Role of activated endocannabinoid system in regulation of cellular cholesterol metabolism in macrophages

Li-sheng Jiang¹,², Jun Pu², Zhi-hua Han², Liu-hua Hu², and Ben He²*

¹Department of Geriatrics, Ren Ji Hospital, Shanghai Jiao tong University School of Medicine, Shanghai, People’s Republic of China; and ²Department of Cardiology, Ren Ji Hospital, Shanghai Jiao tong University School of Medicine, Shanghai, People’s Republic of China

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Aims Evidence from recent studies suggests that the endocannabinoid system participates in the regulation of lipid metabolism and body composition. We hypothesize that the system is activated by oxidized low-density lipoprotein (oxLDL) and regulates cellular cholesterol metabolism in macrophages.

Methods and results Primary peritoneal macrophages isolated from Sprague–Dawley rats and RAW264.7 mice macrophages were cultured. A liquid chromatography/mass spectrometry (LC/MS) system was used to measure the endocannabinoid anandamide (AEA), 2-arachidonoylglycerol (2-AG), and cellular cholesterol levels in macrophages. The regulatory mechanisms of cellular cholesterol metabolism were also investigated by molecular biology methods. The results showed that the endocannabinoid system in macrophages was activated by oxLDL through elevation of the AEA and 2-AG levels and the up-regulation of the cannabinoid CB1 and CB2 receptor expression. Win55,212-2, a synthetic cannabinoid, promotes cellular cholesterol accumulation in macrophages, which was associated with an increase in the expression of CD36 and a decrease in the expression of ATP-binding cassette protein A1 (ABCA1) as mediated by an up-regulated peroxisome proliferator-activated receptor gamma (PPARγ). AM251, a selective cannabinoid CB1 receptor antagonist, impaired the abilities of Win55,212-2-treated macrophages to accumulate cholesterol by down-regulating CD36 receptor expression and up-regulating ABCA1 expression.

Conclusion We have demonstrated, for the first time, that the endocannabinoid system in macrophages is activated by oxLDL and that the activated endocannabinoid system promotes cellular cholesterol accumulation in macrophages. The results also indicate that selectively blocking the CB1 receptor can reduce oxLDL accumulation in macrophages, which might represent a promising therapeutic strategy for atherosclerosis.

KEYWORDS
Cellular cholesterol metabolism; CD36 receptor; Endocannabinoid; ATP-binding cassette protein A1; Peroxisome proliferator-activated receptor gamma

1. Introduction
Atherosclerosis is a chronic inflammatory disease, which is the primary cause of morbidity and mortality in the modern world. The identification of promising novel anti-atherosclerotic therapeutics is, therefore, of great interest and represents a continuing challenge to the medical community.

Oxidized low-density lipoprotein (oxLDL) has been proposed as a key factor in the initiation and progression of atherosclerosis.¹ oxLDL is present in atherosclerotic lesions of humans² and animals.³ It is well established that macrophages take up oxLDL through the scavenger receptor pathways resulting in cholesteryl ester accumulation, foam cell formation,⁴,⁵ and a series of attendant proatherogenic activities.⁶

Synthetic or endogenous cannabinoids and their receptor systems have gained wide interests because of their possible usefulness in multiple chronic inflammatory diseases such as multiple sclerosis,⁷ colonic inflammation,⁸ and atherosclerosis.⁹ There is emerging evidence showing that low doses of Δ9-tetrahydrocannabinol (THC), the major psychoactive cannabinoid compound of marijuana, inhibits the progression of established atherosclerotic lesions. This phenomenon is associated with the reduced proliferation and IFN-γ secretion of lymphoid cells as well as reduced macrophage infiltration into atherosclerotic lesions. The effects of THC, however, are blocked by a specific antagonist of the cannabinoid CB2 receptor.⁹ Established evidence also indicates that the increased endocannabinoid level and the activated CB1 receptor in peripheral tissue are closely linked with multiple cardiometabolic risk factors, including obesity and increased serum lipid production in rodents and humans.¹⁰,¹¹ Consequently, selective antagonists of...
the cannabinoid CB1 receptor such as rimonabant or SR141716 have potential roles in the treatment of multiple cardiometabolic risk factors including abdominal obesity and smoking. Until now, whether the endocannabinoid system is activated by oxLDL, or whether the system participates in the regulation of cellular cholesterol metabolism in macrophages, remains largely unexplored.

In the present study, we investigate (i) whether the endocannabinoid system is activated by oxLDL in cultured macrophages; (ii) whether Win55,212-2, a synthetic cannabinoid, increases cholesterol accumulation in macrophages; and (iii) whether the abilities of Win55,212-2 in the regulation of cellular cholesterol metabolism in macrophages are blocked by the selective antagonist of CB1 receptor.

2. Methods

2.1 Materials

Sprague-Dawley (SD) rats were bought from the Chinese Academy of Sciences, Shanghai Laboratory Animal Center. All cell culture materials were from Corning Life Sciences, USA, while all cell culture reagents, including foetal bovine serum (FBS), were purchased from Gibco Life Technologies. The internal standards anandamide (AEA), 2-arachidonoylglycerol (2-AG), and native low-density lipoprotein (N-LDL) were obtained from Sigma. oxLDL was made of N-LDL by CuSO4 as described earlier. Win55,212-2 and AM251 were obtained from Tocris. The RevertaidTM First Stand cDNA Synthesis Kit was from Fermentas International Inc., Canada, and the SYBR® Premix Ex TaqTM Perfect Real Time kit was from Takara Inc., Japan. The ECL Western Blotting Detection Kit was bought from Amsham Pharmacia Biotech. The antibodies were collected from different corporations: rabbit polyclonal anti-cannabinoid CB1 antibody, rabbit polyclonal anti-cannabinoid CB2 antibody, mouse anti-PPARγ/ -2 monoclonal antibody, and mouse anti-mouse monoclonal CD36 antibody from Chemicon Inc., USA; mouse monoclonal ABCA1 antibody from Abcam Inc., USA; rabbit polyclonal anti-platelet-activating factor (PAF) receptor antibody, rabbit monoclonal anti-β-actin antibody, goat anti-rabbit secondary antibody, and goat anti-mouse secondary antibody from Santa Cruz Inc.; and IRDye 680 Goat Anti-Rabbit IgG and IRDye 800CW Goat Anti-Mouse IgG were purchased from Licor Biosience, Lincoln, NE, USA.

2.2 Cell culture

Peritoneal macrophages were isolated from SD rats (250–350 g) and cultured with Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS, with reference to previously documented studies. The mouse RAW264.7 macrophages (ATCC, Manassas, VA, USA) were cultured on the same culture medium. Before stimulation, the non-adherent cells were removed by washing them twice with DMEM and incubated for 24 h under standard conditions, with oxLDL, Win55,212-2, and AM251 alone or combined. In the experiments with CB1 receptor inhibitor, the cells were pretreated for 2 h with AM251 before adding the other compounds. Finally, the cells were harvested for the following measurements.

For this study, the concentrations of oxLDL, Win55,212-2, and AM251 were chosen by cell viability as evaluated through the MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] assay as described earlier. A low dose of oxLDL (1–12 μg/mL), which does not reduce cell viability in macrophages, was chosen. For Win55,212-2 and AM251, the concentrations from 1 to 20 μM were effective and were therefore chosen.

2.3 Measurement of endocannabinoid levels

In measuring the levels of AEA and 2-AG, RAW264.7 cells were seeded (8 × 106 cells/flask) and cultured overnight in a 75 cm2 flask, then incubated for 24 h with oxLDL (6 and 12 μg/mL) or vehicle under standard conditions. After removing non-adherent cells, the peritoneal macrophages were seeded (2 × 107 cells/dish) and incubated for 24 h with the upper concentrations of oxLDL or vehicle. The cells were then harvested and recounted. After washing with 0.01 M PBS, the cells were dissolved with 1.0 mL PBS and broken in an ice bath with an ultrasonic cell crusher. The samples were mixed with 200 μL of methanol–H2O solution (2:1, v/v), which were agitated for 15 min (240 bpm) by adding with 3.0 mL ethyl acetate–N-hexane solution (9:1, v/v). After centrifuging for 5 min (3500 rpm), the subnatant organic phase was transferred into another tube and dried under a 40 °C air stream. Finally, the residues were re-suspended in 100 μL of methanol for analysis by liquid chromatography, using an Agilent 1100 liquid chromatograph (Agilent Inc., USA), equipped with a thermostated autosampler and column compartment. This was followed by an in-line MS analysis on an API 4000 Triple Quadrupole Mass Spectrometer (Applied Biosystems Inc., CA, USA) equipped with Turbo electrospray ionization. Two selected ions were monitored simultaneously: selected on monitoring m/z 379.2 for 2-AG ions as the internal standards and selected on monitoring m/z 348.2 for AEA ions as described previously. The values of AEA and 2-AG were expressed as pmol/108 cells in triplicate.

2.4 Quantitative determination of cellular cholesterol levels

To determine cellular cholesterol levels, RAW264.7 cells were seeded in 25 cm2 flask (3 × 104 cells/flask) and cultured overnight, then incubated for 24 h with oxLDL (12 μg/mL), Win55,212-2 (1–20 μM), and AM251 (1–20 μM) or vehicle, respectively. The cells were harvested and washed with PBS (0.01 M), then dissolved with 0.1 mL 10% FBS, with reference to previously documented studies. A low percentage of free cholesterol from the total cholesterol level. Chemical ionization. Cholesterol measurements were obtained in the existing literature. Internal standards were selected on monitoring m/z 379.2 for AEA ions as described previously. The values of AEA and 2-AG were expressed as pmol/108 cells in triplicate.

2.5 Quantitative real-time polymerase chain reaction analysis

A quantitative real-time PCR analysis of mouse macrophages CD36, CB1, CB2, PAF-R, PPARγ, ABCA1, and GAPDH was performed with SYBR® Premix Ex TaqTM Perfect Real Time kit in an ABI7900 analyzer.
Cannabinoid promotes cholesterol accumulation in macrophages

(Applied Biosystems Inc.). The following primers were used: mouse CD36 sense primer: 5’ ATGGGCTCGATCGAATGGTCTATT 3’ and anti-sense primer: 5’ ATGAGGCAAGTCTTCCGACGCCCAT 3’, mouse CB1 sense primer: 5’ TTTAGGCGCAATGAACTTGT 3’ and anti-sense primer: 5’ TGGAGAGGGCTGTAAACCCC 3’, mouse CB2 sense primer: 5’ CTTGTGCTTACTTTCTGCT 3’ and anti-sense primer: 5’ ATAGAAGCGCCACCACTGCAG 3’, mouse PAF-R sense primer: 5’ GCCACACACAGGGACTTTG 3’ and anti-sense primer: 5’ TCCATGTCTGCGACAGGGA 3’, mouse CB1, CB2, and PAF-receptor antibodies; 1:1000, for PPAR γ sense primer: 5’ AATAGAAGGCCAGCCCAT 3’, mouse CD36 sense primer: 5’ AAATGCCGACACTCTTTCGAC 3’ and anti-sense primer: 5’ CATACGGAAACTCTTTCACCC 3’, and GAPDH sense primer: 5’ GTGAGCGAGCTGAGTTGC 3’ and anti-sense primer: 5’ ATGGGGCTGCGATCGGAACTGTGGGCTCATT 3’. The total RNA (1 μg) was reverse transcribed to cDNA with a RevertaidTM First Stand cDNA Synthesis Kit. The PCR conditions were: preliminary denaturation at 95 °C for 10 s, 95 °C for 5 s, and 60 °C for 30 s (40 cycles). The real-time PCR data were represented as Ct values, defined as the crossing threshold of PCR using ABI7900 Data Analysis software. The following procedure was used to calculate the relative level of the sample mRNA expression: ΔCt sample = Ct sample – Ct control and ΔΔCt GAPDH = Ct sample – Ct control; ΔΔCt represents the difference between the ΔCt GAPDH and ΔCt sample, calculated by the formula ΔΔCt = ΔCt GAPDH – ΔCt sample. Finally, the N-fold differential expression of the samples compared with the control is expressed as 2ΔΔCt.

2.6 Flow cytometric analysis for CD36 surface expression

An immunofluorescent flow cytometric (FACS) analysis was performed to evaluate the cell-surface expression of the CD36 receptor. After treatment, the cells were harvested and washed twice in PBS. About 1 × 10⁶ cells were suspended in 400 μL of 5% mouse serum/PBS and incubated for 30 min at room temperature while shaking. Anti-CD36 FITC-conjugated antibody (4 μL) was added to the suspension and incubated for 2 h at 4 °C. After washing three times with PBS, the cells were re-suspended into 1.0 mL of PBS before analysis using a Bio-Rad flow cytometer.

2.7 Western blot analysis

Cells were lysed in 100 μL of RIPA buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM EDTA, 1% NP-40, 0.1% SDS, 1 mM PMSF, 10 μg/mL aprotinin, and 2 mM NaF. After boiling for 5 min with 5× laemmli sample buffer, equal amounts of protein (50 μg) were resolved in 10% SDS–polyacrylamide gel electrophoresis and electro-blotted to nitrocellulose at 4 °C. The blots were blocked for 1 h with 5% non-fat dry milk in TB5 containing 0.1% Tween 20 and then incubated overnight at 4 °C with diluted primary antibodies (1:500, for CB1, CB2, and PAF-receptor antibodies; 1:1000, for PPARγ and β-actin antibodies). The blots were then incubated with the

Figure 1 Effects of Win55,212-2 and AM251 on cell viability of RAW264.7 macrophages. Cells were incubated for 24 h with Win55,212-2 or AM251 and underwent cell viability analysis by MTT assay. The data are shown as mean ± SEM with relative optical density values at 570 nM in five duplicate experiments.

Figure 2 Oxidized low-density lipoprotein (oxLDL) increased production of anandamide (AEA) and 2-arachidonoylglycerol (2-AG) in macrophages. Cells were treated for 24 h with oxLDL or vehicle and the levels of AEA and 2-AG were analysed quantitatively through the LC/MS/MS system. Data are shown as mean ± SEM with padmol/10⁶ cells in three duplicate experiments. Con, ox6, and ox12 indicate the control (vehicle), oxLDL (6 μg/mL), and oxLDL (12 μg/mL), respectively. (i) 2-AG levels in RAW264.7 cells were increased significantly by oxLDL (A), but AEA increased slightly (B); (ii) in the primary cultured rat peritoneal macrophages, the levels of 2-AG increased by 1.8- and 3.5-fold (C), and AEA increased by 2.3- and 5.9-fold, respectively (D). Significant differences from control: *P < 0.05, **P < 0.01 for 2-AG; and ***P < 0.01 for AEA, respectively.
secondary antibodies and visualized by chemiluminescence using an ECL Western Blotting Detection Kit. Finally, band intensities were determined by a densitometric analysis of the immunoblots with Quantity One 4.4.0 software (Bio-Rad, Hercules, CA, USA).

2.8 In-cell western assay
Expressions of ABCA1 protein were determined with in-cell western assay as described previously. After treatment, the medium was aspirated and washed. The cells were then fixed for 20 min with 3.7% (v/v) formaldehyde in PBS and permeabilized with 0.1% (v/v) Triton X-100 in PBS on a shaker. After blocking with 100 μL/well Odyssey blocking buffer, the cells were incubated for 2 h with the primary antibodies (ABCA1 1:500, β-actin 1:500, diluted in Odyssey blocking buffer), and the negative control was also incubated with Odyssey blocking buffer. After washing five times with 0.1% Tween 20 (v/v) in PBS, the cells were incubated for 1 h with secondary antibodies diluted in Odyssey blocking buffer (diluted ratio: IRDye 680 Goat Anti-Rabbit IgG, 1:200, IRDye 800CW Goat Anti-Mouse IgG, 1:800). As a final step, the cells were washed followed by the plates undergoing visualization on a Licor’s Odyssey Reader at 700 and 800 nm and analysed for optical density value using Odyssey Software 2.0 version. Data were shown as mean ± SEM in three duplicate experiments.

2.9 Statistical methods
All continuous variables’ data are reported as mean ± SEM. Statistical analysis was performed by one-way ANOVA. The Student–Newman–Keuls test was also used to examine the significant differences between groups. A value of \( P < 0.05 \) was considered significant to reject the null hypothesis.

3. Results
3.1 Win55,212-2 and AM251 tended to reduce cell viability in RAW264.7 macrophages
The results of MTT showed that WIN55,212-2 and AM251 tended to reduce the viability, especially when the concentration was over 5 μM (Figure 1). However, oxLDL at the concentrations from 1 to 48 μg/mL did not reduce cell viabilities in RAW264.7 macrophages.

Figure 3 Oxidized low-density lipoprotein (oxLDL) up-regulated cannabinoid receptors mRNA and protein expressions in RAW264.7 macrophages. The cells were incubated for 24 h with oxLDL (1–12 μg/mL) or vehicle and the expressions of cannabinoid receptors mRNA and protein measured. The results show that: (A) data from real-time PCR are expressed as fold difference from control in three duplicate experiments; (B) the bands were detected by western blot with rabbit polyclonal anti-cannabinoid CB1 or anti-cannabinoid CB2 antibodies. Data are expressed as optical density fold difference related to β-actin in three duplicate experiments. ‘Con, ox1, ox3, ox6, and ox12’ represent vehicle, oxLDL (1 μg/mL), oxLDL (3 μg/mL), oxLDL (6 μg/mL), and oxLDL (12 μg/mL), respectively. Significant differences from control: \( **P < 0.01 \) for CB1; and \( *P < 0.05 \), \( **P < 0.01 \) for CB2, respectively.

Figure 4 Oxidized low-density lipoprotein (oxLDL) increased expressions of platelet-activating factor (PAF) receptor mRNA and protein in RAW264.7 macrophages. The cells were incubated for 24 h with oxLDL (1–12 μg/mL) or vehicle, and expressions of PAF receptor mRNA and protein measured. The results show that: (A) data from real-time PCR are expressed as fold difference from control in three duplicate experiments; (B) the bands were detected by western blot with rabbit polyclonal anti-PAF receptor antibody, and the optical density (OD) value was analysed with Quantity One 4.4.0 software. Data are expressed as OD fold difference related to β-actin in three duplicate experiments. ‘Con, ox1, ox3, ox6, and ox12’ represent vehicle, oxLDL (1 μg/mL), oxLDL (3 μg/mL), oxLDL (6 μg/mL), and oxLDL (12 μg/mL), respectively. Significant differences from control: \( **P < 0.01 \).
3.2 Endocannabinoids system in macrophages was activated by oxidized low-density lipoprotein

3.2.1 Oxidized low-density lipoprotein increased production of anandamide and 2-arachidonoylglycerol in macrophages

The results of LC/MS/MS analysis showed that: after treatment with oxLDL for 24 h, the production of AEA and 2-AG increased by varying degrees in the macrophages. For RAW264.7 cells, oxLDL at the concentrations of 6 and 12 μg/mL resulted in an increase of 2.4- and 4.8-fold in 2-AG levels, respectively (Figure 2A), whereas the AEA levels increased slightly (Figure 2B). In primary rat peritoneal macrophages, both 2-AG and AEA levels were apparently increased by oxLDL (Figure 2C and D). Data were expressed as pmol/10^8 cells in three duplicate experiments.

3.2.2 Oxidized low-density lipoprotein up-regulated expressions of CB1 and CB2 receptors in RAW264.7 macrophages

The results from real-time PCR showed: incubations with oxLDL at the concentrations of 3, 6, and 12 μg/mL resulted in an increase of 3.4-, 3.3-, and 4.1-fold in CB1 receptor mRNA expression, and 1.9-, 3.4-, and 7.4-fold in CB2 receptor mRNA expression, respectively (Figure 3A). Meanwhile, results by western blot showed that the expressions of CB1 receptor protein were up-regulated by 1.8-, 2.2-, and 3-fold, and the expressions of CB2 receptor protein were up-regulated by 1.3-, 1.4-, and 1.7-fold, respectively (Figure 3B).

3.2.3 Mechanisms of the endocannabinoid system activation in RAW264.7 macrophages

The platelet-activating factor receptor mRNA and protein expressions were analysed to explore the mechanisms of the endocannabinoid system activation in RAW264.7 macrophages. The results indicated that the expressions of PAF

Figure 5 Effects of Win55,212-2 and AM251 on cellular cholesterol metabolism in RAW264.7 macrophages. The cells were incubated for 24 h with oxLDL, or combined with Win55,212-2 and AM251, and detections on the cellular cholesterol levels by LC/MS/MS system performed. Data are shown as mean ± SEM of the free, total, and esterified cellular cholesterol content in an amount of cells containing 1 μg of protein (ng/μg.protein) in three duplicate experiments. The results show that: (A) incubation with oxidized low-density lipoprotein (oxLDL) (3–24 μg/mL) apparently increased the levels of free, total, and esterified cellular cholesterol. (B) Compared with the treatment with 12 μg/mL of oxLDL alone, the levels of total and esterified cellular cholesterol were significantly increased by co-incubations with oxLDL and Win55,212-2 (1–20 μM). (C) In contrast to the co-incubations with WIN55,212-2 and oxLDL, when AM251 was pretreated, the levels of free, total, and esterified cellular cholesterol decreased. 'Con, ox1, ox3, ox6, and ox12' represent vehicle, oxLDL (3 μg/mL), oxLDL (6 μg/mL), oxLDL (12 μg/mL), and oxLDL (24 μg/mL), respectively. The black, striped grey, and white bars represent free, total, or esterified cellular cholesterol, respectively, and their significant differences from control are shown as **P < 0.01; ***P < 0.001; and ****P < 0.001, respectively.

Figure 6 Win55,212-2 up-regulated expressions of peroxisome proliferator-activated receptor gamma (PPARγ) receptor mRNA and protein in RAW264.7 macrophages. The cells were incubated for 24 h with Win55,212-2 (1–20 μM) or vehicle (DMSO) and were analysed with PPARγ receptor's mRNA and protein expressions. The results show that: (A) data from real-time PCR are expressed as fold difference from control (vehicle) in three duplicate experiments; (B) the bands were detected by western blotting method with mouse monoclonal anti-PPARγ antibody, and the optical density (OD) value was analysed with Quantity One 4.4.0 software. The data are expressed as OD value fold difference related to β-actin in three duplicate experiments; (C) in contrast to the co-incubations with WIN55,212-2 and oxLDL, when AM251 was pretreated, the levels of free, total, and esterified cellular cholesterol decreased. 'Con, w1, w5, w10, and w20' represent vehicle (DMSO), Win55,212-2 (1 μM), Win55,212-2 (5 μM), Win55,212-2 (10 μM), and Win55,212-2 (20 μM), respectively. Significant differences from control: *P < 0.05, **P < 0.01.
receptor mRNA and protein in RAW264.7 macrophages were significantly up-regulated by oxLDL (Figure 4A and B).

3.3 Regulation of cellular cholesterol metabolism in RAW264.7 macrophages by cannabinoid and its receptor system

3.3.1 Effects of Win55,212-2 and AM251 on cellular cholesterol metabolism in RAW264.7 macrophages

The results of LC/MS/MS analysis showed that when administered for 24 h with oxLDL (3–24 μg/mL), the free, total, and esterified cellular cholesterol levels in RAW264.7 macrophages were apparently increased (Figure 5A). Compared with the treatment with 12 μg/mL of oxLDL alone, the co-incubations with Win55,212-2 (1–20 μM) significantly increased the total and esterified cholesterol levels (Figure 5B). In contrast to the co-incubations with WIN55,212-2 and oxLDL, when pretreated with AM251, the levels of free, total, and esterified cellular cholesterol apparently decreased (Figure 5C).

3.3.2 Regulatory mechanisms of cannabinoid and its receptor system on cellular cholesterol metabolism in RAW264.7 macrophages

The peroxisome proliferator-activated receptor gamma (PPARγ) was investigated to explore the regulatory mechanisms of Win55,212-2 on the cellular cholesterol metabolism in RAW264.7 macrophages. The results showed that after incubations for 24 h with Win55,212-2, the levels of PPARγ mRNA and protein were markedly increased, especially when the concentration was over 5 μM (Figure 6A and B). Accordingly, the CD36 receptor and the ATP-binding cassette protein A1 (ABCA1), two of PPARγ receptor’s downstream transcription factors, were further examined. The results showed that: when treated with oxLDL (1–12 μg/mL), the expressions of CD36 mRNA and protein were up-regulated with a dose-dependent manner (Figure 7A and D), but the ABCA1 mRNA and protein’s expressions were down-regulated by the treatment of 12 μg/mL of oxLDL (Figure 8A and D); compared with the treatment of 6 μg/mL of oxLDL alone, the co-incubations with Win55,212-2 (5–20 μM), the mRNA

![Figure 7](https://academic.oup.com/cardiovascres/article-abstract/81/4/805/727727) Regulation of CD36 receptor expression in RAW264.7 macrophages by Win55,212-2 and AM251. The expression of CD36 mRNA and protein in RAW264.7 macrophages was quantitatively analysed by real-time PCR and FACS analysis. The results show that: (A and D) when administered for 24 h with oxidized low-density lipoprotein (oxLDL) (1–12 μg/mL), the expressions of CD36 mRNA and protein were up-regulated with a concentration-dependent manner; (B and E) compared with the treatment with 6 μg/mL of oxLDL alone, the co-incubations with oxLDL and Win55,212-2 (5–20 μM) increased the CD36 mRNA and protein expression; (C and F) in contrast to the co-incubations with WIN55,212-2 and oxLDL, when AM251 was pretreated, the expressions of CD36 mRNA and protein significantly decreased. Data are shown as mean ± SEM in three experiments in duplicate. Significant differences from control (shown as left bar in each histogram): **P < 0.01.
and protein expression levels of CD36 were further increased (Figure 7B and E), however, the expressions of ABCA1 mRNA and protein were apparently decreased (Figure 8B and E). In contrast to the co-incubations with Win55,212-2 and oxLDL, the expressions of CD36 mRNA and protein significantly decreased when AM251 was pretreated (Figure 7C and F), however, the expressions of ABCA1 mRNA and protein increased (Figure 8C and F).

4. Discussion

There were several major findings in this study: (i) endocannabinoid system in macrophages was activated by oxLDL through elevating the levels of AEA and 2-AG and up-regulating the expressions of cannabinoid CB1 and CB2 receptors. (ii) The increase in cellular cholesterol in response to the synthetic cannabinoid Win55,212-2 was associated with an increase in the expression of CD36 and a decrease in the expression of ABCA1. (iii) AM251, a selective cannabinoid CB1 receptor antagonist impaired the abilities of Win55,212-2 to accumulate oxLDL by down-regulating the expression of CD36 receptor and up-regulating the expression of ABCA1.

To date, at least two major ingredients of endocannabinoids have been identified. One is AEA, and the other is 2-AG.25,26 According to previous reports,27–30 both AEA and 2-AG could be biosynthesized in macrophages in vitro. Upon application of stimulants such as lipopolysaccharide, endocannabinoid AEA and 2-AG levels are increased in macrophage lines or in primary isolated peritoneal macrophages. However, the activation of AEA and 2-AG in macrophages involves different mechanisms: the increase in 2-AG levels is most likely linked with the increased synthesis by up-regulating PAF receptor-mediated phospholipases activation,27 and also a decreased hydrolysis by degrading fatty acid amide hydrolase...
However, the enhancement of AEA levels is mostly related to decreased hydrolysis by FAAH.\textsuperscript{28} as well as increased synthesis by activating the protein tyrosine phosphatase PTPN22.\textsuperscript{30} We also observed that the endocannabinoid system was activated by oxLDL both in RAW264.7 cells and in the primary isolated peritoneal rat macrophages. Consistent with the earlier reports,\textsuperscript{27} oxLDL also up-regulated PAF receptor’s expression, which is linked with elevated 2-AG levels in RAW264.7 cells.

Recent evidence indicates that the cannabinoid system is involved in chronic inflammatory atherosclerotic disease.\textsuperscript{9} In rodents and humans, it has been reported that the endocannabinoid system participates in the regulation of cellular lipid metabolism in macrophages, (ii) whether administration of selective cannabinoid CB1 receptor antagonist will inhibit the atherosclerosis progression in animals by regulation of the cellular cholesterol metabolism.

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