Louis, MO). Human LDL and human oxidized-LDL (low-thiobarbituric acid reactive) were obtained from Biomedical Technologies (Stoughton, MA). Q3GA was obtained from Extrasynthese (Genay, France). 13-Hydroperoxyoctadecadienoic acid (13-HPODE) was obtained from Larodan Fine Chemicals AB (Malmo, Sweden). 13(S)-Hydroxyoctadecadienoic acid (13-HODE) was obtained from Cayman Chemical (Ann Arbor, MI). 4-Hydroxyxenonenal (4-HNE) was obtained from Percipio Biosciences (Burlingame, CA). Rabbit polyclonal antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and anti-phospho-CAV-1 antibody were obtained from

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Cell Signaling Technology (Beverly, MA). Secondary antibody, polyclonal goat anti-rabbit immunoglobulin/horseradish peroxidase was obtained from Dako (Glostrup, Denmark).

**Cells and culture.** HUVEC (Lonza, Basel, Switzerland), were cultured in endothelial cell growth medium, EGM-2 (Lonza), and maintained at 37°C in a humidified atmosphere containing 5% CO₂. Cells were used in experiments from passages 3–10.

**Real-time reverse-transcription polymerase chain reaction (RT-PCR).** HUVEC were plated into 24-well plates (4 × 10⁴ cells/well) and incubated for 24 h. Cells were treated with ox-LDL or related compounds, as described in figure legends. To estimate effects of flavonoids, they were added to cells at the same time as lipids or related compounds. In 6 h pretreatment experiments, flavonoids were washed out with Hank’s balanced salt solution buffer, which was replaced with fresh medium containing lipids or related compounds. Total RNA was isolated from HUVEC using ISOGEN (Nippon Gene, Toyama, Japan). mRNA expression was determined by real-time RT-PCR, as previously described. Primers used to examine genes are listed in Table 1. Primers for CAV-1 and GAPDH were obtained from Takara Bio, Inc. (Otsu, Japan). Primers for ICAM-1 and VCAM-1 were synthesized according to previous report.

Reaction conditions for RT and PCR were based on protocols provided by Applied Biosystems (Foster City, CA). Relative levels of gene expression for each sample were calculated using a comparative Ct method. Expression of target genes (CAV-1, ICAM-1 and VCAM-1) in each sample was normalized to GAPDH Ct values. Data are expressed as mean ± SD of three separate experiments.

**Cell viability assay.** HUVEC were plated into 96-well plates (2 × 10⁴ cells/well). After a 24 h incubation, culture medium was replaced with 100 μl of fresh medium. Ox-LDL-related lipids were dissolved in ethanol and added to culture medium, followed by incubation for an additional 24 h. Cells were treated with 5 μl of Cell Proliferation Reagent WST-1 (Roche, Cat No. 11644807001, Indianapolis, IN) and incubated for 4 h before absorbance at 450 nm was measured. Cell viability is expressed as a percentage of vehicle-treated control.

**Western blotting.** HUVEC were seeded into 60-mm dishes (4 × 10⁴ cells/dish). After incubation for 24 h, cells were treated with lysoPC with or without flavonoid for an additional 24 h. Cell samples were washed twice with phosphate-buffered saline and lysed with lysis buffer [50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 20 mM EDTA, 1% sodium dodecyl sulfate (SDS) polyacrylamide, 0.5% sodium deoxycholate, 0.01% Nonidet P-40, protease inhibitor mixture (Complete EDTA-free), and phosphatase inhibitor tablet (PhosSTOP™)]. Protein concentration was determined by bicinchoninic acid protein assay (Thermo Fisher Scientific, Waltham, MA). Protein samples were boiled with reducing buffer (Nacalai Tesque, Kyoto, Japan) for 5 min.

Samples (10 μg) were separated by 10% SDS polyacrylamide gel electrophoresis. Proteins were transferred onto Immobilon-P® polyvinylidene fluoride transfer membranes (Millipore, Billerica, MA) followed by 1 h blocking of non-specific binding with a commercial blocking buffer (Blocking-One for CAV-1 or Blocking-One P for phospho-CAV-1, Nacalai Tesque, Kyoto, Japan). Membranes were incubated with an anti-CAV-1 antibody (1:5,000 dilution), anti-phospho-CAV-1 antibody (1:200) or anti-GAPDH antibody (1:3,000) for 1 h at room temperature. After washing with Tris-buffered saline containing 0.05% Tween® 20 (TBST), membranes were incubated for 1 h at room temperature with secondary antibody. After washing with TBST, membranes were visualized using Amersham Enhanced Chemiluminescence (ECL™) Prime detection reagents (GE Healthcare, Buckinghamshire, UK). Images were captured with a Lumino Image Analyzer LAS-3000 mini (Fujifilm, Tokyo, Japan).

**Partition of microdomains and non-microdomains in cell membranes.** HUVEC were seeded into 60-mm dishes (4 × 10⁴ cells/dish). After incubation for 24 h, cells were treated with lysoPC or methyl-beta-cyclodextrin (MβCD). Cell lysates were subjected to ultracentrifugation and fractionated as previously described. Isolation of microdomains was confirmed by dot blots, and CAV-1 of each fraction was detected.

**Statistical analyses.** Data are expressed as mean ± SD from at least three independent experiments. Statistical analyses were performed using PASW Statistics 18.0 (IBM, Armonk, NY). Data were analyzed by one-way analysis of variance with Tukey comparison test (p<0.05).

### Results

**Effect of ox-LDL on CAV-1 mRNA expression and suppression by Q and Q3GA.** CAV-1 mRNA expression was upregulated by ox-LDL in the range of 10–100 μg/ml, in a concentration-dependent manner (Fig. 1). In contrast, LDL itself did not exert such an effect. Next, HUVEC were exposed to ox-LDL (100 μg/ml) simultaneously with Q (1, 10 μM) or Q3GA (1, 10 μM) and incubated for 12 h to examine effects of Q and Q3GA on ox-LDL-induced CAV-1 mRNA expression. Significant suppression of ox-LDL-induced CAV-1 mRNA expression was observed with co-administration of 10 μM Q (Fig. 1). Q3GA also tended to suppress CAV-1 mRNA expression, although this effect was not significant.

**Effect of lysoPC and ox-LDL-related lipid peroxidation products on CAV-1 mRNA expression.** First, toxicity of lysoPC and lipid peroxidation products derived from ox-LDL was examined using a WST-1 assay (Fig. 2A). While administration of 0.1 or 1 μM lysoPC did not affect cell viability, 1 μM 4-HNE decreased cell viability by 55%. Neither 13-HPODE nor 13-HODE affected cell viability, even at 10 μM. For our treatment condition, we decided to use a concentration showing non-cytotoxicity. Therefore, concentrations of each compound varied in the following experiments. CAV-1 mRNA expression was upregulated by lysoPC at 0.1 μM, but not 1 μM, whereas 0.1 μM 4-HNE had no effect on expression. 13-HPODE, but not 13-HODE, upregulated CAV-1 mRNA expression at 10 μM (Fig. 2B). CAV-1 mRNA expression induced by 0.1 μM lysoPC increased in a time-dependent manner for 24 h (Fig. 2C); however, lysoPC did not induce phosphorylation of CAV-1 (Fig. 2D).

**Effect of lysoPC on CAV-1 distribution in cell membranes.** Effect of lysoPC on CAV-1 distribution in cell membrane lipid rafts was examined using an ultracentrifugation technique (Fig. 2E). In control cells (only treated with vehicle), CAV-1 existed in the third to fifth fractions (lipid raft region). MβCD, which disrupts lipid rafts by removing cholesterol from cell membranes, broadened observance of CAV-1 through to the membrane lipid rafts.
seventh fraction. After incubation of HUVEC with 0.1 μM
lysoPC, distribution of CAV-1 remained unchanged in the lipid
raft region (third to fifth fractions). Thus, it was confirmed that
lysoPC did not affect location of CAV-1 in cellular membranes.

**Effect of Q and Q3GA on lysoPC-induced CAV-1 expres-
sion.** Both Q and Q3GA suppressed lysoPC-induced CAV-1
mRNA expression (Fig. 3A). CAV-1 protein levels were upregu-
lated by addition of lysoPC for 48 h (Fig. 3B), whereas 10 μM Q
suppressed upregulation of CAV-1 protein levels. Q3GA also
tended to suppress CAV-1 protein upregulation, although this was
not significant. To avoid direct interaction between Q/Q3GA and
lysoPC in culture medium, Q or Q3GA were pre-incubated
with cells for 6 h before addition of lysoPC. After removal of
flavonoids from culture medium, cells were treated with lysoPC.
Pre-incubation experiments also indicated that Q and Q3GA
suppressed lysoPC-induced CAV-1 mRNA expression (Fig. 3C).

**Effect of α-tocopherol on lysoPC-induced CAV-1 mRNA
expression.** We used α-tocopherol as a lipophilic antioxidant
capable of exerting function in biomembranes. Fig. 4 shows co-
administration of both 1 and 10 μM α-tocopherol suppressed
lysoPC-induced CAV-1 mRNA expression.

**Effect of Q and Q3GA on lysoPC- or ox-LDL-induced
ICAM-1 and VCAM-1 mRNA expression.** We first confirmed
that mRNA expression of both ICAM-1 and VCAM-1 were
upregulated by ox-LDL in a time-dependent manner with 24 h
treatment (Fig. 5). After 18 h of incubation with ox-LDL, expres-
sion of both adhesion molecules was significantly upregulated.
Treatment with ox-LDL for 24 h significantly upregulated ICAM-1
mRNA expression (Fig. 6A), which was significantly suppressed by
Q and Q3GA in a concentration-dependent manner in the range
examined (between 0.1 and 10 μM; Fig. 6A). VCAM-1 mRNA
expression tended to be upregulated by ox-LDL, although no
significant difference was observed compared with controls at
24 h (Fig. 6A).

Both ICAM-1 and VCAM-1 mRNA expression were upregu-
lated by addition of ox-LDL, but LDL itself did not show an
elevating effect (Fig. 6B). Both Q and Q3GA were found to signif-
icantly suppress expression of ICAM-1 and VCAM-1 induced by
ox-LDL (Fig. 6B).

**Discussion**

Results from our previous study using cholesterol-fed rabbits
suggested dietary Q accumulates as conjugated metabolites within
the aorta, where it attenuates lipid peroxidation and hyper-
lipidemia. Our immunohistochemical study using a monoclonal
antibody demonstrated that Q metabolites target activated macro-
phages, resulting in selective deposition in human atherosclerotic
arteries. Nevertheless, human epidemiological and interven-
tional studies imply dietary Q exerts multiple mechanisms of
action at diverse targets to protect vascular tissues from athero-
sclerosis. In this study, we focused on vascular endothelial cells as
an alternative target of dietary Q. Endothelial activation and dys-
function are an initial event in atherosclerotic plaque formation,
and endothelial CAV-1 plays a proatherogenic role. Furthermore,
ox-LDL plays a critical role in pathogenesis and progression of
atherosclerosis by several mechanisms including upregulation of
CAV-1 expression. However, little is known about the effects of
dietary Q on ox-LDL-induced endothelial activation through
CAV-1 expression. Therefore, we investigated the effect of ox-
LDL on CAV-1 expression, as well as the modulating effect of
Q and its major metabolite, Q3GA, on ox-LDL-induced CAV-1
expression in HUVEC.

CAV-1-knockout mice showed lower expression of adhesion
molecules, VCAM-1 and ICAM-1. Fernandez-Hernando
et al. observed transgenic mice overexpressing CAV-1 in endo-
thelial cells have increased expression of VCAM-1. Fu et al. suggested CAV-1 co-localizes with ICAM-1 in caveolae struc-
tures and modulates ICAM-1-dependent translocation of mono-
cyes. In addition, an Src family kinase responsible for CAV-1
signaling activates downstream elements of the ICAM-1 pathway,
resulting in ICAM-1 expression throughout caveolae structure.
Fig. 2. Effect of lysoPC and ox-LDL-related lipid peroxidation products on CAV-1 mRNA expression and effect of lysoPC on CAV-1 distribution in cell membranes. (A) Cell viability in lysoPC or ox-LDL-related lipid peroxidation products-treated HUVECs. HUVECs were incubated with lysoPC, 4-HNE, 13-HODE or 13-HPODE for 24 h. (B) CAV-1 mRNA expression in HUVECs. HUVECs were incubated with ox-LDL related lipid for 24 h. (C) Effect of lysoPC (0.1 μM) on CAV-1 expression at 12, 18, and 24 h. (A–C) Values represent mean ± SD (n = 3). Letters represent significant differences among treatment groups. (D) Effect of 24 h lysoPC treatment on CAV-1 phosphorylation in HUVECs in the absence (−) or presence (+) of sodium orthovanadate (Na$_3$VO$_4$). (E) Effect of lysoPC on CAV-1 distribution in microdomain fractions in HUVECs. After incubation of HUVECs with lysoPC for 24 h or MβCD for 30 min, cell lysates were split into nine fractions and CAV-1 distribution was determined by dot blot analysis.
Fig. 3. Effect of Q and Q3GA on lysoPC-induced CAV-1 expression in HUVECs. (A) CAV-1 mRNA expression is induced in HUVECs by lysoPC and co-addition of Q/Q3GA for 24 h. (B) Western blot of CAV-1 and density of each image analyzed. HUVECs were incubated with lysoPC with Q or Q3GA for 48 h. (C) CAV-1 mRNA expression induced in HUVECs by 6 h preincubation with Q/Q3GA and subsequent incubation with lysoPC for 24 h. (A–C) Values represent mean ± SD (n = 3). Letters represent significant differences among treatment groups.
Hydroperoxides are lipid peroxidation products of ox-LDL that include peroxidation products, as well as their degradation products. Lipid peroxidation products of ox-LDL can result in the deposition of lipids into arterial walls, overexpression of adhesion molecules. CAV-1 also promotes the attenuation of CAV-1 expression to protect endothelial cells from atherosclerotic events.

Thus, it is likely CAV-1 expression plays an essential role in the expression of adhesion molecules and their function in endothelial cells. A study in humans showed increased CAV-1 expression in endothelial cells from atherosclerotic patients. CAV-1 expression was also elevated in smokers’ endothelial cells compared with non-smokers.

Our in vitro study indicated expression of ICAM-1 and VCAM-1, along with CAV-1, was promoted by exposure to ox-LDL (Fig. 1 and 6B). That both Q and Q3GA suppressed expression of adhesion molecules and CAV-1 (Fig. 1 and 6B) strongly indicates Q metabolites accumulated within the aorta have the ability to attenuate CAV-1 expression to protect endothelial cells from overexpression of adhesion molecules. CAV-1 also promotes deposition of lipids into arterial walls, by mechanisms of LDL uptake or transcytosis through direct binding to cholesterol and/or fatty acid moieties. Suppression of aortic hyperlipidemia by intake of dietary Q may be derived from attenuation of CAV-1 expression and following LDL uptake or transcytosis.

Oxidative modification of LDL produces various kinds of lipid peroxidation products, as well as their degradation products. Lipid hydroperoxides are lipid peroxidation products of ox-LDL that decompose into aldehydes such as 4-HNE. LysoPC is generated by an LDL-associated, platelet-activating factor-acetylhydrolase-dependent hydrolysis of oxidized phosphatidylcholine.

Thus, it was hypothesized that lysoPC affects function of CAV-1 by changing its location in caveolae structure. However, this hypothesis was ruled out and it can be concluded that lysoPC induces expression of CAV-1 without affecting caveolae.

LysoPC-induced gene expression of CAV-1 was effectively suppressed by simultaneous addition of Q or Q3GA (Fig. 3A). In vascular endothelial cells, lysoPC has been reported to promote O2·− production via enhanced NADPH oxidase activity and induce mitochondrial reactive oxygen species (ROS) production. Here, a lipophilic antioxidant, α-tocopherol, also suppressed CAV-1 expression (Fig. 4), indicating antioxidant activity relates to attenuation of CAV-1 expression and subsequent adhesion molecule expression. CAV-1 expression in fibroblasts was promoted by hydrogen peroxide through activation of the p38 MAPK pathway, resulting from binding of specificity protein 1 (Sp1) to the promoter region, which increased transcription of CAV-1. Interestingly, Q has been shown to suppress promoter region activation and may suppress ROS-dependent signal transduction pathways by scavenging ROS generated during the incubation period. Pre-incubation of cells with Q or Q3GA before addition of lysoPC also resulted in effective inhibition of CAV-1 expression (Fig. 3C), suggesting a direct reaction of Q with lysoPC is not required to exert protective functions. It is noteworthy that both Q3GA and Q suppressed CAV-1 expression (Fig. 3A and C), although cellular uptake of Q3GA seems to barely occur because of its high hydrophilicity.

LysoPC-stimulated ICAM-1 and VCAM-1 expression in astrocytes was time-dependent manner (Fig. 5), although ICAM-1 increased more significantly than VCAM-1 after a 24 h incubation (Fig. 6A). LysoPC is known to promote expression of ICAM-1 and VCAM-1 at physiological concentrations, resulting in rolling and adhesion of monocytes. Activation of the PKC pathway may be involved in lysoPC-induced ICAM-1 expression in porcine coronary arterial endothelium.

Enhancement of ICAM-1 expression was effectively suppressed by Q and Q3GA (Fig. 6A). Q appears to be more effective than Q3GA with regard to lysoPC-induced adhesion molecule expression. Although Q is scarcely present in circulating blood in its aglycone form, circulating conjugated Q metabolites may be deconjugated in the inflammatory site by

Fig. 4. Effect of α-tocopherol on lysoPC-induced CAV-1 expression in HUVECs. HUVECs were incubated with lysoPC with α-tocopherol for 24 h. Values represent mean ± SD (n = 3). Letters represent significant differences among treatment groups.

Fig. 5. Effect of lysoPC treatment on ICAM-1 and VCAM-1 mRNA expression in HUVECs. HUVECs were incubated with lysoPC for indicated time (6, 12, 18 or 24 h). Values represent mean ± SD (n = 3). Letters represent significant differences among treatment groups.
efflux of β-glucuronidase from activated macrophages. Resulting Q aglycone may act as a more effective suppressor of CAV-1 expression than Q metabolites upon exposure to lysoPC and other components present in ox-LDL. Koga and Meydani also suggested the aglycone form is required for Q to exert effective suppression of monocyte cell adhesion to human aortic endothelial cells. Green tea polyphenols and one of their components, epigallocatechin-3-gallate, were reported to protect endothelial cells from CAV-1 expression by modulating MAPK signaling. While it is expected that Q protects endothelial cells in a manner similar to green tea polyphenols, it should be noted that concentrations and structures required for effect vary greatly depending on each polyphenol. This study indicates effective concentrations of Q and Q3GA to modulate CAV-1 expression are around 1 μM, which seems to be within the physiological concentration of circulating blood. Thus, dietary Q may act as a modulator of vascular endothelial functions leading to anti-atherosclerotic effects. This study may support results of previous interventional studies showing beneficial effects of flavonoid intake on reduction of cardiovascular disease risk and improvement of vascular endothelial function.

In conclusion, ox-LDL increased expression of CAV-1 mRNA, as well as expression of mRNA for adhesion molecules ICAM-1 and VCAM-1. Among ox-LDL-related components, lysoPC significantly stimulated CAV-1 and ICAM-1 expression, indicating lysoPC is partly responsible for the role of ox-LDL in mediating CAV-1 expression. Both Q and Q3GA suppressed ox-LDL and lysoPC-induced CAV-1 and adhesion molecule expression. Therefore, Q metabolites may exert anti-atherosclerotic effects by targeting CAV-1 expression in endothelial cells and dietary Q is a promising food factor to prevent atherosclerosis.

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