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4-Methoxycinnamyl p-Coumarate Mediates Anti-Atherosclerotic Effects by Suppressing NF-κB Signaling Pathway and Foam Cell Formation

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Abstract

Vascular inflammation plays a crucial role in atherosclerosis initiation and progression. 4-methoxycinnamyl p-coumarate (MCC), a major bioactive phenylpropanoid found in *Etlingera pavieana* rhizomes, has demonstrated anti-inflammatory activity in lipopolysaccharide-induced macrophages and microglial cells. Therefore, we hypothesized that MCC may also exert anti-inflammatory effects on human vascular endothelial cells, which are key players in the early stages of atherosclerotic plaque formation. To test this hypothesis, we investigated the mechanisms underlying MCC-mediated anti-atherosclerotic effects in tumor necrosis factor-alpha (TNF-α)-treated vascular endothelial cells. An MTT assay was performed to assess the cytotoxicity of MCC on human vascular endothelial EA.hy926 cells. The mRNA expression levels of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) were analyzed using real-time reverse transcription polymerase chain reaction. Foam cell formation induced by oxidized low-density lipoprotein (ox-LDL) in RAW264.7 macrophages was evaluated by Oil Red O staining. Our findings revealed that non-toxic concentrations of MCC (6.25 - 25 μM) significantly downregulated ICAM-1 and VCAM-1 mRNA expression in a concentration-dependent manner. In addition, MCC inhibited the nuclear translocation of the nuclear factor-κB (NF-κB) p65 subunit, as demonstrated by western blot analysis. Moreover, MCC reduced ox-LDL-induced foam cell formation in RAW264.7 macrophages. Collectively, these results suggest that MCC exerts its anti-atherosclerotic effects by downregulating ICAM-1 and VCAM-1 expression via inhibition of the NF-κB signaling pathway in endothelial cells and by suppressing formation of ox-LDL-induced foam cells. These findings highlight the potential of MCC as a promising therapeutic agent for the treatment of vascular inflammatory disorders.

Keywords: 4-Methoxycinnamyl p-coumarate, ICAM-1, VCAM-1, Endothelial cell, Ox-LDL, Foam cell, Vascular inflammation

Introduction

Atherosclerosis is a chronic arterial disease characterized by the formation of atherosclerotic plaques [1]. According to the World Health Organization [2], atherosclerosis is a major risk factor for cardiovascular disease (CVD), which is the leading cause of non-communicable disease-associated death globally. Vascular inflammation is a critical factor in the progression of atherosclerosis, with monocyte adhesion to endothelial cells serving as an early event [1]. This adhesion is predominantly mediated by intracellular signaling, which elevates the expression of endothelial adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). This process facilitates monocyte infiltration into the arterial intima and their subsequent conversion into macrophages [1,3].

Oxidized low-density lipoproteins (ox-LDL), primarily formed as a result of excessive reactive oxygen species (ROS) generation, initiate an inflammatory cascade [4]. Ox-LDL prompt

macrophages to take up lipids and cholesterol, resulting in the formation of foam cells and, ultimately, the development of atherosclerotic plaques [4-6]. Exposure of macrophages to ox-LDL stimulates the release of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6). These cytokines upregulate endothelial adhesion molecules, thereby promoting leukocyte migration to the sites of endothelial damage [3,7]. ICAM-1 and VCAM-1 are crucial in vascular inflammation associated with atherosclerosis, and their expression is up-regulated by TNF-α via the nuclear factor kappa B (NF-κB) transcription factor [8].

4-methoxycinnamyl p-coumarate (MCC), a bioactive phenylpropanoid isolated from the ethanol extract of *Etlingera pavieana* rhizomes, exhibits potent activity in nitric oxide inhibition [9]. Previous studies have demonstrated anti-inflammatory effects of MCC in LPS-induced RAW 264.7, macrophages, and BV2 microglial cell models via the downregulation of pro-inflammatory mediators and cytokines [10,11]. In addition, MCC inhibits acute inflammation in rat models [12]. MCC decreases ICAM-1 and VCAM-1 protein levels in endothelial cells [13]. However, the molecular mechanisms through which MCC inhibits vascular inflammation are not fully understood. Therefore, we investigated the mechanisms underlying the anti-atherosclerotic effect of MCC in TNF-α-induced human vascular endothelial cells, and its effects on ox-LDL-induced foam cell formation in RAW 264.7 macrophages.

Materials and methods

Materials

MTT was obtained from Invitrogen (USA). H_2DCF-DA and Oil Red O were purchased from Sigma-Aldrich (USA). SYBR Green Supermix and iScript Reverse Transcription Supermix were from Bio-Rad (USA). Oxidized LDL, NE-PER extraction reagents, and chemiluminescent substrate were purchased from Thermo Scientific (USA). Antibodies against ICAM-1, VCAM-1, GAPDH, NF-κB p65, and phospho-NF-κB p65 (Ser536) were from Cell Signaling Technology (USA), and the Lamin A antibody was from Santa Cruz Biotechnology (USA).

Methods

Compound preparation

MCC, a kind gift from Dr. E. Srisook (Department of Chemistry, Faculty of Science, Burapha University, Thailand), was isolated from *E. pavieana* rhizomes as described by Srisook *et al*. [9].

Cell culture

This study was designed to investigate the anti-inflammatory and anti-atherosclerotic effects of MCC using human vascular endothelial and murine macrophage cell models. Two cell lines were utilized: EA.hy926 (human vascular endothelial cells) and RAW 264.7 (murine macrophages), both obtained from the American Type Culture Collection (ATCC). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% heat-inactivated fetal bovine serum (FBS), and incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

Cell viability assay

Cell viability was assessed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay as described by Srisook *et al*. [13]. EA.hy926 cells (5×104 cells/well) were seeded in 24-well plates and treated with varying concentrations of MCC for 6 and 24 h. The cells were then washed with phosphate buffered saline (PBS) and incubated in fresh DMEM containing MTT (0.1 mg/mL) for 3 h. The medium was removed and formazan crystals were dissolved in dimethyl sulfoxide (DMSO). The absorbance was measured at 550 nm using a microplate reader.

Real-time reverse transcription-polymerase chain reaction (Real-time RT-PCR)

EA.hy926 cells (3×10^5 cells/plate) were pretreated with MCC for 1 h, followed by TNF-α stimulation (10 ng/mL) for 3 h. Total RNA was extracted using the NucleoSpin® RNA3 Kit (Macherey-Nagel, Germany), and 2 µg of RNA was reverse transcribed using iScript™ Supermix (Bio-Rad). Real-time PCR was performed on a CFX96 Touch system (Bio-Rad) using iTaq™ SYBR Green Supermix. Primer sequences were used as previously described by Srisook *et al*. [13]. GAPDH served as the internal reference gene. Relative

expression levels were calculated using the 2^–ΔΔCt method [14] and analyzed with CFX Manager™ software.

Western blot analysis

EA.hy926 cells were pretreated with MCC for 1 h and then stimulated with TNF-α (10 ng/mL) for 30 min. Whole-cell lysates were prepared using RIPA buffer [13], and cytosolic and nuclear proteins were extracted using NE-PER reagents (Thermo Scientific) following the manufacturer's instructions. Equal amounts of protein were separated by 10% SDS-PAGE and transferred to PVDF membranes. After blocking with 5% nonfat milk, membranes were incubated with primary and HRP-conjugated secondary antibodies. Protein bands were visualized by chemiluminescence and quantified using Image Studio Lite 5.2 software.

Intracellular ROS measurement

To measure intracellular ROS, EA.hy926 cells (1×105 cells/well) growing in 24-well plates were pretreated with MCC for 1 h, followed by stimulation with TNF-α for 12 h. The cells were then washed twice with warm PBS and incubated with H2DCF-DA (2′,7′-dichlorodihydrofluorescein diacetate; 50 μM) for 30 min. Subsequently, cells were rinsed with ice-cold PBS and scraped in ice-cold PBS on ice. The fluorescence intensity was measured using a fluorescence spectrophotometer (Cary Eclipse, Agilent, USA) at excitation and emission wavelengths of 485 and 521 nm, respectively.

Detection of foam cell formation

The effect of MCC on foam cell formation in RAW 264.7 macrophages was evaluated as described by Park *et al*. [15]. RAW264.7 cells (1×105 cells/well) were seeded onto glass coverslips in 24-well plates and pretreated with MCC for 1 h, followed by incubation with ox-LDL (50 μg/mL) for 24 h. The cells were washed with PBS, fixed with 10% formaldehyde for 10 min, and washed again with PBS. Subsequently, the cells were stained with 0.5% (v/v) Oil Red O in isopropanol for 30 min. Excess stain was removed by washing with PBS. Stained lipid droplets were observed under a microscope (Olympus, Tokyo, Japan). The stained cells were dissolved in 100% isopropanol and the absorbance was quantified at 490 nm using a microplate reader.

Statistical analyses

Data are presented as the mean ± standard deviation (SD) of at least 3 independent experiments. Statistical significance was determined using one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons, performed using Minitab 17.1 for Windows. Statistical significance was set at $p < 0.05$.

Results and discussion

Effect of MCC on cell viability

The cytotoxicity of MCC in EA.hy926 cells was assessed using the MTT assay following treatment of cells with various concentrations of MCC for 6 h. MCC (6.25 - 25 μM) did not significantly affect cell viability of the treated cells compared to that of control cells (0.1% DMSO). However, 50 μM MCC significantly decreased cell viability (**Figure 1(A)**). Similarly, MCC (6.25 - 25 μM) did not significantly affect cell viability even after 24 h of treatment (**Figure 1(B)**). Therefore, non-cytotoxic concentrations of MCC (up to 25 μM) were used in subsequent experiments.

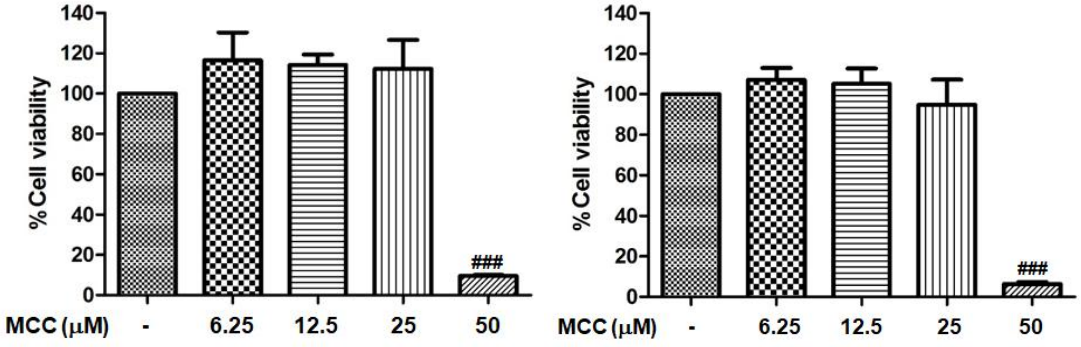
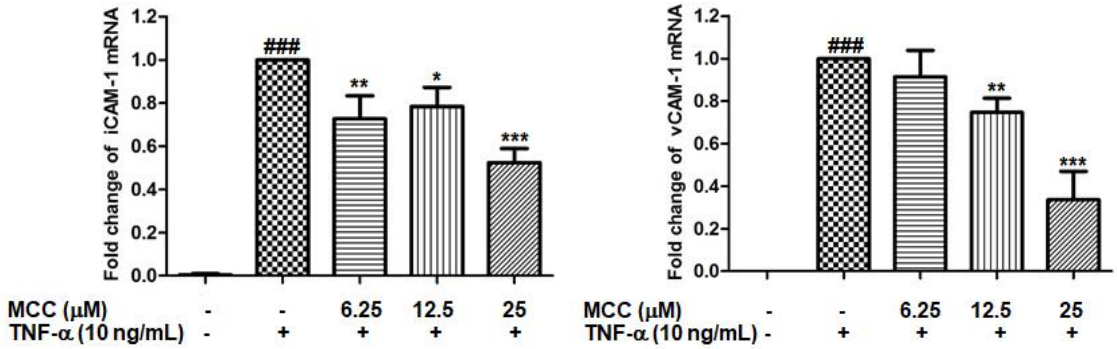


Figure 1 Effect of 4-methoxycinnamyl *p*-coumarate (MCC) on viability of EA.hy926 cells. Cells were treated with the indicated concentrations of MCC for 6 h (A) and 24 h (B). Cell viability was assessed using the MTT assay. Data are presented as mean ± standard deviation (SD) from at least 3 independent experiments conducted in triplicate. DMSO-treated cells were used as the control group. ### $p < 0.001$ compared with control cells.

MCC inhibits ICAM-1 and VCAM-1 expression in TNF-α-induced human endothelial cells

During atherosclerosis, adhesion molecules such as VCAM-1 and ICAM-1 interact with leukocyte-specific adhesion molecules, enhancing leukocyte adherence to the endothelial surface and facilitating their migration across the endothelium [3]. This interaction results in the maturation of macrophages and formation of foam cells, which are essential for the development of atherosclerotic lesions [4,16]. Therefore, reducing the expression of VCAM-1 and ICAM-1 may serve as an effective therapeutic strategy for preventing vascular inflammatory diseases. As shown in **Figures 2(A)** and **2(B)**, the mRNA expression of ICAM-1 and VCAM-1 were markedly elevated in TNF-α-stimulated cells compared with that in the unstimulated control cells. In contrast, pretreatment with 6.25 - 25 μM MCC significantly reduced ICAM-1 and VCAM-1 mRNA expression in a dose-dependent manner compared to that in the TNF-α-stimulated cells ($p < 0.05$). Our previous study demonstrated that ICAM-1 and VCAM-1 protein levels were consistent with the observed mRNA expression [13]. The downregulation of VCAM-1 and ICAM-1, which are involved in leukocyte adhesion to the endothelium, results in reduced monocyte-endothelium adhesion. For instance, Gwon *et al*. [17] found that sargaquinoic acid lowered the expression of ICAM-1, VCAM-1, and MCP-1 in TNF-α-stimulated HUVECs, thereby preventing THP-1 monocyte adhesion to endothelial cells. Similarly, Lin *et al*. [8] demonstrated that andrographolide and 14-deoxy-11,12-didehydroandrographolide inhibited TNF-α-induced interaction between monocytes and endothelial cells by downregulating ICAM-1 and VCAM-1 expression in EA.hy926 cells. Additionally, Lee *et al*. [18] reported that isobavachalcone significantly inhibited TNF-α-induced VCAM-1 and ICAM-1 expression, leading to decreased monocyte adhesion to HUVECs. Our study shows that MCC reduces VCAM-1 and ICAM-1 expression in a dose-dependent manner following TNF-α stimulation at both protein and mRNA levels. These findings indicate that suppression of adhesion molecule expression, which facilitates monocyte adhesion to the vascular endothelium, likely plays a role in MCC-mediated anti-atherosclerotic effects.



(A) (B)

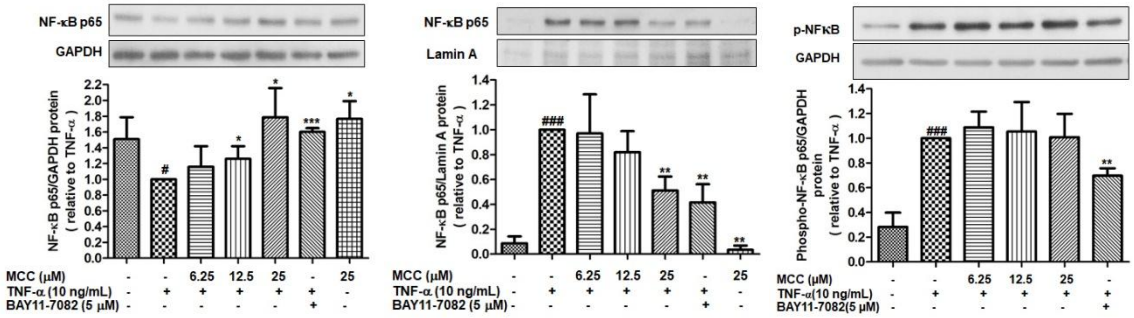
Figure 2 Effect of MCC on intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expression. EA.hy926 cells were pretreated with MCC for 1 h and subsequently stimulated with tumor necrosis factor-alpha (TNF-α) for 3 h prior to mRNA analysis. The mRNA expression of ICAM-1 (A) and VCAM-1 (B) was examined by real-time RT-PCR. Data are presented as mean ± standard deviation (SD; n = 3) of values normalized to GAPDH. DMSO-treated cells were used as the control group. $^{###}p < 0.001$ compared with control cells; $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ compared with TNF-α-stimulated cells.

Effect of MCC on TNF-α-induced NF-κB activation in human endothelial cells

TNF-α, a pro-inflammatory cytokine that triggers the inflammatory activation of vascular endothelial cells, is highly expressed in various inflammatory diseases, including atherosclerosis. TNF-α induces the expression of adhesion molecules on the surface of endothelial cells, primarily through isoform 1 of the TNF receptor (TNFR1), leading to the activation of NF-κB [3]. NF-κB regulates TNF-α-induced expression of adhesion molecules and pro-inflammatory cytokines [19,20]. The primary active form of NF-κB is a heterodimer composed of the p65 and either the p50 or p52 subunits. The activation of NF-κB is transiently regulated by IκBα, which is phosphorylated by inhibitor κB kinase (IKK)-β and subsequent proteolytic degradation of IκBα. This process results in the degradation of IκBα, and translocation of NF-κB into the nucleus [21]. Both ICAM-1 and VCAM-1 promoters contain NF-κB binding sites [19]. To elucidate the molecular mechanism of MCC in TNF-α-induced endothelial cells, the activation of the NF-κB signaling pathway was analyzed by western blotting. Treatment with TNF-α decreased NF-κB p65 cytosolic protein content and increased its nuclear fraction. Furthermore, the phosphorylation of NF-κB p65 protein was markedly increased following TNF-α treatment. We observed that pretreatment with MCC enhanced the level of TNF-α-induced NF-κB p65 protein in the cytoplasm (**Figure 3(A)**) and reduced its level in the nucleus (**Figure 3(B)**) compared to that in TNF-α-stimulated cells. The increase in phosphorylated NF-κB p65 was not significantly inhibited by MCC. However, the addition of BAY 11-7082 led to a decrease in NF-κB p65 phosphorylation (**Figure 3(C)**). These findings demonstrate that MCC may inhibit ICAM-1 and VCAM-1 expression by preventing the nuclear translocation of NF-κB. These results are consistent with the effects of ethanol extracts from *E. pavieana* rhizomes on TNF-α-induced human endothelial cells [13] and align with previous reports on the effects of MCC in LPS-induced BV2 microglial cells [11]. However, MCC did not affect the phosphorylation of Ser536 in the NF-κB p65 subunit. These results suggest that MCC modulates the phosphorylation of NF-κB p65 differently in BV2 microglial cells [11] and endothelial EA.hy926 cells, as demonstrated in the current study.

In addition to IKK/IκB phosphorylation, NF-κB is also induced by ROS, which are key mediators involved in various chronic diseases [22]. ROS production is triggered by TNF-α via TNFR1 activation [3], and leads to injury of endothelial cells and activation of a variety of pro-inflammatory cytokine signaling pathways including NF-κB p65 [8,23]. Therefore, we analyzed the effect of MCC on TNF-α-induced ROS production in human endothelial cells. As shown in **Figure 4**, TNF-α markedly induced intracellular ROS production in EA.hy926 cells compared with that in control cells, and

the production was not attenuated by MCC pretreatment. Gallic acid (50 μM) and trolox (100 μM), which are well-known antioxidants [24,25], significantly decreased TNF-α induced ROS production. These results were different from previous findings of Srisook *et al*. [13], who reported that ethanol extracts of *E. pavieana* rhizomes inhibit TNF-α-induced ROS production in human endothelial cells. However, MCC, a compound isolated from the ethanol extract of *E. pavieana* rhizomes, did not inhibit TNF-α-induced ROS production. This may be because the inhibition of ROS production is mediated by the synergistic effects of various compounds in the ethanol extract of *E. pavieana* rhizomes. Based on these findings, the possible mechanism underlying MCC-mediated inhibitory effects on TNF-α-induced expression ICAM-1 and VCAM-1 may be via downregulation of NF-κB activation and independent of the inhibition of ROS production.



(A) (B) (C)

Figure 3 Effect of MCC on TNF-α-induced NF-κB activation. EA.hy926 cells were pretreated with MCC for 1 h and subsequently stimulated with TNF-α for 30 min. The levels of NF-κB p65 in cytoplasmic (A) and nuclear (B) fractions were detected using western blot analysis. Data are presented as mean ± standard deviation (SD; n = 3) of densitometric values normalized to that of GAPDH and lamin A, respectively. The levels of phosphorylated NF-κB p65 (C) in whole-cell protein extracts were also determined using western blot analysis. Data are presented as mean ± SD (n = 3) of densitometric values normalized to that of GAPDH. DMSO-treated cells were used as the control group. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ compared with control cells; $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ compared with TNF-α-stimulated cells.

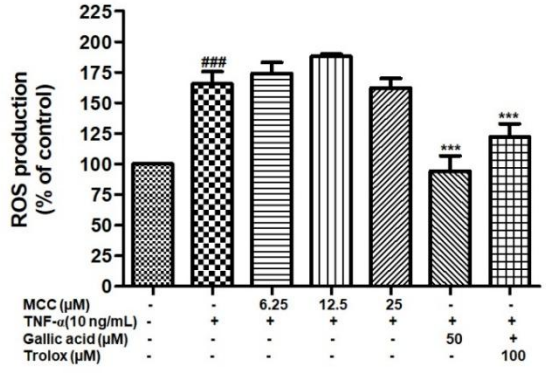
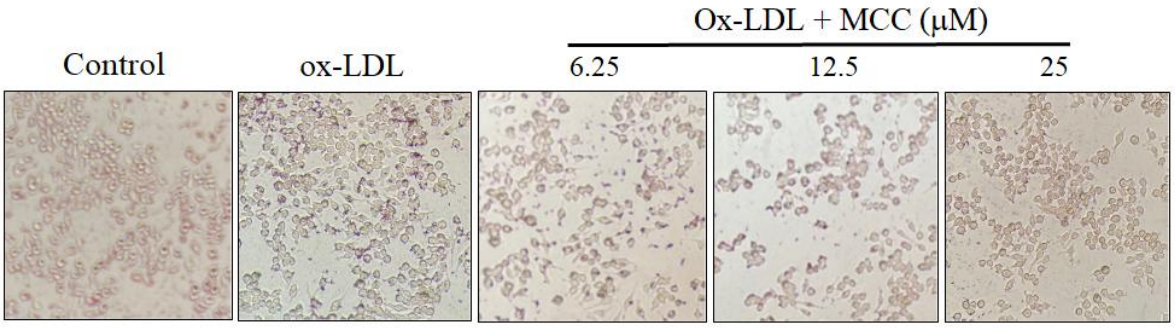


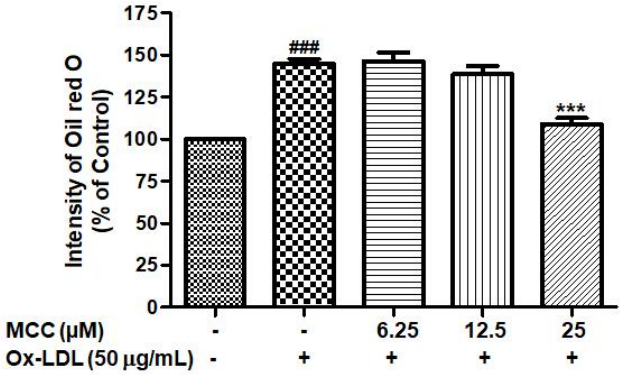
Figure 4 Effect of MCC on TNF-α-induced ROS production. EA.hy926 cells were pretreated with MCC for 1 h, followed by stimulation with LPS for 12 h. Intracellular ROS production was measured using the H2DCF-DA (2′,7′-dichlorodihydrofluorescein diacetate) fluorescent probe. Data are presented as mean ± standard deviation (SD) from 3 independent experiments. DMSO-treated cells were used as the control group. $^{\#\#\#}p < 0.001$ compared with control cells; $***p < 0.001$ compared with TNF-α-stimulated cells.

Effects of MCC on reduction of foam cell formation

As foam cell formation is a critical step in atherosclerosis pathogenesis, we assessed the effects of MCC on foam cell formation in ox-LDL-stimulated RAW264.7 macrophage cells. Intracellular lipid accumulation was examined using the Oil Red O staining assay. Lipid droplets were observed in RAW264.7 cells exposed to ox-LDL, whereas pretreatment with 25 μM MCC resulted in the disappearance of these droplets (**Figure 5(A)**). The quantification of the staining revealed that pretreatment with 25 μM MCC decreased Oil Red O staining compared to treatment with ox-LDL alone (**Figure 5(B)**). These results indicate that MCC pretreatment effectively inhibited foam cell formation in ox-LDL - stimulated RAW264.7 cells. Following ingestion of ox-LDL, macrophages transform into cholesterol-loaded foam cells, which constitute fatty streaks and are hallmarks of early atherosclerotic lesions [4-6,26]. The uptake of ox-LDL is facilitated by a diverse family of scavenger receptors, including scavenger receptor class A (SR-A), cluster of differentiation 36 (CD36) and lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1). These receptors, which are expressed on the macrophage surface, are the 3 most significant contributors to foam cell formation [1,27,28]. Furthermore, ox-LDL induces the expression of SR-A and CD36 [1,29-32]. Cholesterol is removed from macrophages via reverse cholesterol transport, a process mediated by the ATP-binding cassette transporters A1 (ABCA1), ABCG1, and scavenger receptor BI (SR-BI). These transporters facilitate cholesterol efflux from macrophages to apolipoprotein A-I (apoA-I) and high-density lipoproteins (HDL), thereby preventing foam cell formation [5,28].



(A)



(B)

Figure 5 Effect of MCC on formation of foam cells. RAW264.7 macrophage cells were pretreated with MCC for 1 h, followed by stimulation with ox-LDL (50 μg/mL) for 24 h. Following treatment, foam cell formation was detected by Oil Red O staining followed by microscopy (10× magnification). (A) Representative photographs of Oil Red O-stained cells from different groups. (B) Quantification of Oil Red O solution intensity is presented. Data are presented as mean ± standard deviation (SD) from 3 independent experiments. DMSO-treated cells were used as the control group. $^{###}p < 0.001$ compared with control cells; $^{***}p < 0.001$ compared with TNF-α-stimulated cells.

An increasing body of research has indicated that natural compounds contribute significantly to the prevention of foam cell formation by reducing cholesterol uptake and/or enhancing its removal. For example, curcumin suppresses ox-LDL-induced foam cell formation in RAW264.7 cells by downregulating CD36 and peroxisome proliferator-activated receptor gamma (PPARγ) expression, while having no effect on SR-A expression [29]. Similarly, orientin markedly reduces CD36 expression, thereby diminishing ox-LDL-induced foam cell formation in RAW264.7 macrophages [30]. In addition, the ethanol extract of Dan-Lou decreases ox-LDL uptake and lipid accumulation in macrophages by suppressing the protein and mRNA expression of toll-like receptor (TLR) 4 and SR-BI [26]. Li *et al*. [33] reported that quercetin treatment prevents ox-LDL-induced lipid deposition in RAW264.7 cells by enhancing the expression of ABCA1, ABCG1, and liver X receptor-alpha (LXR-α). Moreover, hydroxytyrosol activates the PPAR/LXR pathway, resulting in increased ABCA1 expression, and a subsequent reduction in cholesterol accumulation in THP-1 macrophage-derived foam cells [34]. In the present study, MCC effectively inhibited ox-LDL-induced lipid accumulation in RAW264.7 cells. These findings suggest that the anti-atherosclerotic properties of MCC may be attributed, at least in part, to its role in preventing foam cell formation. However, the precise mechanism by which MCC inhibits macrophage-derived foam cell formation remains unclear. Future studies should focus on investigating the molecular mechanisms through which MCC modulates the expression of proteins involved in cholesterol import and export in macrophages.

Conclusions

In summary, our findings suggest that MCC exerts anti-atherosclerotic effects by reducing the expression of ICAM-1 and VCAM-1 through the inhibition of the NF-κB signaling pathway in human vascular endothelial cells. MCC also prevents the formation of RAW264.7 macrophage-derived foam cells. These results highlight the potential of MCC as a natural therapeutic agent for the treatment of vascular inflammatory diseases, including atherosclerosis.

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Declaration of Generative AI in Scientific Writing

During the preparation of this manuscript, the authors used ChatGPT by OpenAI solely for language editing and grammar correction. All scientific content, interpretation, and conclusions were developed independently by the authors.

CRediT Author Statement

Mayuree Poonasri: Investigation; Writing - Original draft. **Petcharat Sawai**: Investigation. **Klaokwan Srisook**: Funding acquisition; Conceptualization; Methodology; Project administration; Writing – Review and Editing.

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