Inhibitory effects of resveratrol on foam cell formation are mediated through monocyte chemotactic protein-1 and lipid metabolism-related proteins

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Received September 24, 2013; Accepted February 3, 2014

DOI: 10.3892/ijmm.2014.1680

Abstract. Resveratrol has been shown to exert anti-atherosclerotic effects. 5′ AMP-activated protein kinase (AMPK) and monocyte chemotactic protein-1 (MCP-1) play key roles in foam cell formation, which is considered as the initiation of atherosclerosis. Thus, in this study, we investigated whether resveratrol inhibits foam cell formation by regulating lipid accumulation and inflammation. For this purpose, THP-1 cells were treated with 100 nM phorbol 12-myristate 13-acetate (PMA) to induce their differentiation into macrophages. The macrophages were then pre-treated with 2.5 μM resveratrol and subsequently with serum-free (SF) medium alone or SF medium containing lipopolysaccharide (LPS; 100 ng/ml) and oxidized low-density lipoprotein (ox-LDL; 50 μg/ml) for 24 h to detect foam cell formation. To detect the expression of lipid accumulation-related proteins, the macrophages were treated with resveratrol. For the detection MCP-1 expression, the macrophages were treated with LPS and resveratrol, or with resveratrol alone. We incubated the THP-1-derived macrophages in resveratrol (2.5 μM) for 6 h in the presence or absence of 30 µM compound C for 4 h to detect the influence of compound C on the effects of resveratrol. The foam cells were examined using Red O staining. Gene expression levels were determined by qRT-PCR, western blot analysis and ELISA; lipid analysis was carried out by high-performance liquid chromatography (HPLC). The results revealed that resveratrol effectively suppressed foam cell formation induced by LPS. Resveratrol also suppressed lipid accumulation and downregulated the mRNA expression of peroxisome proliferator-activated receptor (PPAR)γ and PPARα, but had no effect on the expression of PPARβ/δ. Resveratrol also upregulated the expression of AMPK and Silent information regulator T1 (SIRT1). However, the effects of resveratrol on SIRT1, PPARγ and PPARα expression and lipid accumulation were reversed when the cells were pre-treated with compound C. Resveratrol downregulated the mRNA expression of MCP-1 in a dose-dependent manner and LPS upregulated its expression in a time-dependent manner. MCP-1 expression induced by LPS was inhibited by resveratrol at both the transcriptional and translational level. These data suggest that resveratrol inhibits foam cell formation by regulating the expression of MCP-1 and activating the AMPK-SIRT1-PPAR signaling pathway; thus, resveratrol may be a novel therapeutic agent for atherosclerosis.

Introduction

Atherosclerosis is a chronic inflammatory disease characterized by inflammatory infiltrates and lipid accumulation (1). It is known that monocytes play an important role in the progression of the disease (2,3). Monocytes first migrate into the arterial tissue in response to locally produced chemokines and then differentiate into macrophages. These macrophages act to augment the expression of several pattern recognition receptors, leading to the accumulation of cholesterol and lipids; these cells then become foam cells (4-6). Therefore, inflammatory infiltrates and the accumulation of cholesterol and lipids in macrophages is a key point in the initiation of atherosclerosis. Over the past decade, the search for natural compounds with the ability to prevent atherosclerosis has been a main focus for many investigators. Several lines of evidence suggest that resveratrol, a plant-derived polyphenol and phytoalexin, exhibits cardioprotective and anti-inflammatory properties (7-10). In the current study, we investigated the potential atheroprotective, anti-inflammatory and lipid-lowering effects of resveratrol. The oral administration of resveratrol has been shown to affect lipid profiles and inflammation markers (11). However, the precise mechanisms involved remain unclear. The balanced flow of lipids into and out of macrophages is necessary to avoid lipid overload, and ultimately, foam cell formation (12). 5′ AMP-activated protein kinase (AMPK) is an

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Key words: atherosclerosis, THP-1 cells, resveratrol, inflammation, lipid metabolism
important serine/threonine kinase well known for regulating cellular energy levels by balancing nutrient availability and energy demand through its control of several proteins involved in glucose and lipid metabolism (13,14). Recent evidence has shown a promising role for AMPK in the attenuation of atherosclerosis and vascular dysfunction (14). Resveratrol can activate the AMPK pathway in adipose tissue (15). In addition, it has been demonstrated that resveratrol exerts cardiometabolic effects by increasing AMPK expression and the level of Silent information regulator T1 (SIRT1) (16).

Conventional risk factors for atherosclerosis trigger an inflammatory response in the artery wall, mediated by complex molecular interactions in which chemokines play a critical role (17). Monocyte chemotactic protein-1 (MCP-1) is a potent chemoattractant for monocytes and plays a pivotal role in early atherogenesis by promoting monocyte infiltration to lesion-prone areas and penetrates between endothelial cells into the inner arterial space (18,19). MCP-1 is synthesized by endothelial cells and monocytes in response to diverse stimuli, including interleukins and oxidized low-density lipoprotein (ox-LDL) (18). Lipopolysaccharide (LPS) is thought to be involved in cardiovascular disease, as it contributes to the development of arterial plaques through activated pro-inflammatory pathways by secreting cytokines, including MCP-1 (20,21). The mechanisms which have been suggested to be responsible for the anti-inflammatory effects of resveratrol, include the inhibition of MCP-1 production (22).

In this study, we investigated the effects of resveratrol on foam cell formation, as well as on the expression of MCP-1 and AMPK in macrophages in order to elucidate the mechanisms involved in its anti-atherosclerotic effects. In addition, the expression of SIRT1 and nuclear peroxisome proliferator-activated receptors (PPARs) was detected. The results of this study provide important information regarding the initiation and prevention of atherosclerosis.

Materials and methods

Materials. Resveratrol, Oil Red O and compound C were purchased from Sigma-Aldrich, St. Louis, MO, USA; antibodies directed against phospho-AMPKα (Thr172), AMPK, SIRT1 and β-actin were from Cell Signaling Technology, Danvers, MA, USA; Texas-Red-conjugated goat anti-mouse secondary antibody was obtained from Molecular Probes, Eugene, OR, USA; LPS, phorbol 12-myristate 13-acetate (PMA) and TRIZol reagent were from Sigma-Aldrich; the cDNA Synthesis kit was obtained from Fermentas, St. Leon-Rot, Germany; the SYBR Premix Ex Taq™ II kit was purchased from Takara Bio Inc., Otsu, Japan; the Human MCP-1 ELISA kit was from R&D Systems, Minneapolis, MN, USA; the BCA kit was from Pierce, Rockford, IL, USA; and Agilent 1100 series HPLC system was from Agilent Technologies, Palo Alto, CA, USA.

Preparation of ox-LDL. Human LDL was purified from the fresh plasma of healthy donors by sequential centrifugation, according to the method described in the study by Feng et al (23) with some modifications. In order to produce ox-LDL, 200 µg/ml LDL were exposed to 20 µM CuSO4 in phosphate-buffered saline (PBS) for oxidation and the reaction was terminated with 40 µM butylhydroxytoluene in ethanol. ox-LDL was then dialyzed against culture medium and sterile-filtered.

Cell culture. The human monocytic cell line, THP-1 (ATCC, Rockville, MD, USA), was grown in RPMI-1640 supplemented with 10% (v/v) fetal bovine serum (FBS), 0.05 mM 2-mercaptoethanol, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/l glucose and 1.5 g/l bicarbonate at 37°C in an atmosphere containing 5% CO2. The THP-1 cells were stimulated by a 48-h exposure to 100 nM PMA to induce their differentiation into adherent macrophages. Differentiated THP-1 macrophages were extensively washed in PBS before being used in experiments.

Resveratrol treatment. A stock concentration of 100 mM resveratrol in 50% DMSO was produced fresh each time and diluted in culture medium to the desired concentration. The controls received the same amount of DMSO.

Foam cell formation assay. The experiments were performed in serum-free (SF) experimental medium. The THP-1 cells were pre-treated with 2.5 µM of resveratrol or the control (DMSO) for 1 h. Subsequently, both the control and resveratrol-treated groups were treated with SF medium alone or SF medium containing LPS (100 ng/ml) and ox-LDL (50 µg/ml) for 24 h. The formation of foam cells was determined by Oil Red O staining. The cells were fixed with 4% formaldehyde for 15 min. Cell lipids were stained with Oil Red O (3 mg/ml in 60% isopropanol) for 10 min, then observed under a microscope.

Quantitative reverse transcription PCR (qRT-PCR). Total cellular RNA was extracted from the THP-1-derived macrophages using TRIZol reagent in accordance with the manufacturer’s instructions, and dissolved in nuclease-free water, prior to being reverse-transcribed to synthesize first-strand cDNA with oligo(dT) primer using the cDNA Synthesis kit. To correct for differences in cDNA loading among the samples, the target PCRs were normalized to a reference PCR involving the endogenous housekeeping gene, β-actin. Non-template controls were included for each primer pair to check for any significant levels of contaminants. A melting-curve analysis was performed to assess the specificity of the amplified PCR products. qRT-PCR was performed using the FastStart SYBR-Green reagent kit according to the manufacturer’s instructions. The reaction conditions followed the instructions provided by the manufacturer of the SYBR Premix Ex Taq II kit using gene-specific primers for MCP-1, SIRT1, PPARs and β-actin (Table I).

Lipid analysis by high-performance liquid chromatography (HPLC). Cellular total cholesterol and triglyceride contents were analyzed by lipid analysis by HPLC. Briefly, the cells were washed 3 times in PBS and lysed by the addition of 10% NaOH solution. Protein concentration was measured using the BCA kit. Masterol was used as a standard curve first, and the extraction procedure was then repeated. Samples were dissolved in 100 µl of isopropanol-acetonitrile (v/v, 20:80), followed by incubation in an ultrasonic water at room temperature for 5 min. Finally, the samples were placed in the Agilent 1100 series HPLC system.
Western blot analysis. Protein concentrations were determined using bovine serum albumin (BSA) as a standard protein with the BCA protein assay. The same amounts of total proteins (15-20 µg/20 µl) were loaded onto a 80 V for 1 h. A 1:1,000 dilution of primary antibody. Following incubation with the corresponding secondary antibody, signals were detected using a chemiluminescent detection system and quantified using Quantity One analysis software.

ELISA. To evaluate the produced levels of MCP-1, the THP-1-derived macrophages were pre-treated with resveratrol (10 µM) for 1 h and then treated with LPS (10 µM) for 6 h. Supernatants from the treated cells were collected and analyzed for MCP-1 using a sandwich ELISA kit according to the manufacturer's instructions.

Data analysis. All results are expressed as the means ± standard deviation (SD). All data were evaluated using SPSS 11.0 software. A typical image from at least 3 similar experiments is presented. Statistical analysis were carried out using t-tests. A value of P<0.05 was considered to indicate a statistically significant difference.

Results

Resveratrol inhibits foam cell formation. We investigated foam cell formation in response to exposure to LPS. As evidenced by Oil Red O staining, in the presence of ox-LDL, the typical formation of foam cells was observed (Fig. 1A-II vs. A-I). Resveratrol effectively suppressed the foam cell formation induced by LPS (Fig. 1A-III vs. A-II). We investigated intracellular lipid accumulation by HPLC. LPS at 100 ng/ml without LDL loading increased lipid accumulation in the macrophages (Fig. 1B, LPS vs. SF medium, solid bars). In the presence of ox-LDL (50 µg/ml) loading, LPS further increased lipid accumulation in the macrophages (Fig. 1B, LPS ox-LDL vs. ox-LDL, solid bars). These observations led us to hypothesize that resveratrol may inhibit foam cell formation by regulating lipid accumulation.

Table I. Primer sequences used in qRT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5′→3′)</th>
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<tbody>
<tr>
<td>β-actin</td>
<td>F: GATCATGTGCTTCTCTGTGAC</td>
</tr>
<tr>
<td></td>
<td>R: ACTCCTGTGCTGATCCAC</td>
</tr>
<tr>
<td>SIRT1</td>
<td>F: GAGTGGAAGAAGGAGCAG</td>
</tr>
<tr>
<td></td>
<td>R: TCTGGCATGCCACATTC</td>
</tr>
<tr>
<td>PPARγ</td>
<td>F: GCAGTTGGGATGTCATCATATGC</td>
</tr>
<tr>
<td></td>
<td>R: CAGGCGGTTATGTTTGA</td>
</tr>
<tr>
<td>PPAR β/δ</td>
<td>F: AAGGCTACCTGAAAAACTCCAAC</td>
</tr>
<tr>
<td></td>
<td>R: GTGCACGCTGATTCCGT</td>
</tr>
<tr>
<td>PPARα</td>
<td>F: GAGAAAGCAAATGGAAACGAGA</td>
</tr>
<tr>
<td></td>
<td>R: GAAAGCGGGTTATTGGCT</td>
</tr>
<tr>
<td>MCP-1</td>
<td>F: AGCCACCTTCATTCCCCCAAG</td>
</tr>
<tr>
<td></td>
<td>R: CTCTGGCCACAAATGGCT</td>
</tr>
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SIRT1, Silent information regulator T1; PPAR, peroxisome proliferator-activated receptor; MCP-1, monocyte chemotactic protein-1; F, forward, R, reverse.
Resveratrol inhibits lipid accumulation through SIRT1 and PPARs. SIRT1 reduces the accumulation of fatty acids by suppressing the expression of PPAR-γ (24). PPAR-γ is one of the PPAR family members, which comprises 3 isotypes: PPARα, PPARγ and PPARβ/δ (25). PPARs play a central role in the regulation of adipogenesis (26). Resveratrol is an activator of SIRT1 (27). Thus, this prompted us to investigate whether resveratrol regulates lipid accumulation through SIRT1-PPARs.

To investigate the effects of resveratrol on SIRT1 and PPAR expression, the THP-1-derived macrophages were treated with resveratrol (2.5 µM) for 6 h. As shown in Fig. 2A, resveratrol significantly upregulated the mRNA expression of SIRT1 (P<0.05), and there were analogous results obtained by western blot analysis (Fig. 2B).

We then detected the expression of PPARs. As shown in Fig. 3, resveratrol significantly downregulated the mRNA expression of PPARγ (P<0.05). As regards PPARα, resveratrol also significantly downregulated the mRNA expression (P<0.05). By contrast, resveratrol had no effect on the mRNA expression of PPARβ/δ (P>0.05), suggesting that resveratrol regulates lipid accumulation through SIRT1-PPARs.

Resveratrol regulates SIRT1-PPARs through AMPK. As shown by our results, resveratrol regulates the expression of SIRT1 and PPARs; however, the mechanisms involved are not yet clear. AMPK, which acts upstream of SIRT1, controls several proteins involved in glucose and lipid metabolism (13,14). The activation of AMPK activates catabolic pathways, generating ATP, and “switches off” a number of processes that consume ATP, such as fatty acid, protein, or cholesterol synthesis (28,29); AMPK also enhances SIRT1 activity (30,31).

To investigate the effects of resveratrol on AMPK, the THP-1-derived macrophages were treated with resveratrol (2.5 µM) for 6 h. The phosphorylated isofrom is the active AMPK form; thus, we determined the phosphorylated AMPK/total AMPK protein ratio. Resveratrol significantly increased the phosphorylated AMPK/total AMPK protein ratio; this increase demonstrated that AMPK was significantly activated by resveratrol (Fig. 4), suggesting that resveratrol regulates SIRT1-PPARs through AMPK.

Effects of AMPK inhibition on SIRT1 and PPAR expression and lipid accumulation. To verify our hypothesis, we evaluated the effects of AMPK inhibition (using compound C) on SIRT1 and PPAR expression and lipid accumulation following incubation of THP-1-derived macrophages in resveratrol (2.5 µM) for 6 h in the presence or absence of 30 µM compound C.
for 4 h. Our results revealed that pre-treatment with 30 µM compound C for 4 h inhibited the resveratrol-induced increase in SIRT1 expression and blocked the suppression of PPARγ/PPARα expression by resveratrol (Fig. 5A and B). We also examined lipid accumulation; the suppression of lipid accumulation following treatment with resveratrol was reversed when the cells were pre-treated with 30 µM compound C (Fig. 5C).

**Resveratrol blocks LPS-induced MCP-1 expression.** Given the well-documented anti-inflammatory effects of resveratrol, we focused on the effects of resveratrol on LPS-induced MCP-1 expression in THP-1-derived macrophages. Resveratrol downregulated the mRNA expression of MCP-1 in a dose-dependent manner (Fig. 6A). As shown in Fig. 6B, MCP-1 mRNA expression increased following treatment with LPS in a time-dependent manner. Pre-treatment with 10 µM resveratrol markedly inhibited the mRNA expression of MCP-1 induced by LPS (Fig. 6C). The induced expression of MCP-1 was determined by ELISA (Fig. 7). Compared to the control group, LPS induced a statistically significant upregulation of MCP-1 expression (P<0.05), and resveratrol downregulated the LPS-induced MCP-1 expression. MCP-1 expression which was induced by LPS was inhibited by resveratrol at both the transcriptional and translational level. These results demonstrate that resveratrol inhibits foam cell formation by regulating inflammatory cytokine (MCP-1) production.

**Discussion**

Cardiovascular disease, which is currently the leading cause of death and illness in developed countries, is a preeminent health issue worldwide (32). Atherosclerosis, a progressive
inflammatory disease, produces arterial plaques characterized by inflammatory infiltrates, lipid accumulation, cell death and fibrosis (2,3,33). Monocytes play an important role in the progression of the disease. Monocytes first migrate, and then differentiate into macrophages. Inflammatory infiltrates and the accumulation of cholesterol and lipids in macrophages allows them to become foam cells; this is a key point in the initiation of atherosclerosis.

Resveratrol is a polyphenol found in grapes, berries and peanuts. It inhibits macrophage activation (34), one of the most important steps in atherosclerosis, as well as lipid accumulation (34,35). In addition, resveratrol has been suggested to exert anti-atherosclerotic effects (36). However, the precise mechanisms responsible for the anti-atherosclerotic effects of resveratrol remain unclear.

In this study, we found that THP-1-derived macrophages treated with LPS and ox-LDL together resulted in the typical formation of foam cells and resveratrol effectively suppressed the foam cell formation induced by LPS. Based on our findings, we suggest that resveratrol exerts anti-atherosclerotic effects by suppressing foam cell formation. Experiments on the effects of lipid deposition in THP-1-derived macrophages treated with resveratrol, revealed that the cellular total cholesterol content was suppressed by resveratrol, suggesting that resveratrol protects against atherosclerosis by inhibiting lipid accumulation.

AMPK regulates cellular energy levels by balancing nutrient availability and energy demand through its control of several proteins involved in glucose and lipid metabolism (13). SIRT1 is one of the 7 mammalian homologs of the Sir2 family that catalyzes NAD⁺-dependent protein deacetylation (37). Both AMPK and SIRT1 have emerged as interesting targets as they are heavily involved in catabolic metabolism, mitochondrial activation, angiogenesis and enhanced cell survival (37-40). PPARs are central regulators of adipogenesis (25). Previous studies have shown that resveratrol negatively modulates PPARγ protein levels in 3T3-L1 adipocytes (41) and it is known that resveratrol affects both SIRT1 and AMPK (42). The data from these studies, as well as ours, suggest that resveratrol regulates lipid metabolism through the AMPK-SIRT1-PPAR signaling pathway.

In this study, we found that AMPK was significantly activated by resveratrol and that resveratrol markedly upregulated the expression of SIRT1 both at the mRNA and protein level. In addition, resveratrol markedly downregulated the mRNA
expression of PPARγ and PPARα, but not that of PPARβ/δ. When the cells were pre-treated with the AMPK inhibitor, compound C, the effects of resveratrol on SIRT1, PPARγ and PPARα expression, as well as on lipid accumulation were reversed, suggesting that resveratrol suppresses lipid accumulation through the AMPK-SIRT1-PPARγ/PPARα signaling pathway.

Inflammatory infiltrates in macrophages are a key to foam cell formation. Thus, we hypothesized that resveratrol may suppress foam cell formation by regulating inflammatory cytokines. We selected MCP-1, a potent chemoattractant for monocytes. MCP-1 has been shown to be overexpressed in human and experimental atheroma, and can recruit mononuclear phagocytes that characteristically accumulate in the nascent atheroma (32,42). A recent study demonstrated that LPS induces the expression of MCP-1 (44). Despite the evidence of a prominent role of MCP-1 in the development of atherosclerosis, and although resveratrol has been shown to inhibit the production of various types of inflammatory cytokines (34,45), few studies have investigated the direct effects of resveratrol on MCP-1. In this study, we found that resveratrol downregulated the expression of MCP-1 in a dose-dependent manner in THP-1-derived macrophages and that LPS induced MCP-1 expression in THP-1-derived macrophages in a time-dependent manner. Most importantly, we found that pre-treatment of THP-1-derived macrophages with resveratrol significantly blocked MCP-1 mRNA expression induced by LPS. Therefore, we speculate that resveratrol plays a role in anti-atherosclerosis by inhibiting the expression of MCP-1.

In conclusion, our results demonstrate that resveratrol suppresses the foam cell formation induced by LPS. Resveratrol inhibits foam cell formation by regulating the expression of the inflammatory cytokine, MCP-1, and by activating the AMPK-SIRT1-PPAR signaling pathway. These results suggest that resveratrol may be a novel therapeutic agent for atherosclerosis.

Acknowledgements

This study was supported by a grant from the Natural Science Foundation of China (no. 81200633).

References


