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Antiatherogenic Effects of Kaempferol and Rhamnocitrin

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Atherosclerosis is a chronic inflammatory disease of the arterial wall. Kaempferol and rhamnocitrin (kaempferol 7-*O*-methyl ether) are two anti-inflammatory flavonoids commonly found in plants. The aim of this study is to investigate the function of kaempferol and rhamnocitrin on prevention of atherosclerosis. Chemical analyses demonstrated that kaempferol and rhamnocitrin were scavengers of DPPH (1,1-diphenyl-2-picrylhydrazyl) with IC_{50} of 26.10 ± 1.33 and 28.38 ± 3.07 μM, respectively. Copper-induced low-density lipoprotein (LDL) oxidation was inhibited by kaempferol and rhamnocitrin, with similar potency, as measured by decreased formation of malondialdehyde and relative electrophoretic mobility (REM) on agarose gel, while rhamnocitrin reduced delayed formation of conjugated dienes better than kaempferol. Cholesterol-laden macrophages are the hallmark of atherogenesis. The class B scavenger receptor, CD36, binds oxidized low-density lipoprotein (oxLDL), is found in atherosclerotic lesions, and is up-regulated by oxLDL. Addition of kaempferol and rhamnocitrin (20 μM) caused significant reductions in cell surface CD36 protein expression in THP-1-derived macrophages ($p < 0.05$). Reverse transcription quantitative PCR (RT-Q-PCR) showed that kaempferol and rhamnocitrin (20 μM) decreased oxLDL-induced CD36 mRNA expression ($p < 0.01$ and $p < 0.05$, respectively). Kaempferol- and rhamnocitrin-treated macrophages also showed reduction in 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanide perchlorate (DiI)-labeled oxLDL uptake. Current evidences indicate that kaempferol and rhamnocitrin not only protect LDL from oxidation but also prevent atherogenesis through suppressing macrophage uptake of oxLDL.

KEYWORDS: Kaempferol; rhamnocitrin; low-density lipoprotein (LDL); atherogenesis; class B scavenger receptor (CD36); DiI-oxLDL

INTRODUCTION

The oxidative hypothesis of atherosclerosis development has attracted extensive investigation of a possible preventive role of antioxidants (*1*). Antioxidants, such as vitamin E or polyphenolic flavonoids, as well as polyphenol-rich foods not only protect low-density lipoprotein (LDL) from oxidation but also reduce the development of atherosclerotic lesions (*2–5*).

There is considerable evidence indicated that atherogenesis is initiated and promoted by lipid oxidation of LDL, ultimately leading to oxidative modification of apolipoprotein B. The oxidized LDL (oxLDL) particles are recognized by macrophage scavenger receptors (SRs) and taken up by the macrophages, forming lipid-laden foam cells in the fatty streak lesions (*6*). SR class B (SR-B) has been identified as the oxLDL receptor. CD36, a member of the SR-B family, plays a quantitatively significant role in oxLDL binding to macrophages (*7*). CD36 is highly expressed on lipid-laden macrophages in human atherosclerotic aorta (*8*) and is up-regulated by oxLDL in HL-60 and THP-1 cells via induction of the peroxisome proliferator-activated receptor γ (PPARγ) (*9*). These findings suggest that CD36 plays a proatherogenic role in foam cell formation and its expression can in turn enhance the uptake of oxLDL.

Kaempferol and rhamnocitrin (kaempferol 7-*O*-methyl ether) (**Figure 1**) are two of flavonoids commonly found in propolis (*10, 11*). In addition, kaempferol and rhamnocitrin have also been isolated from a variety of natural sources and are considered as anti-inflammatory constitutes (*12–16*). Kaempferol is a potent lipophilic antioxidant (*17*); therefore, increased intake of kaempferol appears to be cardioprotective. Rhamnocitrin, however, is abundant in nature and similar to kaempferol in

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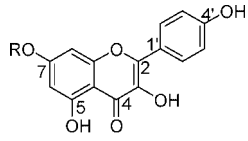

kaempferol R = H
rhamnocitrin R = CH_3

Figure 1. Chemical structures of kaempferol and rhamnocitrin (kaempferol 7-*O*-methyl ether).

chemical structure; no systematic report regarding its antioxidant activity could be found.

In addition to decreasing the oxidative susceptibility of LDL, evidence of the antiatherogenic effects of kaempferol on crucial cells in atherogenesis such as endothelial cells, platelets, smooth muscle cells, and macrophages is mounting (*18–22*). It is well-known that monocyte-derived macrophages are critical cells that are present in all stages of atherogenesis; however, most researches focused only on the anti-inflammatory effects of flavonoids in this type of cell. There is a scarcity of data examining the role of kaempferol or rhamnocitrin in the uptake of oxLDL or the expression of CD36 in human monocyte-derived macrophages. Thus, the aim of this study is to determine the effect of kaempferol and rhamnocitrin on LDL oxidation as well as on the expression and function of CD36 in THP-1-derived macrophages.

MATERIALS AND METHODS

Chemicals. Kaempferol (purity ≥96%, HPLC grade) was from Fluka Chemie (Bushs, Switzerland). Rhamnocitrin was isolated from the resin of *Eucalyptus citriodora*. DPPH and other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO) unless otherwise indicated.

Plant Material. The resin of *Eucalyptus citriodora* was collected in Yung Kang, Tainan, Taiwan, and authentified by Prof. Chang-Sheng Kuoh, Department of Biology, National Cheng Kung University. A voucher specimen (CNACNP0605) was deposited in the natural product laboratory of Department of Applied Chemistry, Chia-Nan University of Pharmacy and Science, Tainan, Taiwan.

Isolation and Structure Elucidation of Rhamnocitrin. Dried powdered resin of *Eucalyptus citriodora* (520 g) was extracted with ethanol (3 × 1.5 L) at room temperature. The concentrated ethanol extract (455 g) was suspended in water. The suspension was extracted with ethyl acetate and *n*-butanol, successively. The ethyl acetate soluble fraction (263 g) was chromatographed on a silica gel column with *n*-hexane–ethyl acetate–methanol (2:1:0 to 0:1:0.5, gradient) as eluent, and 250 mL was collected for each fraction. Fractions 23–32 (6.8 g) containing flavonoids were collected and further purified by silica gel column chromatography, eluting with *n*-hexane–ethyl acetate–methanol (2:1:0.1) to give yellow needle isolate (214 mg). The isolate was identified as rhamnocitrin by comparison of its spectral data with the reported data (*23, 24*).

Rhamnocitrin. Yellow needle crystal; UV (MeOH) λ_{max} (log ε) 266.0 (4.23), 366.0 (4.25) nm. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 12.43 (1H, s, OH-5), 10.18 (1H, s, OH-4′), 9.45 (1H, s, OH-3), 8.05 (2H, d, J = 8.8 Hz, H-2′, 6′), 6.92 (2H, d, J = 8.8 Hz, H-3′, 5′), 6.60 (1H, d, J = 2.0 Hz, H-8), 6.25 (1H, d, J = 2.0 Hz, H-6), 3.80 (3H, s, OCH_3-7). ^{13}C NMR (50 MHz, DMSO-d_6) δ: 176.0 (C-4), 164.7 (C-7), 160.4 (C-5), 159.3 (C-4′), 156.2 (C-8a), 147.2 (C-2), 136.2 (C-3), 129.6 (C-2′, 6′), 121.6 (C-1′), 115.5 (C-3′, 5′), 104.0 (C-4a), 97.4 (C-6), 92.0 (C-8), 56.0 (OCH_3-7).

Purity Analysis of Rhamnocitrin. The purity of rhamnocitrin was determined by HPLC analysis as described below. Purity was determined by comparison of the area of the peak of rhamnocitrin with the area of the peak of standard kaempferol under the same molar concentration. The calibration curves were constructed by plotting the mean peak areas vs the concentrations of the standard. The calculated purity of rhamnocitrin is 96.6% in accordance with the purity of standard kaempferol regarded as 96.0%.

HPLC System and Conditions. The analyses were performed on a Hitachi model L-7100 pump (Tokyo, Japan), a model 7125 injector equipped with a 20 μL sample loop, and a model L-7455 diode array detector. A Phenomenex Luna C18 column (250 × 4.6 mm i.d., 5 μm) at temperature of 30 ± 1 °C was applied for all analyses. Detection wavelength was set at 366 nm. The mobile phase consisted of a mixture of 40% (v/v) K_2HPO_4, 1 mM, pH 3.50, 30% (v/v) CH_3OH, and 30% (v/v) CH_3CN. Three concentrations 2.5, 5.0, and 10.0 μM of the analytes were injected in triplicate. The flow rate was 1.0 mL/min, and 20 μL of each sample solution and 20 μL of standard were injected. Chromatograms were acquired and peak areas calculated by means of a Scientific Information Service Corp. chromatogram data integrator (Taipei, Taiwan). The retention time of kaempferol and rhamnocitrin was 5.8 and 13.7 min, respectively.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) Scavenging Effect. The DPPH scavenging effect was measured according to Dinis et al. (*25*). The reaction was performed in 1 mL of solution containing 0.1 mM of freshly prepared DPPH in methanol and various concentrations of test samples (in DMSO). After incubation at 37 °C for 30 min, the absorbance at 517 nm was measured in triplicate, and the scavenging effect was calculated against vehicle control (DMSO).

Preparation and Oxidation of LDL. LDL (d 1.019–1.063) was prepared from the plasma of healthy donors by sequential ultracentrifugation (*26*). Lipoprotein was desalted and concentrated by filtration (Amicon Ultra-4, Millipore, Billerica, MA) against PBS at 450*g*, 4 °C for 120 min. The protein concentration was measured by the method of Bradford (*27*), using bovine serum albumin as a standard.

Oxidation of LDL was carried out by incubating EDTA-free LDL (0.1 mg/mL) with 10 μM Cu^{2+} in PBS in the presence of indicated test agent or vehicle control (DMSO) at 37 °C.

Analysis of LDL Oxidation. Peroxidation of LDL was measured by the determination of thiobarbituric acid reactive substances (TBARS) and expressed as malondialdehyde (MDA) equivalents (*28*). The quantity of conjugated dienes in LDL was assessed by monitoring the change at A234 (*29*). To measure the electrophoretic mobility, the oxLDL was concentrated by filtration (Microcon YM-3, Millipore). About 1–2 μL of each concentrated sample was loaded onto Titan Lipoprotein Gel (Helena Laboratories, Beaumont, TX) and run at 80 V for 45 min. Gel was then dry and stained with Fat Red 7B according to the manufacturer's instructions. Relative electrophoretic mobility (REM) was calculated as the mobility of oxLDL relative to that of native LDL (nLDL).

Cell Culture. The monocyte-like cell line THP-1 was obtained from Bioresource Collection and Research Center (Hsinchu, Taiwan). The THP-1 cells were cultured in RPMI 1640 medium, which contained 2 mM L-glutamine, 4.5 g/L glucose, 15 mM HEPES (Sigma), 1.0 mM sodium pyruvate, 15% fetal bovine serum (Hyclone, Logan, UT), penicillin (100 IU/mL), and streptomycin (100 μg/mL) (Invitrogen Life Technologies, Carlsbad, CA). Differentiation into macrophages was achieved by treating the cells in six-well plates (1 × 10^5 cells/well) with PMA (phorbol 12-myristate 13-acetate, 200 ng/mL) for 48 h.

Flow Cytometry Analysis of Cell Surface CD36 Expression. Differentiated THP-1 cells were incubated at 37 °C with the test agents or vehicle control (0.1% v/v DMSO) for 24 h. Cells were washed with PBS and then scraped in PBS, pH 8.2. The pellet was incubated with fluorescein (FITC)-conjugated murine antihuman CD36 (clone FA6.152, Immunotech, Beckman Coulter, Fullerton, CA) or isotype controls on ice for 60 min before washing twice with PBS (250*g*, 5 min). The cells were investigated in duplicates by flow cytometry (Coulter EPICS XL, Beckman Coulter). Data were acquired from 15 000 cells (events), and the fluorescent intensity of CD36 expression was determined and expressed as the geometric mean fluorescence intensity (MFI).

RNA Extraction and Analysis. In order to analyze the expression of CD36 mRNAs, differentiated THP-1 cells were incubated at 37 °C with the test substance or vehicle control (0.1% v/v DMSO) for 24 h. Total cellular RNA was prepared using RNA miniprep System (Viogene, Taipei, Taiwan). The expression of each mRNA was analyzed by carrying out TaqMan reverse transcription quantitative polymerase chain reaction (RT-Q-PCR) on a Prism 7300 sequence detection system (Applied Biosystems, Foster City, CA). The sequences of the primers and probes used were as follows: CD36: forward primer, 5′-CTGAG-

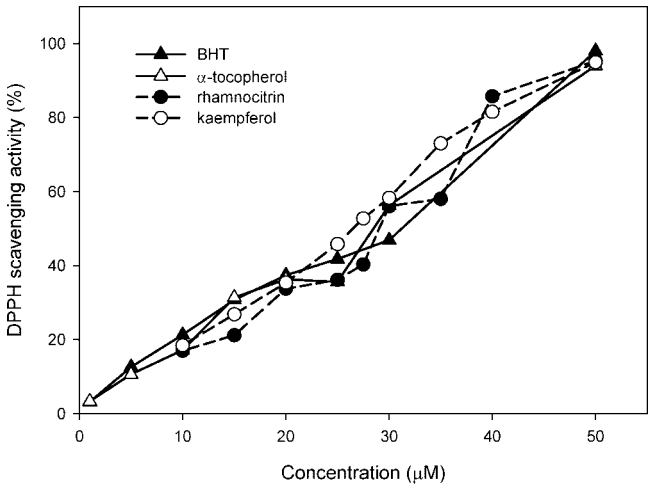

Figure 2. DPPH scavenging effects of kaempferol, rhamnocitrin, BHT, and α-tocopherol. The reaction was performed against 1 mM of freshly prepared DPPH. After incubation at 37 °C for 30 min, the absorbance at 517 nm was measured in triplicate, and the scavenging effect was calculated against vehicle control (DMSO).

GACAACACAGTCTCTTTCC-3′; reverse primer, 5′-ACTGTGAAGT-TGTCAGCCTCTGTTC-3′; and probe, 5′−6FAM-TGGTGCCATCT-TCGAACCTTCACTATCAG-TAMRA-3′ (*30*). The expression level of each mRNA was normalized by reference to the corresponding GAPDH mRNA level (TaqMan GAPDH control reagent, Applied Biosystems).

DiI-oxLDL Uptake by Macrophages. DiI-oxLDL was prepared by oxidization of DiI-LDL (Invitrogen Life Technologies) with 10 μM Cu^{2+} in PBS at 37 °C overnight in dark. THP-1-derived macrophages were incubated with the test substance or vehicle control (0.1% v/v DMSO) for 48 h and then incubated with DiI-oxLDL (50 μg/mL) for another 24 h. The cells were washed and then investigated in duplicates by flow cytometry. Data were acquired from 15 000 cells (events), and the DiI-oxLDL uptake was determined and expressed as the geometric mean fluorescence intensity (MFI).

Statistical Analyses. The Mann–Whitney U test was used to assess significant differences in the presence and absence of test substances, and the level of significance was set at $p < 0.05$. All experiments have been performed at least in three independent trials.

RESULTS

Effects of Kaempferol and Rhamnocitrin on 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging. To evaluate the antioxidant activity of kaempferol and rhamnocitrin, we started with investigating stable free radical (DPPH) scavenging action. **Figure 2** demonstrates that the DPPH scavenging effects of kaempferol and rhamnocitrin were dose-dependent manners. The potencies of kaempferol and rhamnocitrin were compatible with the positive control, α-tocopherol and BHT, at the same concentration. The calculated IC_{50} for kaempferol and rhamnocitrin were 26.11 ± 1.33 and 28.38 ± 3.06 μM, respectively.

Inhibition of LDL Oxidation by Kaempferol and Rhamnocitrin. It is recognized that oxidative modified low-density lipoproteins (oxLDL) play an important role in the generation and progression of the atherosclerotic plaque (*31*). To study the effect of kaempferol and rhamnocitrin as antioxidants in preventing copper-induced oxidation of LDL, three different approaches were employed to measure changes in several parameters known to be associated with LDL oxidation: formation of thiobarbituric acid reactive substances (TBARS), conjugated dienes during lipid peroxidation, and increase in the electrophoretic mobility of LDL due to apolipoprotein B100

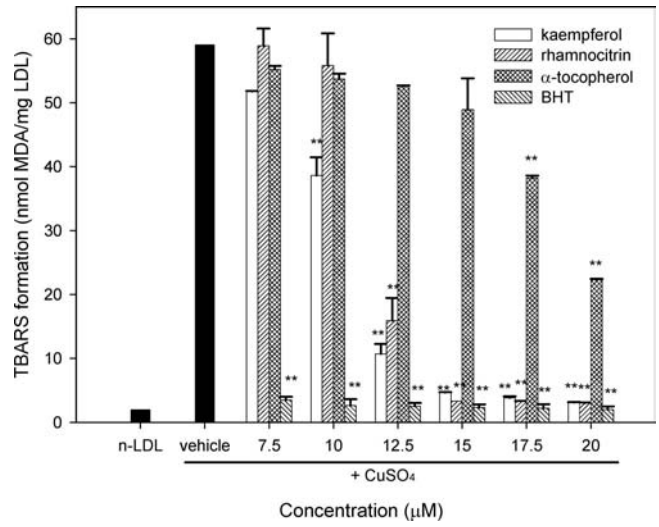

Figure 3. Kaempferol, rhamnocitrin, BHT, and α-tocopherol inhibit TBARS formation in copper-induced low-density lipoprotein peroxidation. EDTA-free LDL (0.1 mg/mL) reacted with 10 μM Cu^{2+} in PBS in the presence of vehicle (DMSO) or different concentrations of indicated reagent at 37 °C for 3 h. Peroxidation of the LDL was expressed as nanomoles of malondialdehyde (MDA) equivalents per milligram of protein. Data represent the mean ± SEM ($n = 3$). **, $p < 0.01$ represents significant differences compared with vehicle control.

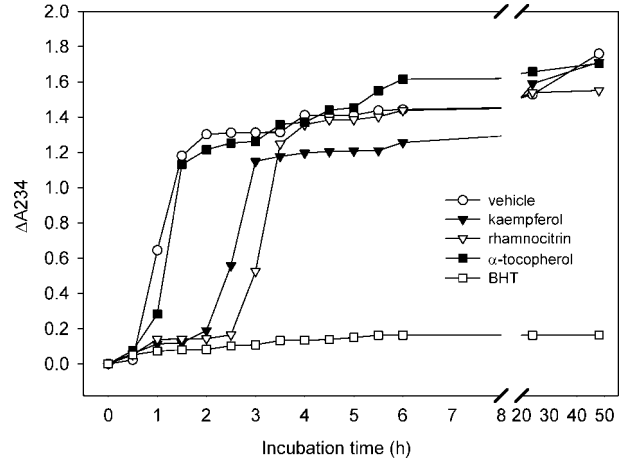

Figure 4. Kaempferol, rhamnocitrin, BHT, and α-tocopherol inhibit conjugated diene formation in copper-induced LDL. EDTA-free LDL (0.1 mg/mL) reacted with 10 μM Cu^{2+} in PBS in the presence of indicated reagent (5 μM) at 37 °C for the indicated period. The formation of conjugated diene was measured by change in absorbance at 234 nm (A_{234}). This experiment was repeated three times with similar results.

modification (*32*). As shown in **Figure 3**, incubation of LDL (0.1 mg/mL) with Cu^{2+} (10 μM) at 37 °C for 3 h, the MDA formation increased from 1.94 ± 0.17 to 59.04 ± 0.28 nmol/mg of LDL. Kaempferol and rhamnocitrin treatment produced dose-dependent reduction in MDA formation with IC_{50} of 10.37 ± 0.72 and11.22 ± 1.56 μM, which are inferior to BHT but better than α-tocopherol.

Oxidation of LDL is accompanied by an increase in absorbance at 234 nm due to the formation of conjugated dienes in constituent polyenoic fatty acids (*33*). As shown in **Figure 4**, 5 μM kaempferol- or rhamnocitrin-treated LDL resulted in an augmented resistance to copper-initiated LDL oxidation, and this was indicated by a prolonged lag phase of 2 and 2.5 h, respectively. The inhibitory effect was inferior to that of BHT but better than of α-tocopherol.

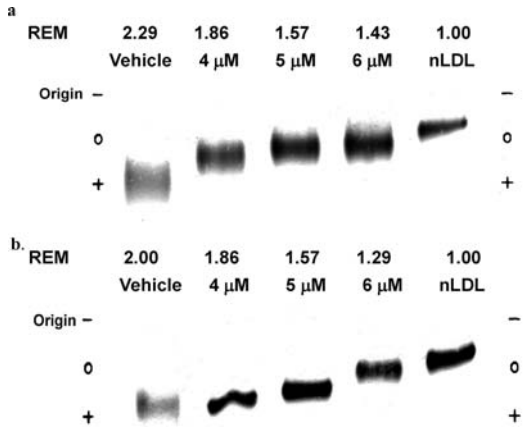

Figure 5. Kaempferol and rhamnocitrin inhibit electrophoretic mobility in copper-induced LDL. Native LDL (nLDL) (0.1 mg/mL) was oxidized with 10 μM Cu^{2+} in PBS in the presence of vehicle or indicated concentration of kaempferol (**a**) or rhamnocitrin (**b**) for 3 h. Concentrated LDL (1–2 μL) was separated on Titan lipoprotein gel and stained with Fat Red 7B. Relative electrophoretic mobility (REM) was calculated as the mobility of oxLDL relative to that of nLDL.

The ability of kaempferol and rhamnocitrin to inhibit the alteration in the surface charge of the apolipoprotein B100 when LDL was incubated with copper ions was monitored by observing the effects on relative electrophoretic mobility (REM). **Figure 5** showed that treatment of native LDL (nLDL, 0.1 mg/mL) with copper (10 μM) increased REM from 1.00 to 2.00–2.29. Cotreatment of nLDL with copper plus indicated concentration of kaempferol or rhamnocitrin compatibly reduced LDL oxidation dose-dependently as indicated by decreased REM.

Effects of Kaempferol and Rhamnocitrin on Cell Surface CD36 Expression. THP-1 cells were used as a model for investigating regulation of CD36 expression on cells of the monocyte/macrophage lineage. These cells can be induced to differentiate into macrophages by a variety of stimuli including phorbol 12-myristate 13-acetate (PMA). Untreated THP-1 cells showed little surface expression of CD36, while PMA-treated THP-1 cells increased in surface CD36 expression that correlated with differentiation process (*34*). **Figure 6a** demonstrated that both kaempferol and rhamnocitrin (20 μM) significantly reduced surface CD36 protein expression in THP-1-derived macrophages (MFI changed from 75.79 $\pm$ 3.87 to 56.64 $\pm$ 1.22 and 56.63 $\pm$ 1.24, respectively, $p < 0.05$). A PPARγ agonist, 15-deoxy-Δ12,14-prostaglandin J_2 (15dPGJ$_2$, 2 μM), used as a positive control, markedly induced CD36 expression (MFI changed to 96.39 $\pm$ 0.42, $p < 0.05$).

Since CD36 protein expression is up-regulated by oxLDL, we further examined the effects of kaempferol and rhamnocitrin when oxLDL was present. **Figure 6b** demonstrates that when THP-1-derived macrophages were cultured with oxLDL (200 μg/mL) plus vehicle for 24 h, CD36 expression increased by about 50% (MFI elevated to 116.41 $\pm$ 3.84). No inhibitory effect on oxLDL-induced CD36 expression was observed when cells were cotreated with oxLDL (200 μg/mL) plus kaempferol or rhamnocitrin (20 μM). On the other hand, cotreatment of cells with oxLDL (200 μg/mL) and 15dPGJ$_2$ (2 μM) synergistically induced CD36 protein expression compared with oxLDL-treated cells ($p < 0.05$).

Effects of Kaempferol and Rhamnocitrin on CD36 mRNA Expression. To further investigate whether kaempferol and rhamnocitrin down-regulated CD36 gene expression in the transcription level, RT-Q-PCR was employed as described in the Materials and Methods section. **Figure 7a** demonstrated that CD36 mRNA expression was induced (11.48 $\pm$ 1.27)-fold by 15dPGJ$_2$ (2 μM) compared with vehicle control ($p < 0.01$). On the other hand, treatment of cells with kaempferol (20 μM) markedly attenuated the CD36 mRNA expression, and the percentage reduction was 47.3 $\pm$ 1.5% compared with vehicle control ($p < 0.05$). On the other hand, no significant effect was observed for rhamnocitrin-treated macrophages.

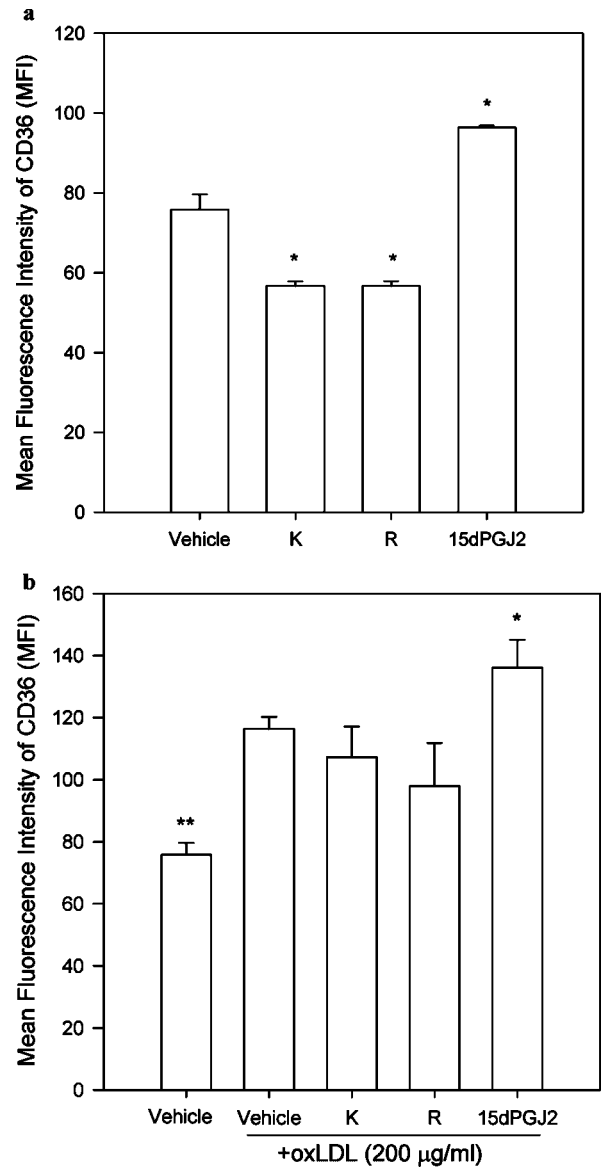

Figure 6. Effects of kaempferol and rhamnocitrin on cell surface CD36 expression in THP-1-derived macrophages. (**a**) THP-1-derived macrophages were treated with vehicle (0.1% DMSO), kaempferol (K, 20 μM), rhamnocitrin (R, 20 μM), or 15-deoxy-Δ12,14-prostaglandin J_2 (15dPGJ$_2$, 2 μM) for 24 h. (**b**) THP-1-derived macrophages were cotreated with oxLDL (200 μg/mL) plus vehicle (0.1% DMSO), kaempferol (K, 20 μM), rhamnocitrin (R, 20 μM), or 15-deoxy-Δ12,14- prostaglandin J_2 (15dPGJ$_2$, 2 μM) for 24 h. Cells were then stained with FITC labeled-mouse antihuman CD36 IgG1 (FA6.152). Cell surface CD36 expression was measured by flow cytometry. Data represent the mean $\pm$ SEM of three independent experiments performed in duplicate. *, $p < 0.05$ represent significant differences compared with vehicle control in (**a**). *, $p < 0.05$ and **, $p < 0.01$ represent significant differences compared with oxLDL plus vehicle in (**b**).

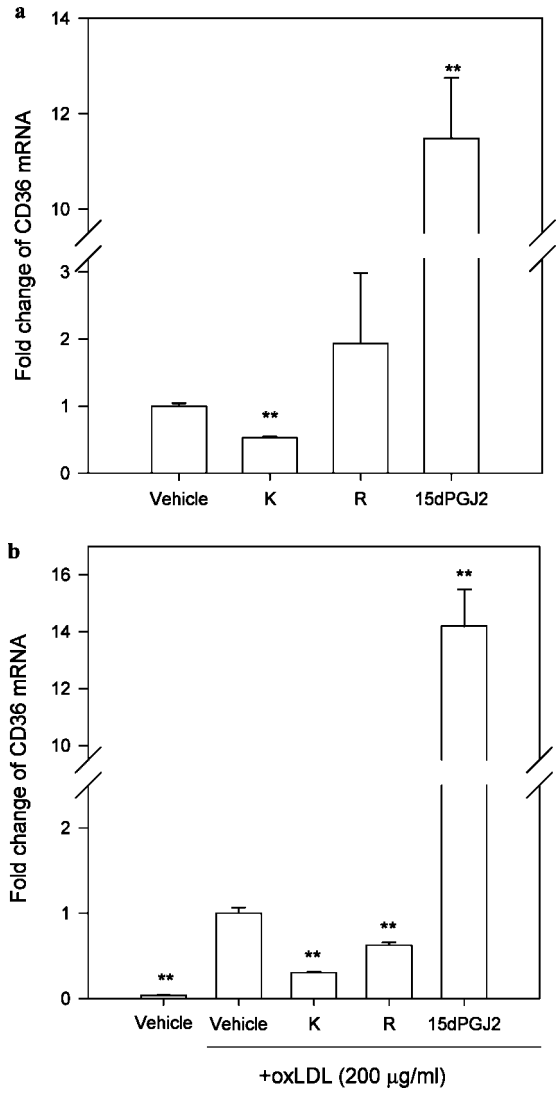

Figure 7. Effects of kaempferol and rhamnocitrin on CD36 mRNA expression in THP-1-derived macrophages. (**a**) THP-1-derived macrophages were treated with vehicle (0.1% DMSO), kaempferol (K, 20 μM), rhamnocitrin (R, 20 μM), or 15-deoxy-Δ12,14-prostaglandin J_2 (15dPGJ$_2$, 2 μM) for 24 h. (**b**) THP-1-derived macrophages were cotreated with oxLDL (200 μg/mL) plus vehicle (0.1% DMSO), kaempferol (K, 20 μM), rhamnocitrin (R, 20 μM), or 15-deoxy-Δ12,14-prostaglandin J_2 (15dPGJ$_2$, 2 μM) for 24 h. Total cellular RNA was prepared, and the expression of each mRNA was analyzed by TaqMan reverse transcription quantitative polymerase chain reaction (RT-Q-PCR) as described in the Materials and Methods section. The data were normalized with reference to the expression levels of the corresponding GAPDH mRNAs. Data in (**a**) represent the mean ratio $\pm$ SEM of three independent experiments performed in duplicate relative to the value of the vehicle. **, $p < 0.01$ represents significant differences compared with vehicle control. Data in (**b**) represent the mean ratio $\pm$ SEM of three independent experiments performed in duplicate relative to the value of oxLDL plus vehicle. **, $p < 0.01$ represents significant differences compared with oxLDL plus vehicle.

Treatment of THP-1-derived macrophages with oxLDL (200 μg/mL) plus vehicle for 24 h induced dramatic increase in CD36 mRNA expression as compared with vehicle-treated cells ((33.4 $\pm$ 2.1)-fold, $p < 0.01$). **Figure 7b** shows that cotreatment of cells with oxLDL (200 μg/mL) plus 15dPGJ$_2$ (2 μM) synergistically induced CD36 mRNA expression as compared with oxLDL-treated cells ((14.2 $\pm$ 1.3)-fold, $p < 0.01$). On the other hand, both kaempferol and rhamnocitrin (20 μM) significantly attenuated oxLDL-induced CD36 mRNA expression by 70 $\pm$ 1% and 27.6 $\pm$ 3%, respectively, as compared with oxLDL-treated cells ($p < 0.01$).

Effects of Kaempferol and Rhamnocitrin on DiI-oxLDL Uptake by Human Macrophages. The above results led us to examine the effects of kaempferol and rhamnocitrin on cellular uptake of oxLDL. DiI-oxLDL is known to be bound to and/or taken up via CD36. THP-1-derived macrophages were pretreated with kaempferol, rhamnocitrin (20 μM), 15dPGJ$_2$ (2 μM), or vehicle for 48 h. Next, the cells were exposed to DiI-oxLDL for 24 h at 37 °C. **Figure 8** demonstrates that pretreatment of cells with 15dPGJ$_2$ (2 μM) for 48 h significantly enhanced DiI-oxLDL uptake (MFI value increased from12.86 $\pm$ 1.77 to 28.31 $\pm$ 3.71). On the other hand, both kaempferol and rhamnocitrin (20 μM) markedly inhibited DiI-oxLDL uptake (MFI values decreased to 9.81 $\pm$ 0.17 and 8.62 $\pm$ 0.55, respectively; $p < 0.05$).

DISCUSSION

Oxidative modification of LDL is believed to be an important event in atherogenesis (*35*), and several studies have reported on the antioxidant effect of kaempferol and other flavonoids, that is, decreasing the susceptibility of LDL to oxidation (*17*). Current results demonstrated that kaempferol and rhamnocitrin were equally strong in DPPH bleaching (**Figure 2**) as well as inhibiting TBARS formation (**Figure 3**) and LDL surface net charge change (**Figure 5**). However, rhamnocitrin, probably due to being more lipophilic, delayed conjugated diene formation more effectively than kaempferol (**Figure 4**).

Because of the high antioxidant capacity of flavonoids *in vitro*, it has been suggested that flavonoids act as antioxidants in human plasma and other extracellular fluids and protect LDL from oxidation. However, studies of *ex vivo* oxidation of plasma and LDL obtained from human subjects before and after short- or long-term consumption of flavonoid-rich foods have yielded conflicting results (*18*). Therefore, it is unlikely that the health benefits of dietary flavonoids are explained merely by their antioxidant activity in plasma or LDL. Macrophage scavenger receptors are thought to play a significant role in atherosclerotic foam cell development because of their ability to bind and internalize oxLDL. To better understand the beneficial effects of kaempferol and rhamnocitrin, we used the monocytic leukaemia cell line THP-1, which differentiates to macrophages in response to phorbol 12-myristate 13-acetate (PMA), to investigate their effects on the expression and activity of CD36, a class B scavenger receptor, in human monocyte/macrophages.

PMA, a protein kinase C (PKC) activator, is a potent inducer of CD36 (*36*). We found that kaempferol and rhamnocitrin (20 μM) reduced surface CD36 protein expression in PMA-activated THP-1 cells by about 25% (**Figure 6a**). However, RT-Q-PCR revealed that only kaempferol, but not rhamnocitrin, significantly attenuated CD36 mRNA expression (**Figure 7a**). These results suggested that kaempferol and rhamnocitrin, with similar chemical structure, may regulate PMA-stimulated CD36 expression in different levels, namely transcription vs post-transcription, or they may exert different effects on cell signaling pathway. Further investigation is required to understand the exact mechanism.

It has been shown that oxLDL up-regulates CD36 expression through initial PKC and subsequent PPARγ activation (*37*). In this study we further stimulated PMA-activated THP-1 cells with oxLDL (200 μg/mL) and found that oxLDL up-regulated surface CD36 protein expression by about 50% compared with PMA-activated THP-1 cells (**Figure 6b**). RT-Q-PCR revealed that

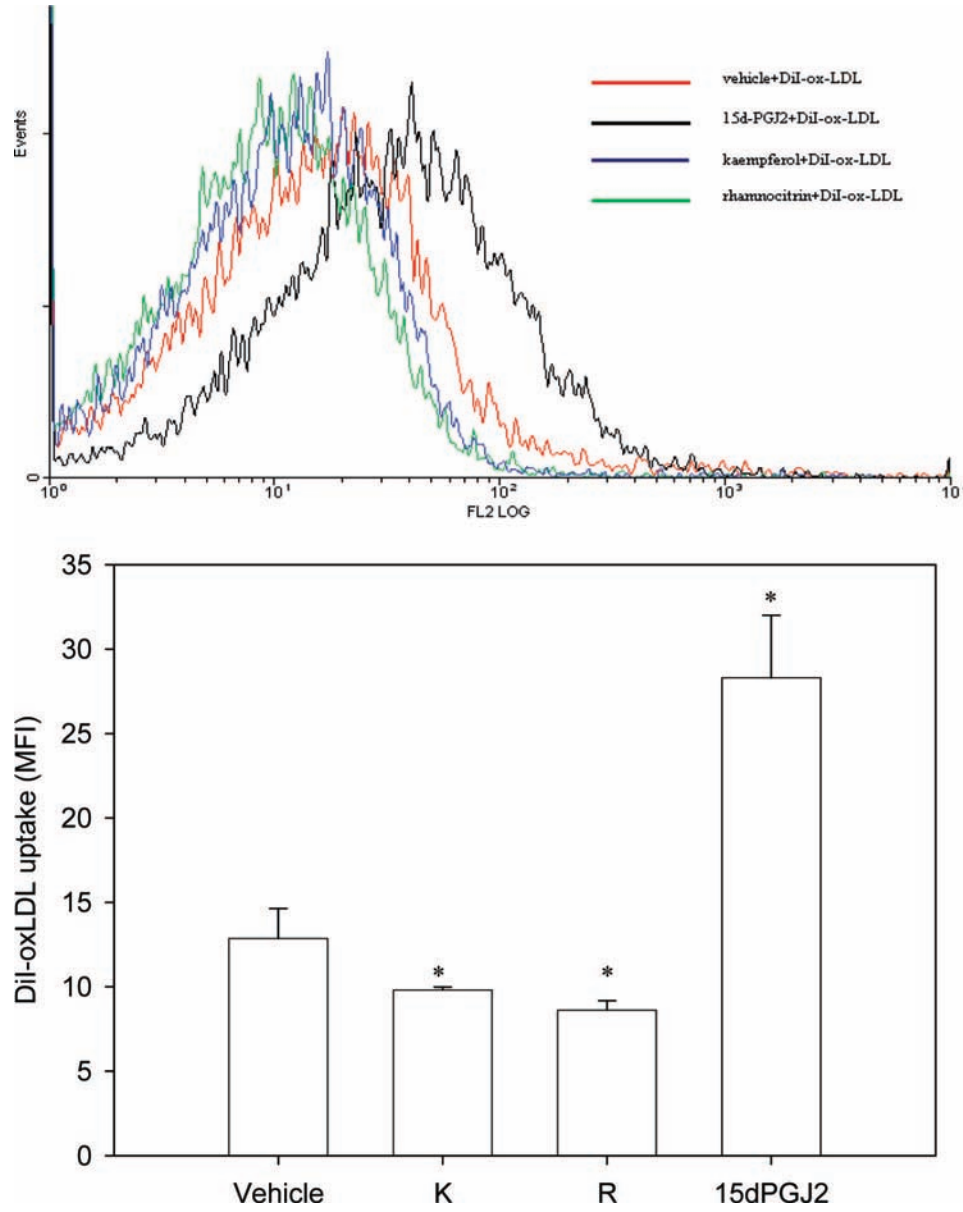

Figure 8. Effects of kaempferol and rhamnocitrin on DiI-oxLDL uptake in THP-1-derived macrophages. THP-1-derived macrophages were pretreated with kaempferol or rhamnocitrin (20 μM) for 2 days. DiI-oxLDL (50 μg/mL) was added to cells and incubated for another 24 h. Cells were subjected to flow cytometry analysis as described in the Material and Methods section. Data represent the mean ± SEM of three independent experiments performed in duplicate. *, $p < 0.05$ represents significant differences compared with vehicle control.

oxLDL stimulated CD36 mRNA expression by more than 30-fold compared with PMA-activated cells (**Figure 7b**). Coincubation oxLDL-treated macrophages with either kaempferol or rhamnocitrin (20 μM) did not change surface CD36 protein expression (**Figure 6b**) but reduced mRNA expression by about 70% and 30%, respectively, compared with oxLDL-incubated macrophages (**Figure 7b**). The discrepancy of the effects on oxLDL-stimulated CD36 protein and mRNA expression may result from the detection sensitivity difference between flow cytometry and RT-Q-PCR. It is also possible that the expression of cell surface receptor is regulated in several stages, including transcription, translation, and post-translational processing; as a result, the dramatic increase in oxLDL-induced CD36 mRNA expression cannot be completely shown on surface protein expression. Nevertheless, kaempferol seems to be more effective than rhamnocitrin in inhibiting CD36 mRNA expression regardless of the presence of oxLDL, indicating that it may interfere with both PKC and PPARγ activation. In comparison with their antioxidant activity, it is likely that the inhibitory effects of kaempferol and rhamnocitrin on CD36 expression may not directly result from their antioxidant activity.

To elucidate whether there were any functional consequences for kaempferol-or rhamnocitrin-mediated inhibition of CD36 expression, we examined DiI-labeled oxLDL uptake in THP-1-derived macrophages. Current results demonstrated that preincubation of macrophages with kaempferol or rhamnocitrin (20 μM) for 48 h was able to block DiI-oxLDL uptake over 24 h by 20–30% (**Figure 8**). This coincided with the reduction in CD36 surface expression following kaempferol or rhamnocitrin treatment in THP-1-derived macrophages (**Figure 6a**).

It has been reported that natural antioxidants, such as α-tocopherol and grape powder polyphenols, significantly decreased CD36 expression and oxLDL uptake in human monocyte-derived macrophages and animal model, respectively (*2*,*38*). The current report demonstrates that flavonol and its derivative, kaempferol and rhamnocitrin, not only prevent LDL oxidation

but also reduce macrophage uptake of oxLDL via down-regulation of CD36 expression.

ABBREVIATIONS USED

15dPGJ$_2$, 15-deoxy-Δ12,14-prostaglandin J$_2$; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanide perchlorate; DPPH, 1,1-diphenyl-2-picrylhydrazyl; LDL, low-density lipoprotein; MDA, malondialdehyde; nLDL, native LDL; oxLDL, oxidized low-density lipoprotein; PMA, phorbol 12-myristate 13-acetate; REM, relative electrophoretic mobility; RT-Q-PCR, reverse transcription quantification polymerase chain reaction; TBARS, thiobarbituric acid reactive substances.

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LITERATURE CITED

(1) Aviram, M. Macrophage foam cell formation during early atherogenesis is determined by the balance between pro-oxidants and anti-oxidants in arterial cells and blood lipoproteins. *Antioxid. Redox Signaling* **1999**, *1*, 585–94.

(2) Fuhrman, B.; Volkova, N.; Coleman, R.; Aviram, M. Grape powder polyphenols attenuate atherosclerosis development in apolipoprotein E deficient (E0) mice and reduce macrophage atherogenicity. *J. Nutr.* **2005**, *135*, 722–728.

(3) Fuhrman, B.; Aviram, M. Anti-atherogenicity of nutritional antioxidants. *IDrugs* **2001**, *4*, 82–92.

(4) Fuhrman, B.; Aviram, M. Flavonoids protect LDL from oxidation and attenuate atherosclerosis. *Curr. Opin. Lipidol.* **2001**, *12*, 41–8.

(5) Munteanu, A.; Zingg, J. M.; Ricciarelli, R.; Azzi, A. CD36 overexpression in ritonavir-treated THP-1 cells is reversed by alpha-tocopherol. *Free Radical Biol. Med.* **2005**, *38*, 1047–56.

(6) Meydani, M. Vitamin E and atherosclerosis: beyond prevention of LDL oxidation. *J. Nutr.* **2001**, *131*, 366S–8S.

(7) Endemann, G.; Stanton, L. W.; Madden, K. S.; Bryant, C. M.; White, R. T.; Protter, A. A. CD36 is a receptor for oxidized low density lipoprotein. *J. Biol. Chem.* **1993**, *268*, 11811–6.

(8) Nakata, A.; Nakagawa, Y.; Nishida, M.; Nozaki, S.; Miyagawa, J.; Nakagawa, T.; Tamura, R.; Matsumoto, K.; Kameda-Takemura, K.; Yamashita, S.; Matsuzawa, Y. CD36, a novel receptor for oxidized low-density lipoproteins, is highly expressed on lipid-laden macrophages in human atherosclerotic aorta. *Arterioscler. Thromb. Vasc. Biol.* **1999**, *19*, 1333–9.

(9) Nagy, L.; Tontonoz, P.; Alvarez, J. G.; Chen, H.; Evans, R. M. Oxidized LDL regulates macrophage gene expression through ligand activation of PPARgamma. *Cell* **1998**, *93*, 229–40.

(10) Popravko, S. A.; Gurevich, A. I.; Kolosov, M. N. Flavonoid components of propolis. *Chem. Nat. Compd.* **1972**, *5*, 397–401.

(11) Scheller, S.; Wilczok, T.; Imielski, S.; Krol, W.; Gabrys, J.; Shani, J. Free Radical Scavenging by Ethanol Extract of Propolis. *Int. J. Radiat. Biol.* **1990**, *57*, 461–5.

(12) Comalada, M.; Ballester, I.; Bailon, E.; Sierra, S.; Xaus, J.; Galvez, J.; Medina, F. S. d.; Zarzuelo, A. Inhibition of pro-inflammatory markers in primary bone marrow-derived mouse macrophages by naturally occurring flavonoids: Analysis of the structure-activity relationship. *Biochem. Pharmacol.* **2006**, *72*, 1010–21.

(13) Zhang, X.; Hung, T. M.; Phuong, P. T.; Ngoc, T. M.; Min, B. S.; Song, K. S.; Seong, Y. H.; Bae, K. Anti-inflammatory activity of flavonoids from Populus davidiana. *Arch. Pharm. Res.* **2006**, *29*, 1102–8.

(14) Martini, N. D.; Katerere, D. R.; Eloff, J. N. Biological activity of five antibacterial flavonoids from Combretum erythrophyllum (Combretaceae). *J. Ethnopharmacol.* **2004**, *93*, 207–12.

(15) Kempuraj, D.; Madhappan, B.; Christodoulou, S.; Boucher, W.; Cao, J.; Papadopoulou, N.; Cetrulo, C. L.; Theoharides, T. C. Flavonols inhibit proinflammatory mediator release, intracellular calcium ion levels and protein kinase C theta phosphorylation in human mast cells. *Br. J. Pharmacol.* **2005**, *145*, 934–44.

(16) Wang, J.; Mazza, G. Effects of anthocyanins and other phenolic compounds on the production of tumor necrosis factor alpha in LPS/IFN-gamma-activated RAW 264.7 macrophages. *J. Agric. Food Chem.* **2002**, *50*, 4183–9.

(17) Brown, J. E.; Khodr, H.; Hider, R. C.; Rice-Evans, C. A. Structural dependence of flavonoid interactions with Cu2+ ions: implications for their antioxidant properties. *Biochem. J.* **1998**, *330*, 1173–8.

(18) Lotito, S. B.; Frei, B. Dietary flavonoids attenuate tumor necrosis factor alpha-induced adhesion molecule expression in human aortic endothelial cells. Structure-function relationships and activity after first pass metabolism. *J. Biol. Chem.* **2006**, *281*, 37102–10.

(19) Tzeng, S. H.; Ko, W. C.; Ko, F. N.; Teng, C. M. Inhibition of platelet aggregation by some flavonoids. *Thromb. Res.* **1991**, *64*, 91–100.

(20) Ruiz, E.; Padilla, E.; Redondo, S.; Gordillo-Moscoso, A.; Tejerina, T. Kaempferol inhibits apoptosis in vascular smooth muscle induced by a component of oxidized LDL. *Eur. J. Pharmacol.* **2006**, *529*, 79–83.

(21) Kowalski, J.; Samojedny, A.; Paul, M.; Pietsz, G.; Wilczok, T. Effect of kaempferol on the production and gene expression of monocyte chemoattractant protein-1 in J774.2 macrophages. *Pharmacol. Rep.* **2005**, *57*, 107–12.

(22) Liang, Y. C.; Huang, Y. T.; Tsai, S. H.; Lin-Shiau, S. Y.; Chen, C. F.; Lin, J. K. Suppression of inducible cyclooxygenase and inducible nitric oxide synthase by apigenin and related flavonoids in mouse macrophages. *Carcinogenesis* **1999**, *20*, 1945–52.

(23) Sakakibara, M.; Difeo, D.; Nakatani, N.; Timmermann, B. N.; Mabry, T. J. Flavonoid methyl ethers on the external leaf surface of Larrea tridentata and L. Divaricata. *Phytochemistry* **1976**, *15*, 727–31.

(24) Angrawal, P. K. *Carbon-13 NMR of Flavonoids*; Elsevier: Amsterdam, 1989; pp 152–3.

(25) Dinis, T. C.; Maderia, V. M.; Almeida, L. M. Action of phenolic derivatives (acetaminophen, salicylate, and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. *Arch. Biochem. Biophys.* **1994**, *315*, 161–9.

(26) Havel, R. J.; Eder, H. A.; Bragdon, J. H. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **1955**, *34*, 1345–53.

(27) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–54.

(28) Hermann, M.; Gmeiner, B. Altered susceptibility to in vitro oxidation of LDL in LDL complexes and LDL aggregates. *Ann. N. Y. Acad. Sci.* **1993**, *683*, 363–4.

(29) Esterbauer, H.; Striegl, G.; Puhl, H.; Rotheneder, M. Continuous monitoring of in vitro oxidation of human low density lipoprotein. *Free Radical Res. Commun.* **1989**, *6*, 67–75.

(30) Hirakata, M.; Tozawa, R.; Imura, Y.; Sugiyama, Y. Comparison of the effects of pioglitazone and rosiglitazone on macrophage foam cell formation. *Biochem. Biophys. Res. Commun.* **2004**, *323*, 782–8.

(31) Steinbrecher, U. P.; Zhang, H. F.; Lougheed, M. Role of oxidatively modified LDL in atherosclerosis. *Free Radical Biol. Med.* **1990**, *9*, 155–68.

(32) Steinbrecher, U. P.; Witztum, J. L.; Parthasarathy, S.; Steinberg, D. Decrease in reactive amino groups during oxidation or endothelial cell modification of LDL. Correlation with changes in receptor-mediated catabolism. *Arteriosclerosis* **1987**, *7*, 135–43.

(33) Kleinveld, H. A.; Hak-Lemmers, H. L.; Stalenhoef, A. F.; Demacker, P. N. Improved measurement of low-density-lipoprotein susceptibility to copper-induced oxidation: application of a short procedure for isolating low-density lipoprotein. *Clin. Chem.* **1992**, *38*, 2066–72.

(34) Han, S.; Sidell, N. Peroxisome-proliferator-activated-receptor gamma (PPARgamma) independent induction of CD36 in

THP-1 monocytes by retinoic acid. *Immunology* **2002**, *106*, 53–9.

(35) Witztum, J. L. The role of oxidized LDL in atherosclerosis. *Adv. Exp. Med. Biol.* **1991**, *285*, 353–65.

(36) Yesner, L. M.; Huh, H. Y.; Pearce, S. F.; Silverstein, R. L. Regulation of monocyte CD36 and thrombospondin-1 expression by soluble mediators. *Arterioscler. Thromb. Vasc. Biol.* **1996**, *16*, 1019–25.

(37) Feng, J.; Han, J.; Pearce, S. F. A.; Silverstein, R. L.; Gotto, A. M., Jr.; Hajjar, D. P.; Nicholson, A. C. Induction of CD36 expression by oxidized LDL and IL-4 by a common signaling pathway dependent on protein kinase C and PPAR-{gamma}. *J. Lipid Res.* **2000**, *41*, 688–696.

(38) Devaraj, S.; Hugou, I.; Jialal, I. Alpha-tocopherol decreases CD36 expression in human monocyte-derived macrophages. *J. Lipid Res.* **2001**, *42*, 521–7.

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