Original Article

L-4F Inhibits Oxidized Low-density Lipoprotein-induced Inflammatory Adipokine Secretion via Cyclic AMP/Protein Kinase A-CCAAT/Enhancer Binding Protein β Signaling Pathway in 3T3-L1 Adipocytes

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Abstract

Background: Adipocytes behave like a rich source of pro-inflammatory cytokines including monocyte chemoattractant protein-1 (MCP-1). Oxidized low-density lipoprotein (oxLDL) participates in the local chronic inflammatory response, and high-density lipoprotein could counterbalance the proinflammatory function of oxLDL, but the underlying mechanism is not completely understood. This study aimed to evaluate the effect of apolipoprotein A-I mimetic peptide L-4F on the secretion and expression of MCP-1 in fully differentiated 3T3-L1 adipocytes induced by oxLDL and to elucidate the possible mechanisms.

Methods: Fully differentiated 3T3-L1 adipocytes were incubated in the medium containing various concentration of L-4F (0–50 μg/ml) with oxLDL (50 μg/ml) stimulated, with/without protein kinase A (PKA) inhibitor H-89 (10 μmol/L) preincubated. The concentrations of MCP-1 in the supernatant, the mRNA expression of MCP-1, the levels of CCAAT/enhancer binding protein α (C/EBPα), and CCAAT/enhancer binding protein β (C/EBPβ) were evaluated. The monocyte chemotaxis assay was performed by micropore filter method using a modified Boyden chamber.

Results: OxLDL stimulation induced a significant increase of MCP-1 expression and secretion in 3T3-L1 adipocytes, which were inhibited by L-4F preincubation in a dose-dependent manner. PKA inhibitor H-89 markedly reduced the oxLDL-induced MCP-1 expression, but no further decrease was observed when H-89 was used in combination with L-4F (50 μg/ml) ($P > 0.05$). OxLDL stimulation showed no significant effect on C/EBPα protein level but increased C/EBPβ protein level in a time-dependent manner. H-89 and L-4F both attenuated C/EBPβ protein level in oxLDL-induced 3T3-L1 adipocytes.

Conclusions: OxLDL induces C/EBPβ protein synthesis in a time-dependent manner and enhances MCP-1 secretion and expression in 3T3-L1 adipocytes. L-4F dose-dependently counterbalances the pro-inflammatory effect of oxLDL, and cyclic AMP/PKA-C/EBPβ signaling pathway may participate in it.

Key words: Adipocytes; CCAAT/Enhancer-Binding Proteins; L-4F; Monocyte Chemoattractant Protein-1; Oxidized low-density Lipoprotein; Protein Kinase A

Introduction

Migration of monocytes into the subendothelium is the initial step of inflammation and atherosclerotic plaque formation, which plays a key role in the atherosclerotic plaque formation. Monocyte chemoattractant protein-1 (MCP-1) facilitates the migration of inflammatory cells by chemotaxis and is the trigger signal of inflammation in atherosclerosis.[1,2]

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Adipocyte and adipose tissue not only play a role in lipid synthesis and storage, but also are associated with a low-grade chronic inflammation, supporting the notion that obesity plays an important role in the development of atherosclerosis as well as insulin resistance. Adipocytes behave like a rich source of pro-inflammatory cytokines including MCP-1.[3] Thus, therapies that target in macrophage accumulation may be beneficial in decreasing the atherosclerotic risk associated with obesity, and a potential therapeutic target is MCP-1.[4,5] Since MCP-1 promotes atherosclerosis, inhibition of MCP-1 may be effective in lowering atherosclerotic risk associated with obesity.

Oxidized low-density lipoprotein (oxLDL) participates in the local chronic inflammatory response. High-density lipoprotein (HDL) and apolipoprotein A-I (apoA-I), the main proteins of HDL, are antiatherogenic, cardioprotective, and exhibit anti-inflammatory properties. ApoA-I mimetic peptide 4F, which contains only 18 amino acids and contains a Class A amphipathic helix with a polar and a nonpolar face that allows it to bind lipids similar to apoA-I.[6] ApoA-I mimetic peptide can convert HDL from pro-inflammation to anti-inflammation in young mice,[7] as well as decrease macrophage traffic into the aortic arch and innominate arteries in a mouse model of influenza and atherosclerosis.[8] Previous reports suggest that apoA-I and apoA-I mimetic peptide are potent anti-inflammatory agents that may have therapeutic potential. But whether apoA-I mimetic peptide shows anti-inflammatory effects in adipocytes is not understood.

In this study, we investigated the extent to which adipocytes contribute to overall increases in oxLDL-mediated MCP-1 levels in 3T3-L1 adipocytes. We further investigated whether L-4F down-regulates MCP-1 levels and mRNA expression and attempted to explore the possible mechanism.

Methods

Materials

Mouse 3T3-L1 preadipocytes were purchased from the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. HDLs were purchased from Sigma (USA). OxLDL were purchased from the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. L-4F was synthesized by Shenzhen Hybio Engineering Co., Ltd., (China). Primers were synthesized by Beijing Augct Biotechnology Co., Ltd., (China). Blood samples were taken from healthy volunteers in the graduate school of The Second Xiangya Hospital of Central South University.

Cell culture

Mouse 3T3-L1 preadipocytes were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (fetal bovine serum [FBS]). At the 3rd day after confluence, adipocytes differentiation was induced by adding 3-Isobutyl-1-Methylxanthine (IBMX; 0.5 mmol/L), dexamethasone (0.25 μmol/L), and insulin (10 mg/L) for 2 days. Then, cells were maintained in DMEM supplemented with 10% FBS and insulin alone for an additional 2 days. The medium was then changed to DMEM containing 10% FBS for an additional 4 days. At that time, more than 90% adipocytes differentiation was achieved.[9]

For the experiment, cells were plated in 6-well plates at a density of 1.5×10^6 cells/ml. When appropriate, fully differentiated adipocytes were shifted to a serum-free medium containing DMEM and 0.2% bovine serum albumin and were incubated for 18 h with various concentrations of L-4F (0, 1, 10, and 50 μg/ml) separately, with/without protein kinase A (PKA) inhibitor H-89 (10 μmol/L) preincubated for 1 h before incubated with 50 μg/ml oxLDL, accompanied with oxLDL (50 μg/ml) for the last 1, 6, or 12 h.

Monocyte chemoattractant protein-1 protein measurement

MCP-1 concentrations in 3T3-L1 adipocytes supernatants were measured by using enzyme-linked immunoabsorbent assay. Each sample was assayed in duplicate. Intra-assay and inter-assay precision variability were <8%.

RNA isolation and amplification with reverse transcription-polymerase chain reaction

Total RNA was extracted from 3T3-L1 adipocytes using TRIzol reagent (Invitrogen, USA) according to manufacturer's instructions. First strand cDNA was synthesized using reverse transcription kits (Promega, USA), and the cDNA obtained was used for polymerase chain reaction (PCR) amplification to estimate mRNA expression. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous control gene for normalization. Specific primer sequences used were as follows: MCP-1: 5'-ACTGAAGCCAGCTCTCTCTTCCTC-3' (forward) and 5'-TTCCTTCTTGGGGTCAGCACAGAC-3' (reverse), GAPDH: 5'-ACCACAGTCCATGCCATCAC-3' (forward), and 5'-TCCACCACCCTGTTGCTGTA-3' (reverse). The sizes of PCR products were 274 bp for MCP-1 and 439 bp for GAPDH. The PCR thermocycling conditions for the genes were as follows: an initial denaturation for 3 min at 94°C, 36 cycles with denaturation at 94°C for 50 s, primer annealing at 60°C (MCP-1), or 58°C (GAPDH) for 30 s, and extension at 72°C for 50 s, and then a final extension step at 72°C for 10 min. Five microliters of each PCR product was electrophoresed on 1% agarose gel electrophoresis and ethidium bromide-stained bands were scanned by Gel Doc 2000 Imaging System (Bio-Rad Inc., Hercules, CA, USA). Optical density was analyzed through Quantity One 4.03 analysis software (Bio-Rad Inc., Hercules, CA, USA). The results of MCP-1 levels were presented relative to the expression of GAPDH.

Nuclear protein extraction and analysis of CCAAT/enhancer binding protein α, β proteins by Western blot

Total nuclear protein was extracted from 3T3-L1 adipocytes using nuclear protein extraction kits (Beyotime Institute of Technology, China) according to manufacturer's instructions and quantitated by BCA method. After 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, protein was

transferred to PVD membranes. CCAAT/enhancer-binding proteins (C/EBPs) were identified by a rabbit-anti-mouse polyclonal C/EBPα or C/EBPβ antibody (Santa Cruz, USA) for 2 h, followed by the goat-anti-rabbit-IgG antibody (Santa Cruz) for 1 h. Mouse β-actin was used as the internal control.

Human peripheral monocyte chemotactic activity assay

Heparinized venous blood was obtained from normal volunteers at the age of 20–30 years. Mononuclear cells were obtained by the Ficoll-Hypaque (Sigma-Aldrich, St. Louis, MO, USA) gradient centrifugation method. The resultant leukocyte suspensions containing more than 95% neutrophils were resuspended in Hank's balanced salt solution (HBSS)-fetal calf serum at a final concentration of 10^6 neutrophils/ml. Chemotaxis assays were performed in modified Boyden chambers separated with three groups: (1) random migration groups: monocytes suspension with DMEM were added to the upper well and DMEM were added to the lower well; (2) chemotaxis groups: monocytes suspension were added to the upper well and supernatants of 3T3-L1 adipocytes incubated with oxLDL for 12 h or different concentrations of L-4F (1, 10, and 50 μg/ml) were added to the lower well; and (3) positive control group: monocytes suspension were added to the upper well, and active serum was added to the lower well. Migration distances were detected.

Statistical analysis

Statistical analyses were performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). The data were presented as mean ± standard deviation (SD). Comparisons among several samples were tested by one-way analysis of variance. All tests were two-sided, and a $P < 0.05$ was considered statistically significant.

Results

L-4F decrease monocyte chemoattractant protein-1 levels in supernatant

MCP-1 levels in supernatant with oxLDL stimulation for 12 h increased to 2.13 folds compared to fully differentiated adipocytes. The 1, 10, and 50 μg/ml L-4F decreased MCP-1 levels by 8%, 29%, and 91%, separately [Figure 1].

L-4F inhibit oxidized low-density lipoprotein-induced monocyte chemoattractant protein-1 mRNA expression

L-4F dose dependently inhibited MCP-1 mRNA expression. Compared with oxLDL stimulation, L-4F at 10 and 50 μg/ml significantly decreased MCP-1 mRNA expression to about 29 ± 8% ($P = 0.002$) and 91 ± 6% ($P = 0.0005$), respectively. PKA inhibitor H-89 (10 μmol/L) significantly reduced oxLDL-induced MCP-1 expression to 71 ± 7% ($P = 0.0008$), but no further decreasing occurred when H-89 (10 μmol/L) adding with the existing of L-4F (50 μg/ml) ($P = 0.397$) [Figure 2].

Effect of L-4F on monocyte chemotactic activity

The migration distances markedly increased after oxLDL stimulated for 12 h. There was a significant difference even between the oxLDL stimulated group and the positive control group ($P = 0.030$). L-4F reduced the monocyte chemotactic activity in a dose-dependent manner [Table 1].

Effect of L-4F on CCAAT/enhancer binding protein α and β expression

OxLDL (50 μg/ml) stimulation increased C/EBPβ level in a time-dependent manner but had no marked influence on C/EBPα level during the 1st h to the 12th h. On the time of 1, 6, and 12 h, the level of C/EBPβ protein all decreased after incubated with 50 μg/ml L-4F and showed the inhibition of L-4F on C/EBPβ protein level. In 3T3-L1 adipocytes with oxLDL stimulated for 12 h, C/EBPβ protein level slightly reduced after incubated with 50 μg/ml L-4F, compared with that after 10 μmol/L H-89 intervention [Figure 3].

Discussion

In this study, we demonstrated that fully differentiated 3T3-L1 adipocytes secreted MCP-1, and oxLDL stimulation induced a significant increase of MCP-1 secretion and

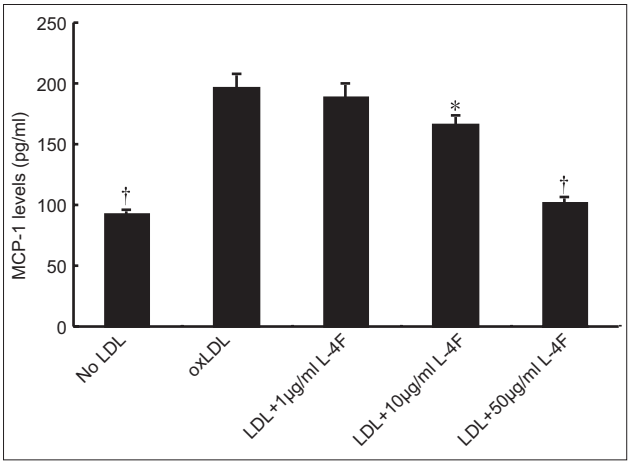

Figure 1: Effect of L-4F on MCP-1 level in the supernatant. Fully differentiated 3T3-L1 adipocytes were incubated for 18 h with L-4F (0, 1, 10, and 50 μg/ml), and co-incubated with oxLDL (50 μg/ml) for the last 12 h. Then, MCP-1 levels in supernatant were evaluated by using enzyme-linked immunoabsorbent assay kits. Data representative of 3–5 independent determinations were expressed as mean ± standard deviation. *$P < 0.05$, †$P < 0.001$, compared with the oxLDL group. MCP-1: Monocyte chemoattractant protein-1; oxLDL: Oxidized low-density lipoprotein; LDL: Low-density lipoprotein.

Table 1: Effect of L-4F on monocyte chemotactic activity

Groups	Numbers	Migration distances (μm), mean ± SD
Random migration group	30	69.88 ± 8.19
oxLDL stimulated group	30	136.75 ± 8.03*
1 μg/ml L-4F group	30	125.67 ± 6.25
10 μg/ml L-4F group	30	109.03 ± 7.88†
50 μg/ml L-4F group	30	85.37 ± 10.44‡
Positive control group	30	108.33 ± 5.04*,†

*P<0.001, versus random migration group; †P<0.05, versus oxLDL stimulated group; ‡P<0.001, versus oxLDL stimulated group. SD: Standard deviation; oxLDL: Oxidized low-density lipoprotein.

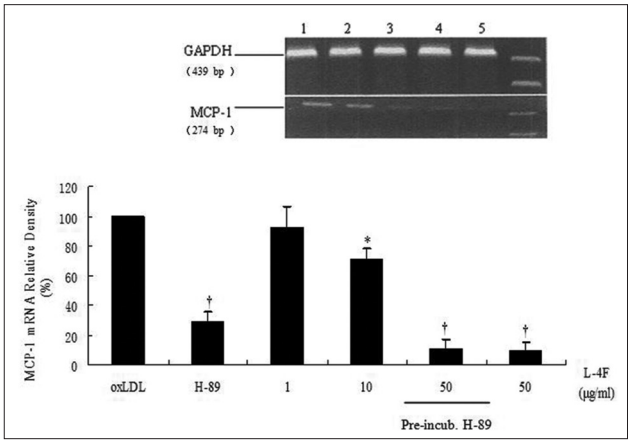

Figure 2: Effect of L-4F on MCP-1 mRNA expression induced by oxLDL. Fully differentiated 3T3-L1 adipocytes were incubated for 18 h with L-4F (0, 1, 10, and 50 μg/ml), with/without protein kinase A inhibitor H-89 (10 μmol/L) preincubated for 1 h before incubated with 50 μg/ml oxLDL, and co-incubated with oxLDL (50 μg/ml) for the last 12 h. The expression of MCP-1 mRNA was assessed by reverse transcription-polymerase chain reaction, and GAPDH was used as the housekeeping gene for normalization. Data were expressed as mean ± standard deviation from at least three independent determinations. *$P < 0.05$, †$P < 0.001$, compared with the oxLDL group. 1: 1 μg/ml L-4F; 2: 10 μg/ml L-4F; 3: 10 μmol/L H-89; 4: 50 μg/ml L-4F; 5: 50 μg/ml L-4F + 10 μmol/L H-89; MCP-1: Monocyte chemoattractant protein-1; oxLDL: Oxidized low-density lipoprotein; LDL: Low-density lipoprotein; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

expression, indicated that oxLDL is one of the potent inducer of MCP-1.[10]

HDL has been shown to exert identified antiatherosclerotic function, which is partially associated with its anti-inflammatory properties[11,12] and is mainly depended on the effect of its main apolipoprotein, apoA-I.[13-15] In this study, we observed that apoA-I mimetic peptide L-4F, dose dependently inhibited oxLDL-induced MCP-1 secretion in 3T3-L1 adipocytes, and significantly suppressed monocyte migration. These were consistent with the find that HDL could counterbalance the proinflammatory function of oxLDL.[16-18] But the molecular mechanism for the inhibition of MCP-1 secretion by L-4F is not understood. Zoico *et al*.[19] showed that in adipocytes exposed to lipopolysaccharide (LPS), adiponectin pretreatment suppressed MCP-1 production through inhibition of NF-κB mediated by attenuated IκB-α and IKK gene expression, as well as through increased PPARγ expression. Our another study observed that HDL could suppress tumor necrosis factor-α (TNF-α) mRNA expression in oxLDL-stimulated 3T3-L1 adipocytes by activating cyclic AMP-PKA (cAMP-PKA) signaling pathway, reducing IκB degradation and inhibiting NF-κB activation. But the anti-inflammatory property of HDL was only partially associated with PKA-IκB-α-NF-κB signaling pathway (not published). Hence, we assumed that perhaps other unknown signaling pathways participated in it. Further understanding is needed to explore the comprehensive mechanism.

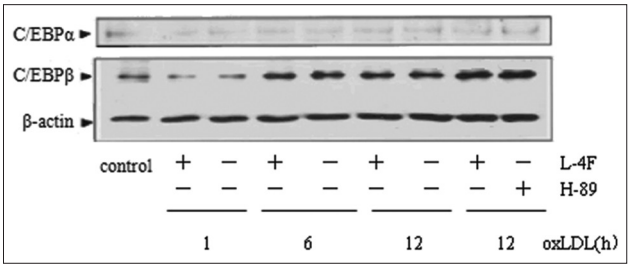

Figure 3: Effect of L-4F with/without H-89 on C/EBP α and β expression after oxLDL-stimulated. Fully differentiated 3T3-L1 adipocytes were incubated for 18 h with L-4F (50 μg/ml), with/without protein kinase A inhibitor H-89 (10 μmol/L) preincubated for 1 h before incubated with 50 μg/ml oxLDL, and co-incubated with oxLDL (50 μg/ml) for the last 1, 6, or 12 h. The C/EBP α and β proteins were assessed by Western blot. C/EBP: CCAAT/enhancer binding protein; oxLDL: Oxidized low-density lipoprotein.

C/EBPs were another critical transcription factors except NF-κB. In 3T3-L1 adipocytes, the transcriptional activity of C/EBPβ was repressed when its transactivation function was inhibited.[20] MCP-1 mRNA expression in aortas was significantly correlated with the expression of C/EBPβ and C/EBPα mRNA in hyperinsulinaemic rat aortas.[21] TNF-α up-regulated the expression of C/EBPβ in 3T3-L1 adipocytes, thereby led to an increasing product of MCP-1.[22] LPS decreased C/EBPα mRNA levels and significantly increased MCP-1 mRNA levels as well as its secretion in aging adipocytes *in vitro*,[23] and macrophage migration is concerned with the interaction between C/EBPβ and TNF-α.[24] Based on the above, we proposed a hypothesis that C/EBPs may regulate oxLDL-induced MCP-1 expression in adipocytes, and participate in the anti-inflammatory function of L-4F.

In this study, we observed that during 12 h with oxLDL stimulation, there was no marked change on C/EBPα protein levels, but C/EBPβ protein levels increased with time. L-4F pretreatment reduced oxLDL-induced C/EBPβ protein levels in adipocytes, indicated that L-4F might inhibit C/EBPβ transcriptional function with/without the inhibition of C/EBPβ promoter activation, resulting the reduction of C/EBPβ protein synthesis.

It had been demonstrated that the activation of C/EBPβ promoter is mediated through incomplete cAMP-response element-binding protein (CREB) sites located close to the TATA box of the C/EBPβ gene. C/EBPβ promoter activity might be completely blocked by the PKA inhibitor H89. Cotransfection experiments with CREB and PKA expression plasmids further supported that C/EBPβ transcription is mediated via PKA-dependent CREB activation.[25] We also found that C/EBPβ protein levels decreased after H-89 pretreatment, which indicated that there was a relationship between oxLDL-induced C/EBPβ expression and PKA in adipocytes. Furthermore, we found that treatment with L-4F or H-89 either attenuated MCP-1 mRNA expression. The decreased magnitude of MCP-1 mRNA expression with L-4F treatment in adipocytes was greater than that with

H-89 treatment, but it had no further reduction of MCP-1 mRNA expression in adipocytes co-incubated with L-4F and H-89 compared with that with L-4F treatment only. Hence, we deduced that the inhibition of oxLDL-induced MCP-1 expression with L-4F in adipocytes may mainly through the PKA-C/EBPβ signaling pathway, that is, L-4F exhibited its anti-inflammatory property in adipocytes exposed to oxLDL by blocking PKA activity, inhibiting the activation of C/EBPβ promoter, and then attenuating C/EBPβ transcription and protein synthesis, and ultimately resulting in the reduction of MCP-1 mRNA expression and secretion.

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Conflicts of interest

There are no conflicts of interest.

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