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Dietary Compound Quercitrin Dampens VEGF Induction and PPARγ Activation in Oxidized LDL-Exposed Murine Macrophages: Association with Scavenger Receptor CD36

Jung-Suk Choi,[†] Ji-Young Bae,[†] Dong Shoo Kim,[†] Jing Li,[†] Jung-Lye Kim,[†] Yong-Jin Lee,[‡] and Young-Hee Kang*,[†]

[†]Department of Food and Nutrition and Korean Institute of Nutrition, Hallym University, Chuncheon, 200-702 Korea and [‡]Chuncheon Bioindustry Foundation, Chuncheon, 200-161 Korea

Oxidized LDL (oxLDL) has been implicated in the pathogenesis of atherosclerosis accompanying lipid-laden cell appearance, inflammatory responses, and vascular dysfunction. This study examined the potentials of polyphenol quercitrin to inhibit oxLDL induction of scavenger receptor A (SR-A) and CD36 involving activation of peroxisome proliferator-activated receptor gamma (PPARγ). J774A1 murine macrophages were cultured with 10 μg/mL Cu^{2+}-oxLDL for various times in the presence of 1−10 μmol/L quercitrin. Cu^{2+}-oxLDL at the given concentration facilitated macrophage proliferation and enhanced oxLDL uptake. Quercitrin dampened oxLDL uptake and lipid accumulation elevated in macrophages exposed to oxLDL. Western blot analysis revealed that 10 μg/mL oxLDL upregulated expression of SR-A and CD36, which was rapidly abolished at the transcriptional levels by 10 μmol/L quercitrin within 4 h. Quercitrin diminished production of proinflammatory and proatherogenic vascular endothelial growth factor that augmented through the oxLDL binding to CD36. Similarly, quercitrin repressed expression of macrophage inflammatory protein-2 and monocyte chemoattractant protein-1 involved in monocyte trafficking and macropahage migration. In addition, quercitrin attenuated oxLDL-induced transcriptional activation of PPARγ leading to CD36 induction. Furthermore, quercitrin alleviated macrophage uptake of oxLDL through interfering with PKC-PPAR signaling cascades. These results demonstrate that quercitrin blocked oxLDL uptake, cholesterol influx and lipid-laden foam cell formation through inhibiting induction of SR and VEGF linked to PKCα-PPARγ-responsive pathways. Therefore, quercitrin may be an antiatherogenic agent blocking foam cell formation pertaining to induction of SR and VEGF.

KEYWORDS: Macrophage CD36; oxidized LDL; PPARγ; quercitrin; VEGF

INTRODUCTION

Oxidized LDL (oxLDL) is a potent inducer of inflammatory molecules and stimulates release of chemotactic proteins from endothelium. Since atherosclerosis is an inflammatory disorder of the arterial wall, therapeutic interventions targeting atherosclerosis should focus on blocking LDL oxidation. OxLDL is believed to be the most atherogenic form of LDL. Macrophages taking up oxLDL are transformed into lipid-laden foam cells, forming atherosclerotic plaques in the fatty streak lesions (*1*). OxLDL particles are recognized by macrophage scavenger receptor (SR) class A (SR-A), SR-B CD36 antigen (CD36), and macrophage antigen CD68 (*2*, *3*). Active components present in oxLDL including lipid hydroperoxides, oxysterols and lysophosphatidylcholine have been proposed to be formed by various cellular mediators such as reactive oxygen species (ROS) and myeloperoxidase regulate LDL oxidation (*4*).

*To whom correspondence should be addressed. Mailing address: Department of Food and Nutrition, Hallym University, Chuncheon, Republic of Korea. Phone: 82-33-248-2132. Fax: 82-33-254-1475. E-mail: yhkang@hallym.ac.kr.

It has been suggested that vascular endothelial growth factor (VEGF) well recognized as an angiogenic factor can promote macrophage migration critical for atherosclerosis (*5*). VEGF was upregulated in macrophages by oxLDL through increasing p38 mitogen-activated protein kinase (MAPK) activity and VEGF mRNA stability, suggesting the significance of VEGF in the progression of atherosclerosis (*6*). Moreover, VEGF high in atherosclerotic plaques increased monocyte recruitment and activation through enhanced expression of inflammatory monocyte chemoattractant protein (MCP)-1 (*7*). Peroxisome proliferator-activated receptor (PPAR) is involved in inflammatory responses (*8*). PPARγ is expressed in macrophage foam cells and atherosclerotic lesions and regulates the transcriptional process of several genes related to lipid metabolism (*9*, *10*). In addition, PPARγ was believed to play important roles in the macrophage cholesterol trafficking and the regulation of VEGF by oxLDL (*6*). Uptake of oxLDL by CD36 leads to generation of endogenous PPAR ligands from oxLDL such as hydroxyoctadecadienoic acid (HODE) (*6*).

The tea polyphenol epigallocatechin gallate (EGCG) reduced in vivo levels of ROS, important markers for inflammatory

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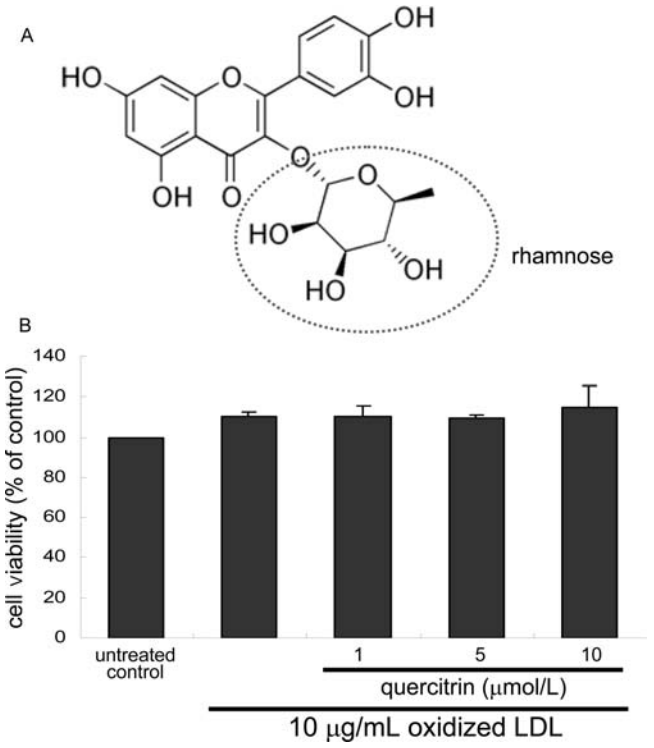

Figure 1. Chemical structure of quercitrin (**A**) and viability (**B**) of murine macrophages exposed to oxLDL in the absence and presence of quercitrin. J774A1 murine macrophages were pretreated with 1−10 μmol/L quercitrin and exposed to 10 μg/mL Cu^{2+}-oxLDL for 24 h. Cell viability was measured using MTT assay and presented as means ± SEM (n = 4) with multiple estimations. Values not sharing a letter are different at $P < 0.05$.

diseases (*11*). Berberine, natural constituent of the plant berberis, protected against oxLDL-induced endothelial apoptosis by inhibiting ROS generation (*12*). Oxidized lipids triggered ROS production and impairment of antioxidant systems, which effects were counterbalanced by dietary phenols (*13*). Quercitrin (quercetin 3-*O*-α-L-rhamnoside) is a glycoside extracted from the oak bark as a bitter and yellow dye citron. Quercitrin showed a stronger peroxyl radical-scavenging capacity than Trolox, a water-soluble vitamin E analogue (*14*). In addition, quercitrin effectively downmodulated proanaphylactic inducing immune responses and prevented allergic reaction (*15*). Cherries are rich in two important flavonoids, isoquercitrin and quercitrin, as well as anthocyanins. Consuming foods such as cherries has been shown to help neutralize cancer-causing agents, decreasing oxidative stress (*16*).

Based on literature evidence that quercitrin (**Figure 1A**) showed antioxidative activity (*14*), this study examined induction of CD36 and SR-A and VEGF, when 10 μg/mL oxLDL was applied to J774A1 murine macrophages treated with 1−10 μmol/L quercitrin. This study clarified a response network between CD36, VEGF and PPAR all enhanced in oxLDL-exposed macrophages. Furthermore, inhibitory mechanism(s) of quercitrin for the oxLDL uptake and cholesterol influx via CD36 induction were elucidated in relation to activation of PPARγ and protein kinase C (PKC) α.

MATERIAL AND METHODS

Materials. Fetal bovine serum (FBS), trypsin−EDTA and penicillin−streptomycin were purchased from Lonza Walkersvillle (Walkersvillle, MD). Flavonol quercitrin was obtained from Sigma-Aldrich (St. Louis, MO). Antibodies against SRA, CD36, VEGF, PPARγ and PKCα were obtained from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated goat antirabbit IgG and donkey antigoat IgG were provided by Jackson ImmunoResearch Laboratories (West Grove, PA). Quercitrin was dissolved in dimethyl sulfoxide (DMSO) for live culture with cells; a final culture concentration of DMSO was ≤0.05%.

Preparation and Oxidation of Human Plasma LDL. Human plasma LDL was prepared by a discontinuous density gradient ultracentrifugation as previously described (*17*). Pooled human normolipidaemic plasma LDL fraction was dialyzed overnight against 0.154 mol/L NaCl and 0.01% EDTA (pH 7.4) at 4 °C and used within 4 wks. Protein concentration of the plasma LDL fraction was determined by the Lowry method (*18*), and the concentrations of triacylglycerol, total cholesterol and phospholipids were measured using diagnostic kits (Asan Pharmaceuticals, Hwasung, Korea). The concentrations of total protein, total cholesterol, triacylglycerol and phospholipid in prepared LDL fractions were all within the appropriate ranges.

Fully Cu^{2+}-oxLDL was prepared by incubating with 5 μmol/L CuSO4 in F-10 medium at 37 °C for 24 h. The extent of LDL oxidative modification was regularly determined using thiobarbituric acid reactive substances and electrophoretic mobility assay (*19*). Aliquots of prepared LDL fraction were run on a 0.8% agarose electrophoresis gel in barbital buffer (pH 8.6) to measure electrophoretic mobility. The gel was fixed in a 5% trichloroacetic acid solution and rinsed in 70% ethanol. Gel photographs were obtained using a Polaroid film (Polaroid, Wayland, MA).

Cell Culture and Toxicity. The macrophage-like cell line J774A1 (mouse histocytic lymphoma cells) was grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS at 37 °C in a humidified atmosphere of 5% CO_2 in air. Macrophages were pretreated with 1−10 μmol/L quercitrin and exposed to 10 μg/mL cholesterol-oxLDL for various times.

After the incubation of J774A1 macrophages with Cu^{2+}-oxLDL in the absence and presence of quercitrin, the 3-(4,5-dimethylthiazol-yl)diphenyl tetrazolium bromide (MTT) assay was carried out to quantitative cellular viability. Cells were incubated in a fresh medium containing 1 mg/mL MTT for 3 h at 37 °C. The resultant purple formazan product was dissolved in 0.5 mL of isopropanol with gentle shaking. Absorbance of the formazan dye was measured at λ = 570 nm with background subtraction using λ = 690 nm.

Lipid Uptake. To identify foam cell formation of macrophages, Oil Red O staining (Sigma-Aldrich) was carried out. Oil Red O is a fat-soluble diazo dye used for staining of neutral triglycerides and lipids and some lipoproteins. After culture of J774A1 with 10 μg/mL oxLDL, cells were washed with PBS containing 0.05% Tween 20 and fixed in 4% ice-cold formaldehyde for 15 min. Thereafter, 0.5% Oil Red O dissolved in 2-propanol was added to cells and incubation allowed for 4 h. After mounting with aqueous mounting medium, images were obtained by using an optical microscope.

Western Blot Analysis. Western blot analysis was performed using whole cell extracts from J774A1 macrophages as previously described (*17*). Equal protein amounts of cell lysates were electrophoresed on 10% SDS−PAGE and transferred onto a nitrocellulose membrane. Nonspecific binding was blocked by soaking the membrane in 0.5 mol/L Tris-HCl (pH 7.5) buffer containing 1.5 mol/L NaCl and 1% Tween 20 (TBS-T) with 5% skim milk for 3 h. The membrane was incubated overnight at 4 °C with a polyclonal goat antibody of SR-A or VEGF and with a polyclonal rabbit antibody of CD36, PPARγ or PKCα. After three washes with TBS-T, the membrane was incubated for 1 h with a donkey antigoat IgG or goat antirabbit IgG conjugated to horseradish peroxidase. The individual protein level was determined using Super Signal West Pico chemiluminescsnce detection reagents (Pierce Biotechnology, Rockford, IL) and Agfa X-ray film (Agfa-Gevaert, Belgium). Incubation with monoclonal mouse β-actin antibody (Sigma-Aldrich Chemical) was also performed for comparative controls.

Analyses of Real-Time-Polymerase Chain Reaction (PCR). Following culture protocols, total RNA was isolated from J774A1 using a commercially available Trizol reagent kit (Molecular Research Center, Cincinnati, OH). The RNA (2 μg) was reversibly transcribed with 200 units of reverse transcriptase and 0.5 mg/mL oligo-$(dT)_{15}$ primer (Bioneer, Korea). The levels of mRNA transcripts of SR-A (forward primer, 5′-CAT GAA GAG GAT GCT GAC T-3′; reverse primer, 5′-GGA AGG GAT GCT GTC ATT GAA-3′) and CD36 (forward

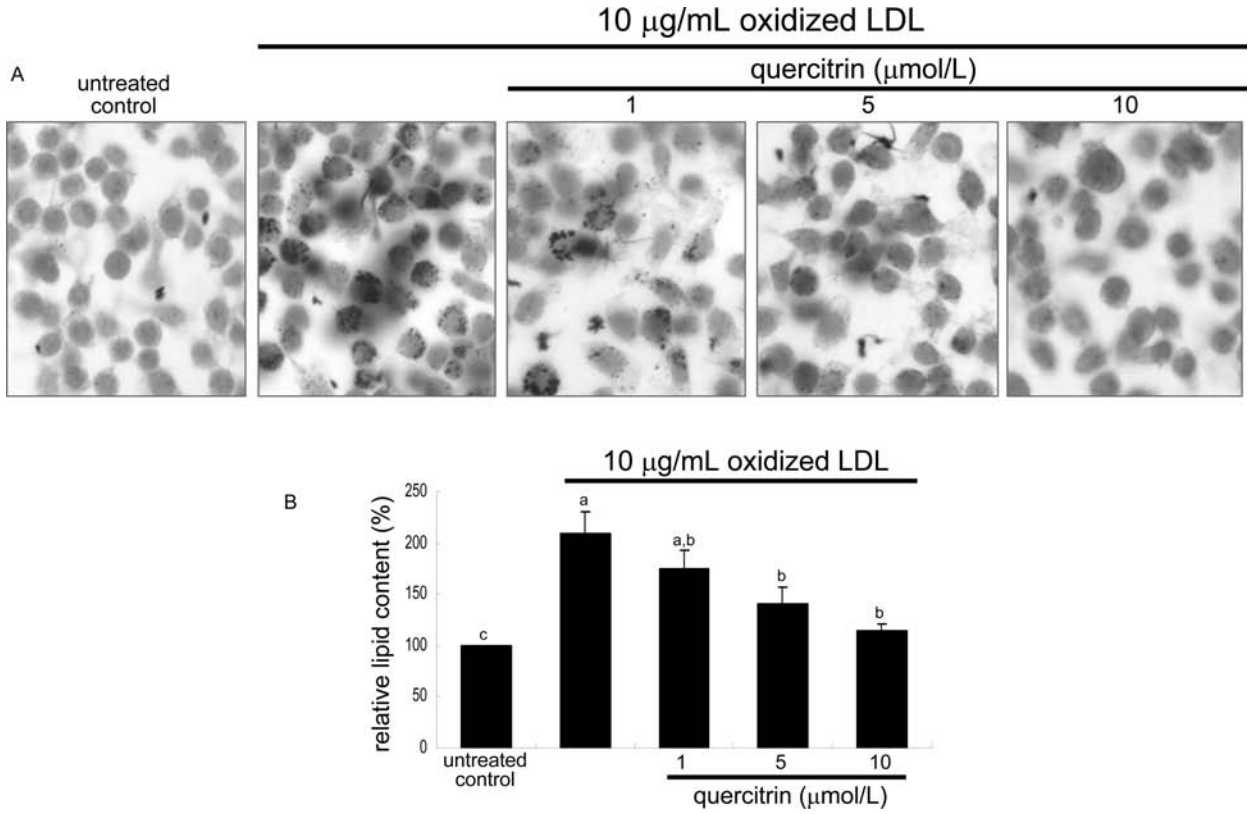

Figure 2. Inhibitory effects of quercitrin on oxLDL uptake of murine macrophages exposed to 10 μg/mL Cu^{2+}-oxLDL. J774A1 macrophages were pretreated with 1−10 μmol/L quercitrin and exposed to 10 μg/mL Cu^{2+}-oxLDL for 4 h (**A**). Cells were stained with 5 μg/mL Oil Red O, and microphotographs were obtained using an optical microscope (3 separate experiments). Magnification: 200×. For the measurement of lipid accumulation, cells were incubated with 10 μg/mL Cu^{2+}-oxLDL for 24 h (**B**). Data represent means ± SEM from 5 independent experiments with multiple estimations. Values not sharing a letter refer to significant different at $P < 0.05$.

primer, 5′-GCT TGC AAC TGT CAC AT-3′; reverse primer, 5′-GCC TTG CTG TAG CCA AGA AC-3′) were quantified by real-time RT-PCR using SYBR Green PCR kit (Quagen, Valencia, CA). The standard PCR conditions were 95 °C for 10 min, and 40 cycles at 95 °C (10 s), 60 °C (15 s) and 72 °C (20 s), followed by melting curve analysis. Analysis of real-time RT-PCR results and calculation of the relative quantitations were performed using the Delta CT analysis of Rotor-gene software version 6.0 (Corbett Research, Australia). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH; forward primer, 5′-TTG TCA AGC TCA TTT CCT GGT ATG-3′; reverse primer, GCC ATG TAG GCC ATG AGG TC-3′) was used for internal normalization.

Enzyme-Linked Immunoadsorbent Assay (ELISA). After culture protocols culture medium supernatants were collected and stored at −20 °C. The secretion of VEGF, MCP-1 and MIP-2 was measured in duplicate with a mouse ELISA QuantiGlo Kit (R&D systems, Minneapolis, MN) using a microplate reader (Molecular Devices, Sunnyvale, CA).

Data Analysis. The results are presented as mean ± SEM. Statistical analyses were conducted using the Statistical Analysis statistical software package from SAS Institute (Cary, NC). One-way ANOVA was used to determine inhibitory effects of quercitrin on the receptor induction of oxLDL in macrophages. Differences among treatment groups were analyzed with Duncan's multiple-range test and were considered to be significant at $p \leq 0.05$.

RESULTS

Inhibitory Effects of Quercitrin on Foam Cell Formation. During the 24 h incubation of J774A1 macrophages with 10 μg/mL Cu^{2+}-oxLDL, the cell viability substantially but insignificantly increased by ≈10%. Quercitrin at nontoxic doses of 1−10 μmol/L rendered the cell viability unchanged (**Figure 1B**). It should be noted that ≤10 μmol/L quercitrin *per se* did not result in lipid peroxidation (data not shown).

There was a heavy Oil Red O staining observed in macrophages exposed to 10 μg/mL oxLDL (**Figure 2A**). When 1−10 μmol/L quercitrin was applied to macrophages prior to treating oxLDL, the lipid droplet staining dwindled in a dose-dependent manner. Consistently, cellular lipid accumulation increased in oxLDL alone-treated macrophages, whereas quercitrin dose-dependently attenuated the cellular lipid accumulation (**Figure 2B**). These data show that ≥5 μmol/L quercitrin interrupted oxLDL uptake and foam cell formation in macrophages.

Inhibition of Macrophage Induction of CD36 and SR-A by Quercitrin. Macrophage uptake of oxLDL is known to entail induction of CD36 and SR-A. This study investigated whether quercitrin blocked induction in oxLDL-exposed J774A1 macrophages. The addition of oxLDL to cells instigated CD36 expression rapidly within 2−4 h, which was sustained up to 6 h (**Figure 3A**). Induction of SR-A was initiated by oxLDL in a similar manner to that of CD36 (data not shown). On the contrary, CD36 expression was dampened in cells incubated with oxLDL in the presence of 10 μmol/L quercitrin (**Figure 3B**). The SR-A induction by 4 h incubation with oxLDL was repressed by ≥5 μmol/L quercitrin. This indicates that quercitrin is effective in blocking a receptor-mediated uptake of oxLDL required for cholesterol influx.

The effects of quercitrin on oxLDL-elicited mRNA expression of CD36 and SR-A were determined by using real-time RT-PCR. Treatment of macrophages with oxLDL increased mRNA levels of CD36 and SR-A, compared to those of untreated control (**Figure 3C**). OxLDL-elevated mRNA expression of CD36 was downmodulated by ≥5 μmol/L quercitrin, In addition, the SR-A induction was abolished by 10 μmol/L quercitrin (**Figure 3C**).

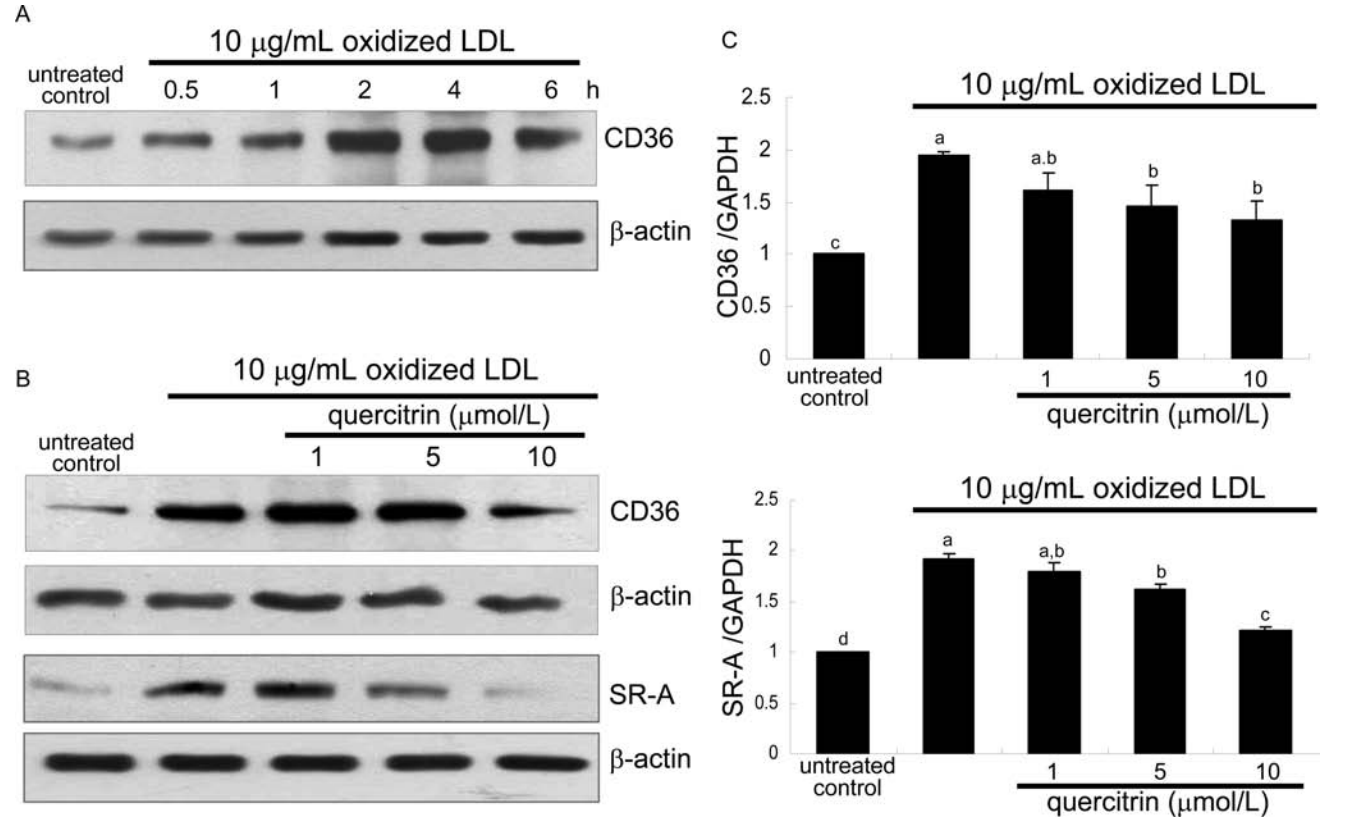

Figure 3. Western blot data showing the time course response of CD36 induction to oxLDL in macrophages (**A**) and the inhibition of oxLDL-induced expression of CD36 and SR-A by quercitrin (**B**). J774A1 cells were treated with 1−10 μmol/L quercitrin and exposed to 10 μg/mL Cu^{2+}-oxLDL. Total cell lysates were subjected to Western blot analysis with a primary antibody against CD36 and SR-A. Bands are representative of 3 independent experiments. β-Actin was used as an internal control. Real time RT-PCR (**C**) showing mRNA transcriptional levels of CD36 and SR-A in quercitrin-treated and oxLDL-stimulated macrophages for 2 h. GAPDH was used as a housekeeping gene for the coamplification with CD36 and SR-A. The bar graphs (means ± SEM, $n = 3$) represent quantitative mRNA transcript levels of CD36 and SR-A relative to GAPDH. Ratio values not sharing a letter refer to significant different at $P < 0.05$.

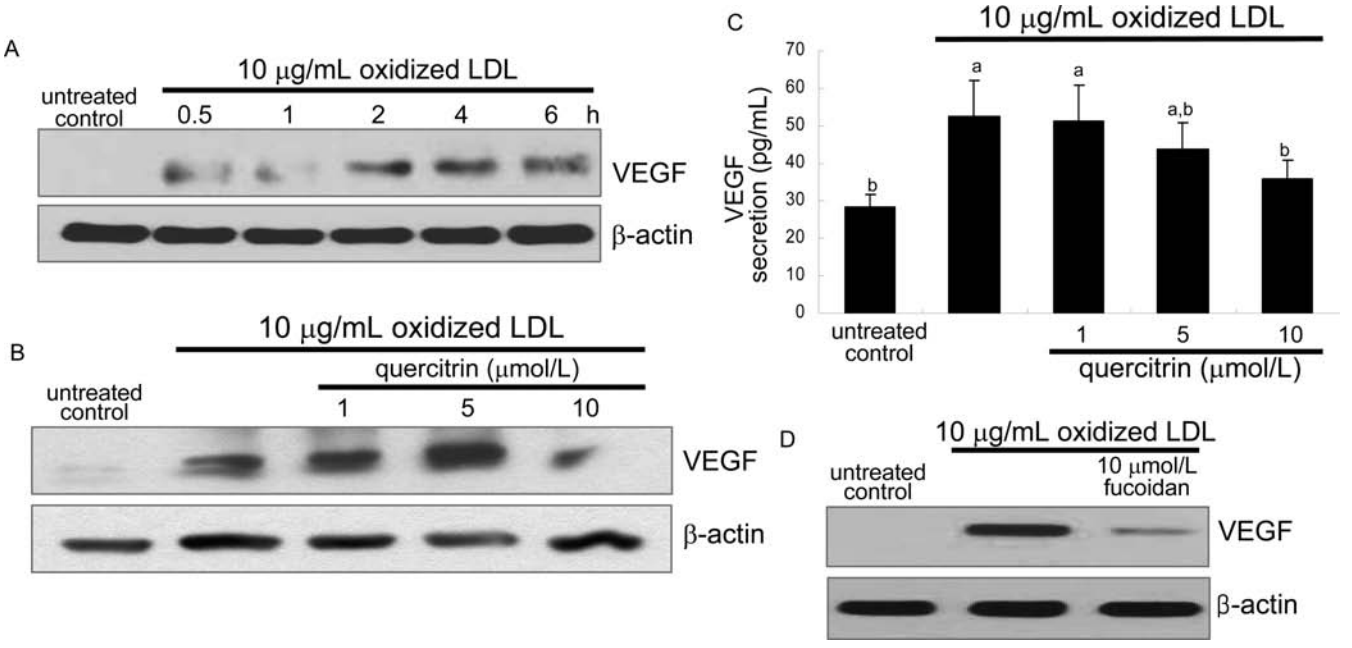

Figure 4. Time course response of VEGF expression (**A**) and effect of quercitrin on oxLDL-induced VEGF expression (**B**) and secretion (**C**). Cells were treated with 1−10 μmol/L quercitrin with and without a stimulation of 10 μg/mL Cu^{2+}-oxLDL. The VEGF production was measured 4 h after treatment with Cu^{2+}-oxLDL in the presence of quercitrin. For the measurement of VEGF cellular expression, total cell lysates were subjected to Western blot analysis with a primary antibody against VEGF (**B**). For the measurement of VEGF secretion, cell culture media collected were run using ELISA kits (**C**). Western blot analysis (**D**) showing involvement of oxLDL binding to CD36 in the VEGF expression. The CD36 inhibitor fucoidan at 10 μmol/L was added to oxLDL-treated cells for 4 h. Bands are representative of 3 independent experiments. β-Actin was used as an internal control. Bar graph data (**C**) represent means ± SEM from 3 separate experiments. Values not sharing a letter are different at $P < 0.05$.

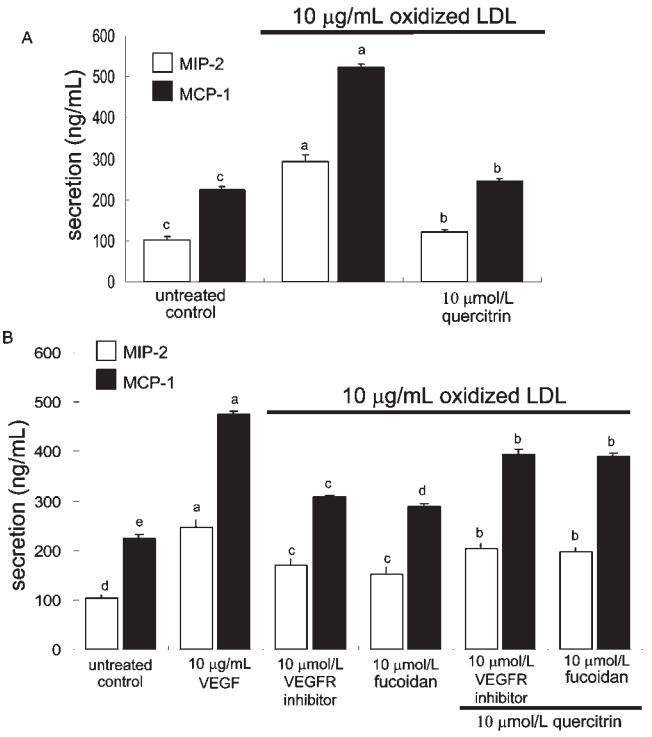

Figure 5. Secretion of MIP-2 and MCP-1 in macrophages exposed to oxLDL in the presence of quercitrin (**A**). J774A1 macrophages were treated with 10 μmol/L quercitrin in the absence and presence of 10 μg/mL Cu^{2+}-oxLDL for 6 h. For the measurement of secretion of MIP-2 and MCP-1, cell culture media were collected and run using ELISA kits. Effects of VEGF and CD36 on secretion of MIP-2 and MCP-1 in oxLDL-exposed macrophages (**B**). VEGF protein (10 μg/mL) alone was treated for 6 h for the secretion of MIP-2 and MCP-1 in macrophages. Each inhibitor of VEGFR (VEGF receptor tyrosine kinase inhibitor II) and CD36 (fucoidan) at 10 μmol/L was added to cells and exposed to oxLDL for 6 h in the absence and presence of quercitrin. Respective bar graph data represent means ± SEM from 3 separate experiments. Respective values not sharing a letter are different at $P < 0.05$.

These results imply that quercitrin inhibited induction of CD36 and SR-A via a direct modulation at their gene transcriptional levels.

Effect of Quercitrin on Production of VEGF and Inflammatory Chemokines. Proinflammatory cytokine VEGF has been shown to be involved in the monocyte recruitment and activation through enhancing expression of inflammatory MCP-1 (*7*, *20*). The cellular level of VEGF was rapidly increased following 4 h exposure of J774A1 cells to Cu^{2+}-oxLDL (**Figure 4A**). In contrast, 10 μmol/L quercitrin blocked VEGF expression upregulated by Cu^{2+}-oxLDL (**Figure 4B**). Consistently, quercitrin significantly attenuated the VEGF secretion followed by 6 h treatment with oxLDL (**Figure 4C**). This study further investigated whether VEGF expression was mediated via the oxLDL-upregulated CD36. The oxLDL-enhanced VEGF expression was abolished in the presence of 10 μmol/L CD36 inhibitor fucoidan (Sigma-Aldrich, **Figure 4D**).

The secretion of MIP-2 and MCP-1 was examined in macrophages exposed to oxLDL (**Figure 5A**). The secretion of MIP-2 and MCP-1 was markedly augmented in concomitance with production of VEGF while incubating macrophages with oxLDL. However, the increased production was significantly attenuated by preaddition of 10 μmol/L quercitrin. In addition, the secretion of MIP-2 and MCP-1 was elevated by VEGF but dampened by 10 μmol/L VEGFR inhibitor (VEGF receptor tyrosine kinase inhibitor II, Calbiochem, San Diego, CA) or 10 μmol/L CD36 inhibitor fucoidan. Secretion of MIP-2 and MCP-1 appeared to be mediated through the oxLDL-upregulated VEGF most likely via the CD36 receptor (**Figure 5B**). When the VEGFR inhibitor or fucoidan was added to macrophages in the presence of 10 μmol/L quercitrin, the accumulation of MIP-2 and MCP-1 was rather enhanced. This might be attributed to a masking effect of quercitrin on the inhibitors of VEGF and CD36, thus disturbing a receptor-mediated upregulation of production of MIP-2 and MCP-1.

Interruption of PPARγ Activation by Quercitrin. It has been shown that PPARγ is expressed in macrophage foam cells and atherosclerotic lesions (*9*, *10*). As expected, 10 μg/mL oxLDL stimulated PPARγ expression in macrophages (**Figure 6A**). The PPARγ activation was upmodulated in oxLDL-exposed macrophages as well, evidenced by nuclear levels of PPARγ. However, the treatment of ≥5 μmol/L quercitrin diminished the activation of PPARγ elevated in the presence of oxLDL (**Figure 6A**). The PPARγ expression was enhanced by oxLDL at its transcriptional level, which was highly attenuated by 10 μmol/L quercitrin (**Figure 6B**). It was investigated whether the oxLDL-elicited CD36 induction involved PPARγ activation in lipid-laden macrophages. The oxLDL-augmented CD36 was abrogated by addition of 10 μmol/L PPARγ inhibitor (GW9662, Sigma) to cells (**Figure 6C**).

Involvement of PKCα in PPAR Signaling for Induction of SR-A and CD36. OxLDL enhanced cellular PKCα level of murine macrophages, which was markedly diminished by 10 μmol/L quercitrin (**Figure 7A**). This study determined whether quercitrin alleviated macrophage uptake of oxLDL through interfering with PKC-PPAR signaling cascades. When 10 μg/mL PKCα inhibitor peptide (Santa Cruz Biotechnol., Santa Cruz, CA) was added to macrophages exposed to 10 μg/mL oxLDL, the induction of CD36 and SR-A was apparently attenuated in parallel with decreased PPARγ level (**Figure 7B**). Accordingly, the transcriptional induction of CD36 and SR-A involved instigation of PKCα-PPARγ signaling transduction. Quercitrin appeared to block the regulation of PKCα-PPARγ signaling pathways leading to induction of CD36 and SR-A involved in oxLDL uptake and cholesterol influx.

DISCUSSION

LDL oxidation is thought to be a critical early event in the pathogenesis of atherosclerosis (*1*). Macrophages taking up oxLDL are transformed into lipid-laden foam cells, dying and forming part of atherosclerotic plaques in the fatty streak lesions (*1*). Several cellular receptors involved in binding and internalizing oxLDL have been characterized as SR that unlike the LDL receptor is not subject to negative regulation by high levels of intracellular cholesterol. Macrophage SR include SR-A, CD36 and CD68 (*2*, *3*), in which SR-A was referred to as acetylated LDL receptor expressed on macrophages but not on peripheral monocytes (*21*). OxLDL is the natural ligand for SR-A, and partially competes for the binding of acetylated LDL to macrophages (*22*). CD36 has been identified as a receptor on murine macrophages that recognize oxLDL but not acetylated LDL (*2*), and it accounts for as much as 70% of oxLDL uptake in human macrophages. The SR of CD36 has been shown to be upregulated by cytokines present in atheromatous lesions (*23*). This study attempted to examine whether quercitrin modulated the induction of both CD36 and SR-A and their subsequent internalization of oxLDL in murine macrophages exposed to oxLDL. The possibility that quercitrin disturbed the access of oxLDL to SR proteins on macrophages cannot be ruled out.

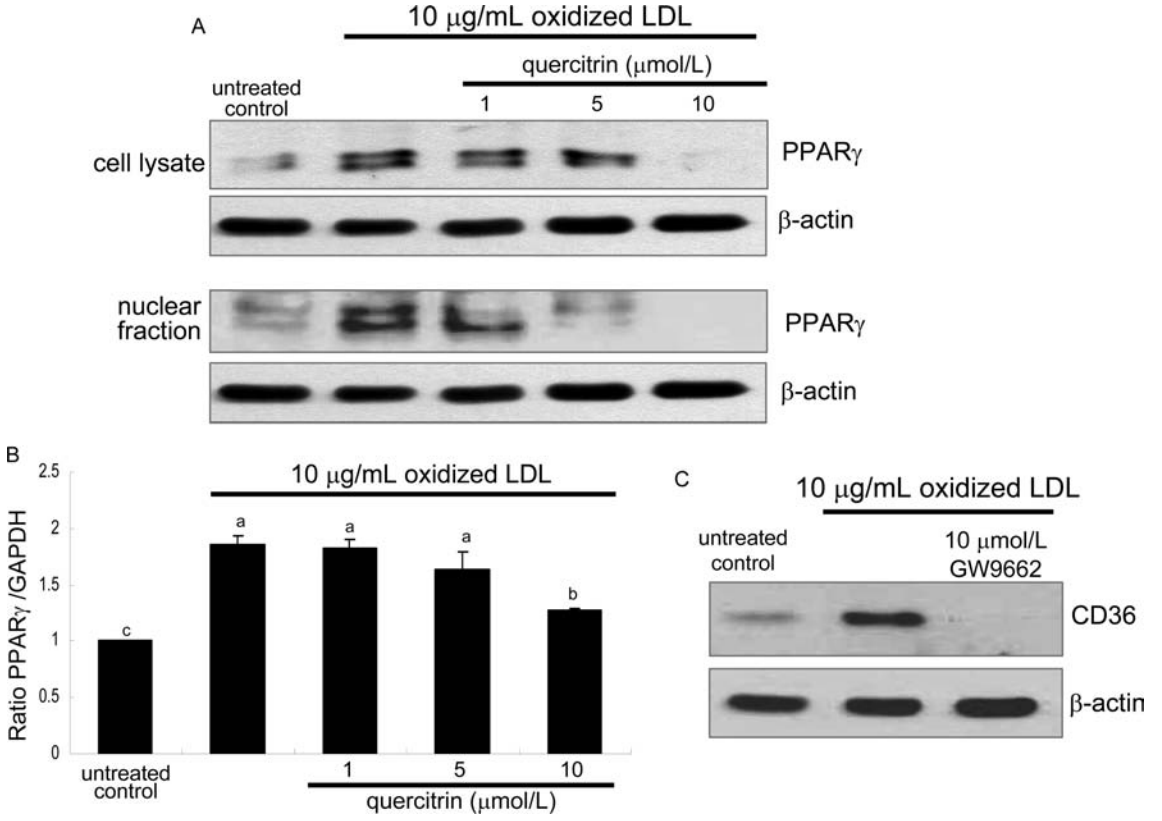

Figure 6. Inhibition of Cu^{2+}-oxLDL-induced PPARγ activation (**A**) and PPARγ mRNA expression (**B**) in quercitrin-treated J774A1 murine macrophages. Cells were treated with 1−10 μmol/L quercitrin and exposed to Cu^{2+}-oxLDL. Total cell lysates were electrophoresed on 10% SDS−PAGE and immunoblotted with a primary antibody against PPARγ. The mRNA expression PPARγ was determined by real time RT-PCR analysis. GAPDH was used as a housekeeping gene for the coamplification with CD36 and SR-A. The bar graphs (means ± SEM, $n = 3$) represent quantitative mRNA transcript level of PPARγ relative to GAPDH. Values not sharing a letter are different at $P < 0.05$. Inhibition of CD36 induction by blockade of PPARγ in the presence of 10 μmol/L CD36 inhibitor GW9662 added to oxLDL-treated cells (**C**). Total cell lysates were immunoblotted with a primary antibody against CD36. β-Actin was used as an internal control. Bands (**A** and **C**) are representative of 3 separate experiments.

Consequently, the oxLDL internalization via SR might be diminished by adding quercitrin regardless of demoted SR induction.

Previous studies have shown that consumption of polyphenol compounds mitigates the risk of cardiovascular diseases including atherosclerosis (*24*). Submicromolar flavonoids isolated from *Albizia julibrissin* foliage water extracts revealed their potential to inhibit LDL oxidization and monocyte adherence, where quercitrin was one of the major components of the water extracts (*25*). This study examined whether the natural compound quercitrin modulated the induction of CD36 and SR-A promoted by oxLDL. Quercitrin dampened oxLDL-enhanced induction of both SR-A and CD36 at the transcriptional levels. Additionally, resveratrol repressed expression of lipoprotein lipase and SR-AII involved in the lipid uptake, indicating that this polyphenol may potentially limit cholesterol accumulation in human macrophages (*26*).

It has been suggested that VEGF recognized as an angiogenic factor can promote macrophage migration critical for atherosclerosis (*5*). Monocyte chemotaxis in response to VEGF high in atherosclerotic plaques was shown to be mediated by VEGF-receptor flt-1 (*7*). Increased VEGF delivery in cholesterol-fed apoB/apoE double knockout mice and in cholesterol-fed rabbits elevated the extent of atherosclerosis (*27*). Furthermore, VEGF was upregulated in macrophages by oxLDL through increasing p38 MAPK activity and VEGF mRNA stability, indicating the significance of VEGF in the progression of atherosclerosis (*6*). This study disclosed that oxLDL induced VEGF production rapidly along with the induction of CD36 in murine macrophage, in which quercitrin blunted both CD36 expression and VEGF secretion. These results enlighten that the oxLDL binding to CD36 and receptor-mediated uptake of oxLDL are required for VEGF production by macrophages. It is also deemed that the diminution in VEGF secretion was attributed to the inhibition of SR induction in quercitrin-treated macrophages. However, the regulatory mechanisms involved in increased VEGF secretion in response to oxLDL have not been clearly dissected. In a recent study (*28*), the VEGF upregulation was mediated through PI3K and PKCζ, but not through low-density lipoprotein receptor type 1 (LOX-1), SR-A and CD36.

The production of MCP-1 as well as VEGF markedly diminished in lectin-like oxidized LOX-1-deficient mice, indicative of an important role of LOX-1 in the formation of choroidal neovascularization (*29*). MCP-1 was involved in the inflammatory process of atherosclerosis through the induction of CD36 expression via the ERK pathway, differentiating monocytes into foam macrophages and inducing monocyte migration (*30*). Similarly, this study showed that the secretion of MIP-2 and MCP-1 like VEGF was enhanced in concomitance with CD36 induction in oxLDL-exposed macrophages. It is also believed that the secretion of the chemokines of MIP-2 and MCP-1 involved the receptor-mediated macrophage uptake of oxLDL, which was inversely responsive to quercitrin.

Mechanisms responsible for the quercitrin inhibition of SR and VEGF in response to oxLDL are not yet established. Several studies suggested a partial role of PPARγ and p38 MAPK in the

regulation of VEGF by oxLDL (*6*). The oxLDL−LOX-1 system was involved in upregulation of VEGF expression in articular cartilage through activation of PPARγ (*30*, *31*). In this study quercitrin inhibited oxLDL-boosted PPARγ activation via a PKCα-dependent pathway. It should be noted that the PKCα blockade nearly abolished oxLDL-triggered activation of PPARγ and induction of CD36 and SR-A. It has been reported that oxLDL was found to markedly increase nuclear PPARγ levels and CD36 induction, which was attenuated by a treatment with hibiscus anthocyanin (*32*). Additionally, the flavonols of fisetin, morin and myricetin blocked oxLDL uptake by macrophages at least in part through reducing CD36 expression on U937-derived macrophages (*33*). Such inhibition was mediated by interference with PPARγ activation thus counteracting the deleterious autoamplification loop of CD36 expression stimulated by PPARγ ligand. Therefore, quercitrin appeared to act as an inhibitor of PKC or PPARγ that limited induction of SR and VEGF.

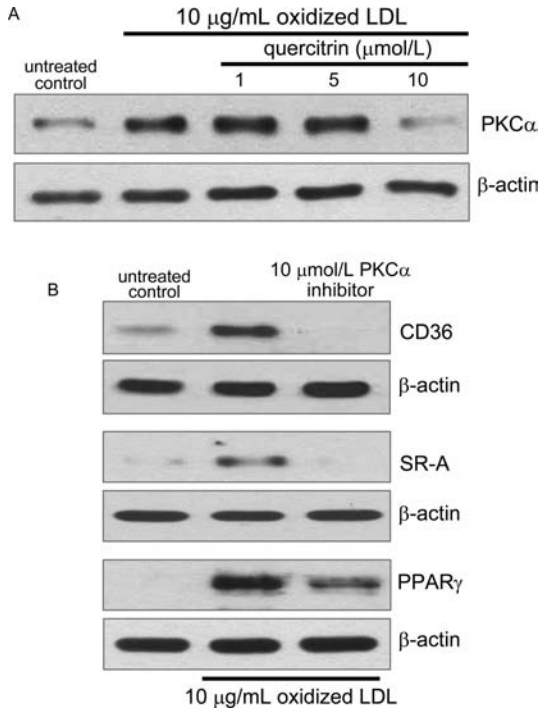

Figure 7. Blockade of Cu^{2+}-oxLDL-induced phosphorylation of PKCα in quercitrin-treated J774A1 murine macrophages (**A**). Cells were treated with 1−10 μmol/L quercitrin in the absence and presence of 10 μg/mL Cu^{2+}-oxLDL for 4 h. Western blot analysis of total cell lysates was carried out, using a primary antibody against phosphorylated PKCα. Inhibition of CD36, SR-A and PPARγ by PKCα blockade in oxLDL-stimulated macrophages (**B**). Cells were treated with 10 μmol/L PKCα inhibitor peptide and exposed to 10 μg/mL oxLDL. β-Actin was used as an internal control. Bands (**A** and **B**) are representative of 3 separate experiments.

In summary, the current report demonstrated that quercitrin reduced oxLDL uptake and cholesterol influx in relation to atherogenesis by J774A1 murine macrophages through reducing expression of both CD36 and SR-A on macrophages (**Figure 8**). Production of VEGF as an antiatherogenic factor entailed the oxLDL-upregulated CD36 and subsequent increase in the cholesterol influx, which was interrupted by quercitrin. The SR inhibition by quercitrin was mediated by hindering PKCα-dependent pathways, thus counteracting PPARγ-responsive cellular signaling. Accordingly, quercitrin works as a PKC inhibitor or a PPARγ antagonist so as to inhibit SR activation and may be an antiatherogenic agent blocking foam cell formation pertaining

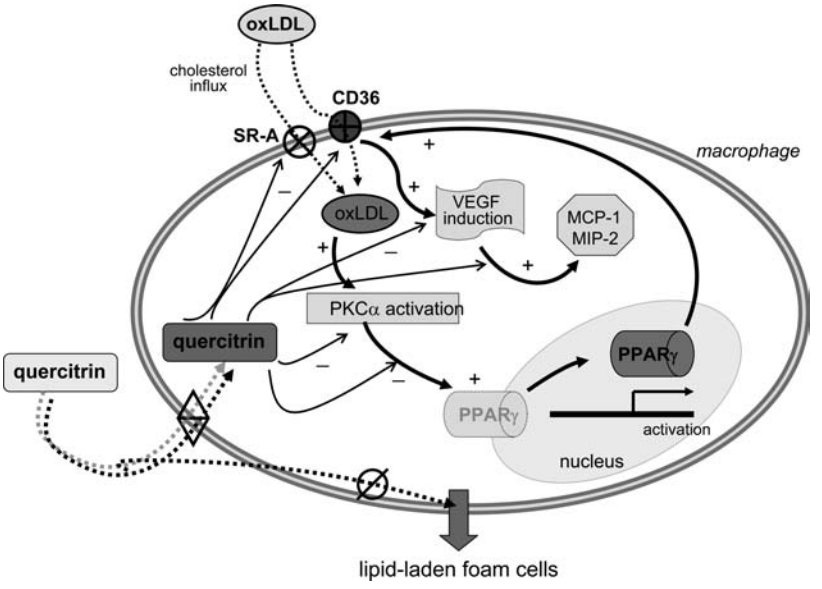

Figure 8. Schematic diagram showing antiatherogenic actions of quercitrin blocking foam cell formation pertaining to induction of SR and VEGF. The symbol indicates inhibition or blockade due to a quercitrin treatment.

to induction of SR and VEGF. However, it should be pointed out that most of the dietary polyphenols undergo extensive modifications during transfer across the small intestine and then again in the liver (*35*). Although quercitrin may serve as modulators against atherogenesis *in vitro*, its role as an antiatherogenic agent *in vivo* remains unclear.

ABBREVIATIONS USED

DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; 9-HODE, 9-hydroxy-(*S*)-10,12-octadecadienoic acid; LOX-1, low-density lipoprotein receptor type 1; MAPK, mitogen-activated protein kinases; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; MTT, 3-(4,5-dimethylthiazol-yl)diphenyl tetrazolium bromide; NF-κB, nuclear factor-κB; NO, nitric oxide; oxLDL, oxidized low-density lipoprotein; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PKC, protein kinase C; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; SR, scavenger receptors; TBS-T, Tris buffered saline-Tween 20; VEGF, vascular endothelial growth factor.

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