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The Effects of Highexpression and Knockdown Adipophilin in The Activity of ERK1/2 and Expression of PPARγ and Lipid Accumulation in Cells*

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Abstract Our previous studies have showed that adipophilin promoted intracellular lipids accumulation through ERK1/2-PPARγ signaling pathway. In the study we explored that whether highexpression and knockdown adipophilin affects the activity of ERK1/2 and the expression of PPARγ and lipid accumulation in RAW264.7 cells, and further certified that adipophilin promoted intracellular lipids accumulation through this pathway. The recombinant retroviral vetors pQCXIP-HA-Adipophilin and pSuper-retro-adipophilin siRNA were verified by the methods of enzyme-digesting. The recombinant retroviral vetors were transfected into PA317 cell by mediating Sofast™, which can induce retroviruses release. Then we used the collected retroviruses to infect RAW264.7 cells and achieved adipophilin gene highexpression and knockdown RAW264.7 cell lines applying puromycin screening. After the infected RAW264.7 cells incubated with 50 mg/L Ox-LDL for 24 h, the lipids accumulation were measured by Oil red O staining and HPLC, the expression of mRNA and proteins of adipophilin and PPARγ were detected by semi-quantitative RT-PCR and Western blot respectively, and phosphorylation of ERK1/2 was analyzed by Western blot too. The results of enzyme-digesting confirmed the recombinant retroviral vectors pQCXIP-HA-Adipophilin and pSuper-retro-adipophilin siRNA as expected. In the situation of Dutch fat, transfected cells with pQCXIP-HA-Adipophilin significantly increased the accumulation of lipids, but reduced the expression of PPARγ and the phosphorylation of ERK1/2, which were reversed in cells with pSuper-retro-adipophilin siRNA transfection. Our results showed that ERK1/2 and PPARγ may be related with lipids accumulation caused by adipophilin expression in macrophages incubated with modified LDL. Therefore, adipophilin might contribute, *in vivo*, to lipid accumulation in the intima of the arterial wall through the ERK1/2-PPARγ signaling pathway.

Key words adipophilin, extracellular signal-regulated kinase1/2, peroxisome proliferator-activated receptor gamma, retroviruses
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Oxidized low density lipoprotein (Ox-LDL) is known to play a key role in the induction of monocyte-derived macrophages into foam cells, and foam cell deposition in the vessel wall is one of the key characteristics in the development of human atherosclerotic lesions. Adipose differentiation-related protein (adipophilin, ADFP or ADRP) is a 50 ku protein encoded by a gene initially isolated from

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differentiating adipocytes. Initial tissue distribution of adipophilin mRNA expression in mice revealed its expression to be limited to adipose tissue [1]. Its expression has now been found in diverse cell types, such as fibroblasts, endothelial cells and epithelial cells in culture and in particular, adipophilin is associated with the lipid fractions in the cells [2-3]. Adipophilin was also localized to specific cell types in tissues, such as lactating mammary epithelial cells, adrenal cortex cells, Sertoli and Leydig cells of the male reproductive system, and steatotic hepatocytes in alcoholic liver [3]. These data suggest that adipophilin may be a specific marker for lipid accumulation in the cells [4]. Adipophilin also augmented TNF-α, MCP-1, and IL-6 expression in acetylated LDL induced macrophages, which might be one role of adipophilin in atherosclerosis[5]. These data showed that adipophilin can promote lipid storage in cells and may be relevant to atherosclerosis.

Peroxisome proliferator-activated receptors(PPARs) are a family of ligand-activated transcription factors that belong to the nuclear hormone receptor superfamily and form heterodimers with retinoid X receptor (RXR).Three distinct PPARs, termed α, γ and δ, have been identified. Recently, PPARγ is induced in monocytes and/or macrophages following exposure to Ox-LDL and is expressed at high levels in the foam cells of atherosclerotic lesions[6-9]. Furthermore, there is a peroxisome proliferator activated receptor response element (PPRE) in the promotor gene of adipophilin.

Mitogen-activated protein kinases (MAPKs) are serine/threonine protein kinases that can phosphorylate their target proteins. Three major subfamilies have been: extracellular signal regulated kinases 1/2 (ERK1/2), p38 MAPKs, and Jun N-terminal kinases/ stress-activated protein kinases (JNKs/SAPKs). MAPKs are activated by a family of MAPK kinases (MKKs) and they are important mediators for signal transduction from the cell surface to the nucleus [10-11]. JNKs and p38 mediate signals in response to cytokines and environmental stress, whereas the ERK subtypes are classically recognized as key transducers in the signaling cascade that mediates cell proliferation in response to growth factors such as platelet-derived growth factor and endothelial growth factor. It is becoming increasingly clear that the ERK pathway, like those of p38 and JNK, is activated by environmental stresses, including reactive oxygen species such as H_2O_2 [12], and hyperglycemia enhanced the adipogenic induction of lipid accumulation through an ERK1/2-activated PI3K/Akt-regulated PPARγ pathway[13].

All of the above data showed that adipophilin, PPARγ and ERK1/2 are involved in the lipid accumulation, and the results of our previous studies manifested that Ox-LDL activates adipophilin through ERK1/2-PPARγ signal pathway in RAW264.7 cells[14]. However, the effect of adipophilin on PPARγ and ERK1/2 in the condition of Ox-LDL treatment is not fully understood. For further investigate the mechanism of adipophilin in lipid accumulation, in this study, we used adipophilin gene highexpression and siRNA mediated by recombinant retroviral vectors pQCXIP-HA-Adipophilin and pSuper-retro-adipophilin siRNA respectively to observe the effect of adipophilin on the expression of PPARγ, the activity of ERK1/2 and the lipid accumulation in RAW264.7 cells.

1 Materials and methods

1.1 Materials

Rabbit polyclonal anti-ERK1/2 (total-ERK), anti-phospho ERK1/2 (p-ERK), and anti-PPARγ antibodies were purchased from ABZOOM, rabbit polyclonal anti-adipophilin was purchased from Bejing Biosynthesis Biotechnology Co. Ltd. Mouse polyclonal anti-β-actin antibodies was purchased from BOSTER. Peroxidase-Conjugated AffiniPure Goat Anti-mouse IgG (H+L) and Peroxidase-Conjugated Goat anti-Rabbit IgG (H+L) were purchased from ZSGB-BIO and MULTISCIENCES respectively. RevertAid™ First Strand cDNA Kit was purchased from Fermentas. Pancreatin, EB, DMSO and Oil Red O were purchased from Amresco. RPMI 1640 and Dulbecco's modified Eagle's medium with 4.5 g/ml of glucose (DMEM-high) medium was purchased from GIBCO. TRIzol was purchased from Sangon. 100 bp Ladder Marker was purchased from GENERAY BIOTECH. All primers were synthesized by the Shanghai Bioengineering of Biological Engineering Service Co. Ltd. Agarose was purchased from GENE TECH Limited. Acetonitrile, isopropanol and *n*-hexane were all chromatographic pure and purchased from Chemical Reagent Factory of Tianjin Fu Chen. BCA kit was purchased from Beyotime Institute of

Biotechnology. Retroviral vetor pQCXIP was purchased from Invitrogen. Retroviral vetor pQCXIP-HA-Adipophilin, pSuper-retro-scramble siRNA and pSuper-retro-adipophilin siRNA were constructed and stored by our members. DH5α was a gift from Institute of Pathobiology of University of South China. Fast extraction kit of plasmid subsample was purchased from Solarbio in Beijing. *Eco*R Ⅰ was purchased from Beijing BioDev Genomics Co.Ltd. *Bam*H Ⅰ and *Not* Ⅰ were purchased from TAKARA Biotechnology(Dalian) Co.Ltd. *Hin*dⅢ was purchased from Fermentas. EZNAR Fastfilter Endo-free Plasmid Maxi Kit was purchased from Omega. Gene transfection reagent of sohua-Sofast™ was purchased from Xiamen Sunma Biotechnology Co.Ltd. Polybrene was purchased from Chemicon. Puromycin was purchased from Amresco. All other chemicals were of the best grade available from commercial sources.

1.2 Cell cultures

A Safe Packaging Line PA317 cells, a gift from Department of Biochemistry of Central South University, were cultured in DMEM-high containing 25 mmol/L HEPES buffer and 10% fetal calf serum (FCS) at 37℃ in 5% CO_2. A mouse macrophage-like cell line RAW264.7 cells, purchased from Cell Bank in Shanghai Institute of Cell Biology of The Chinese Academy of Sciences, were maintained in RPMI 1640 medium containing 25 mmol/L HEPES buffer and 10% FCS at 37℃ in 5% CO_2.

1.3 Plasmids assay

Transformed the stored recombinant retroviral vectors pQCXIP, pQCXIP-HA-Adipophilin, pSuper-retro-scramble siRNA and pSuper-retro-adipophilin siRNA to *Escherichia coli* DH5α, picked out three clones randomly for amplification, and then extracted plasmids. Digested vector pQCXIP-HA-Adipophilin with *Bam*H Ⅰ or *Eco*R Ⅰ, digested pSuper-retro-scramble siRNA and pSuper-retro-adipophilin siRNA with *Eco*R Ⅰ and *Hin*dⅢ. The digestion products was shown in Figure 1a. There were two bands of 1.3 kb and 7.2 kb, which were in the expected sizes of HA-adipophilin and empty vector pQCXIP respectively. And the digestion products of pSuper-retro-scramble siRNA and pSuper-retro-adipophilin siRNA were consistent with the expected size too (Figure 1b). The results of digestion showed that the vectors were correctly constructed.

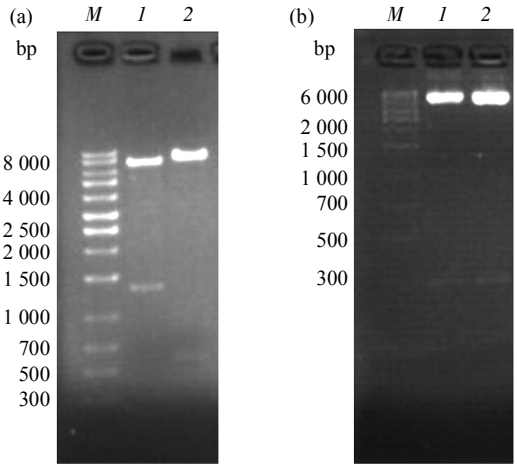

Fig. 1 The analysis of digestion products of recombinant retroviral vectors pQCXIP-HA-Adipophilin, pSuper-retro-scramble siRNA and pSuper-retro-adipophilin siRNA

M: Marker. (a) Digestion product of pQCXIP-HA-Adipophilin by *Bam*H Ⅰ (*1*) and pQCXIP-HA-Adipophilin by *Not* Ⅰ (*2*). The target size of Lane *1* is 1 351 bp. (b) Digestion product of pSuper-retro-scramble siRNA and pSuper-retro-adipophilin siRNA by *Eco*R Ⅰ (*1*) and *Hin*dⅢ (*2*) respectively. The target sizes of Lane*1* and Lane*2* are 275 bp.

1.4 Transfect retroviral vectors to PA317 cells

One day before transfection, PA317 cells were grown in 6-well culture dishes at a density of 4×10^5 cells/well in DMEM-high supplemented with 10% FCS. After overnight incubation, PA317 cells were transfected using gene transfection reagent of sohua-Sofast™ when the cells presented monolayer cell and fused up to 95%. First, diluted 2 μg vectors pQCXIP, pQCXIP-HA-Adipophilin, pSuper-retro-scramble siRNA or pSuper-retro-adipophilin siRNA using 100 μl serum-free DMEM-high medium respectively, and then mixed softly. Second, added 6 μl sohua-Sofast™ to 100 μl serum-free DMEM-high medium and mixed softly in new EP tubes. Third, added the dilution of sohua-Sofast™ to the dilution of vectors, then incubated 20 min at room temperature. Next, the media were removed, and cells were rinsed twice with serum-free DMEM-high medium. After removal of the medium, added sohua-Sofast™/DNA mixture to every well and mixed softly, then incubated the cells for 4 h at 37℃. After that, cultured the cells for 48 h with changed DMEM-high medium containing

10% FCS. Finally, collected the medium which contained retroviruses.

1.5 Retrovirus infections

One day before puromycin screening, RAW264.7 cells were grown in 24-well culture dishes at a density of 4×10^4 cells/well in RPMI 1640 supplemented with 10% FCS. Added 0 mg/L, 1.5 mg/L, 3 mg/L, 4.5 mg/L, 6 mg/L, 7.5 mg/L and 9 mg/L puromycin to each well when the cells presented monolayer cell and fused up to 70%～80% and cultured at 37℃ in 5% CO_2. And changed the medium every 2 ～3 days without changing the concentration of puromycin and observed the state of cells any time. We selected the concentration of puromycin when there was a great quantity mortality of the cells within 5 days and died totally within 14 days. In the study, we found that the best screening concentration of puromycin was 3 mg/L and maintained in 1.5 mg/L.

RAW264.7 cells were grown in 6-well culture dishes at a density of 4×10^5 cells/well in RPMI 1640 supplemented with 10% FCS at 37℃ . Next day the cells were infected with 1 ml mixed liquor which contained retroviruses of pQCXIP, pQCXIP-HA-Adipophilin, pSuper-retro-scramble siRNA or pSuper-retro-adipophilin siRNA at dilutions of 1×10^{-2}, 1×10^{-3} and 1×10^{-4} with RPMI 1640 medium respectively, in the presence of polybrene (8 g/L). And supplemented RPMI 1640 medium and 10% FCS after the cells cultured for 6 h at 37℃ with the retrovirus solution, which diluted the concentration of polybrene (4 g/L). Then passaged the cells from 1 to 2 after cultured 24 h and added 3 mg/L puromycin for screening the cells. Changed the medium every 1～2 days. Used 1.5 mg/L puromycin when the cells died mostly. And there were cell clones after incubation for 14 days. So we digested the cells with pancreatin and seeded in 96-well culture dishes with 100 μl/well. Then made marks of wells that contained one cell with invert microscope after incubation for 24 h. Changed the medium to continue cultivation and passaged the cells to 24-well, 6-well and culture flask gradually. Then changed with serum-free RPMI 1640 medium containing 2 g/L BSA and Ox-LDL modified followed our previous procedure [14] for treatment. Cells were harvested and splited for analysis of adipophilin protein, total RNA was prepared according to the manufacturer's suggestions, and was dissolved in DEPC-treated water.

1.6 Total RNA extraction, cDNA synthesis and reverse transcription polymerase chain reaction (RT-PCR) analysis

Reconstructive RAW264.7 cells(2×10^6 cells/well) were incubated with or without the indicated treatment. Total RNA was extracted using TRIzol, isopropyl alcohol and chloroform at various treatments of factor. For RT-PCR analyses, The first strand cDNA synthesis containing 2 μg of total RNA was primed with oligo(dT) and RevertAid™ First Strand cDNA Kit under optimized conditions following the manufacturer's protocol. PCRs were performed using 2 ×Taq PCR master mix from TIANGEN and specific primers for mouse PPARγ, adipophilin and GAPDH(Table 1). The quantitative results for PPARγ and adipophilin were normalized by the levels of GAPDH mRNA. The resulting products were separated on a 1.2% agarose gel and stained with ethidium bromide.

Table 1 The specific primers for mouse PPARγ, adipophilin and GAPDH

Gene	Length/bp	T_m/℃	Cycle	Primer sequence
PPARγ	277	57	35	F: 5′ CCGAAGAACCATCCGATTGA 3′
				R: 5′ CGGGAAGGACTTTATGTATGA 3′
Adipophilin	523	55	35	F: 5′ CCAAGGATTCTGTAGCCAGCA 3′
				R: 5′ ACAGTGGGACTCATCGGTGTC 3′
GAPDH	354	60	35	F: 5′ CAGTCCATGCCATCACTGCCA 3′
				R: 5′ AGGTGGAGGAGTGGGTGTCGC 3′

1.7 SDS-PAGE and Western blot analysis

Reconstructive RAW264.7 cells (2×10^6 cells/well in 6-well plates) were immediately lysed in ice-cold lysis buffer containing 50 mmol/L Tris, 150 mmol/L sodium chloride, 2 mmol/L ethylene diaminetera acetic acid(EDTA), 1 mmol/L phenylmethyl sulfonyl

fluoride (PMSF), 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mmol/L sodium fluoride, 1 mmol/L sodium ortho-vanadate, 15 mmol/L sodium pyrophosphate, and 10 mmol/L β-glycerophosphate, and then centrifuged(10 000 r/min at 4℃ for 10 min). Supernatants were used as sample proteins. Protein concentrations were determined by Coomassie Brilliant Blue (Nanjing Jiancheng Bioengineering Institute), according to the protocol recommended by the manufacturer. Samples were applied to 10% SDS gels, followed by electrophoretic transfer to a polyvinylidene difluoride (PVDF) membrane (Millipore) using wet blotting, the transfer membrane was blocked at 4℃ for 4～6 h and then washed with Tris-buffered saline containing Tween 20 (TBS-T). For immunodetection, the blocked membrane was incubated at room temperature for 3 h with anti-PPARγ, anti-ERK1/2, anti-p-ERK1/2, anti-adipophilin and anti-β-actin primary antibodies at a dilution of 1∶1 000. After washing with TBS-T for three times, the membranes were incubated at room temperature for 1 h with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibodies at a dilution of 1∶3 000, washed with TBS-T. The immunoreactive proteins were visualized by chemiluminescence using an ECL plus kit with exposure of the transfer membrane to X-ray film. Immunoreactive bands were quantified by using AlphaImager2200 analysis software, and the data represent the protein variation after treatment of the factors.

1.8 Lipid staining

Reconstructive RAW264.7 cells, measured of lipid accumulation through staining of neutral fats and cholesterol esters with Oil Red O, were placed into 6-well plates with slides at a density of 4×10^5 cells/cm^2 and cultured for 24 h, then treated with 50 mg/L Ox-LDL for 24 h, after that the cells were rinsed with PBS and fixed with 10% formalin for 5 min at room temperature, and incubated in fresh formalin for at least 1 h. The cells were then rinsed again with 60% isopropanol and then incubated with fresh filtered Oil Red O solution (60% saturated Oil Red O/40% deionized water) for 20 min. For analysis, slides were then washed in isopropanol for 10 min, rinsed in tap water, counterstained with hematoxylin and mounted in glycerol/gelatin solution. And then the cells were took photographs in the light microscope.

1.9 Intracellular total cholesterol (TC) and free cholesterol (FC) assay by high performance liquid chromatogram (HPLC)

Detected intracellular TC and FC by HPLC of highexpression and knockdown adipophilin RAW264.7 cells without or with treatment with 50 mg/L Ox-LDL for 24 h. Took stigmasterol as the internal standard and got standard curve. After treatment, the cells were washed three times with PBS, next the cells were lysed in solution same as lysing for proteins and the samples were centrifuged (3 000 r/min at 4℃ for 10 min), then the supernatants were collected. After proteins quantification with BCA kit, proteins included in the remaining supernatants were precipitated with 7.2% trichloroacetic acid, then collected the supernatants for cholesterol test after centrifuged (800 r/min at 4℃ for 10 min). For obtaining TC, add potassium hydroxide solution (8.9 mol/L) 200 μl to 100 μl supernatant to hydrolyse cholesterol ester (CE), and add sodium hydroxide(1 mol/L) 200 μl for obtaining FC. All of the samples were mixed with the internal standard solution respectively, then extracted with *n*-hexane and ethanol and oxidized with 2 mol/L chromium trioxide oxidation, then dried in a vacuum and dissolved in 200 μl acetonitrile-isopropanol (80∶20). The samples determined by HPLC.

1.10 Statistical analysis

The data were analyzed by ANOVA with SPSS16.0 statistical software and were expressed as the $\bar{x} \pm s$. Differences were considered significant when $P < 0.05$.

2 Results

2.1 Construction of the stable highexpression adipophilin and adipophilin siRNA RAW264.7 cells successfully

To determine whether construction of the stable highexpression adipophilin and adipophilin siRNA RAW264.7 cells successfully, we determined the mRNA and protein of adipophilin in transfected RAW264.7 cells with recombinant retroviral vector pQCXIP-HA-Adipophilin and the results showed obvious increase of adipophilin mRNA and protein expression in comparison to cells transfected with the empty vector and the control(Figure 2, Figure 3). And the expression of adipophilin in transfected RAW264.7 cells with recombinant retroviral vector adipophilin siRNA were suppressed obviously.

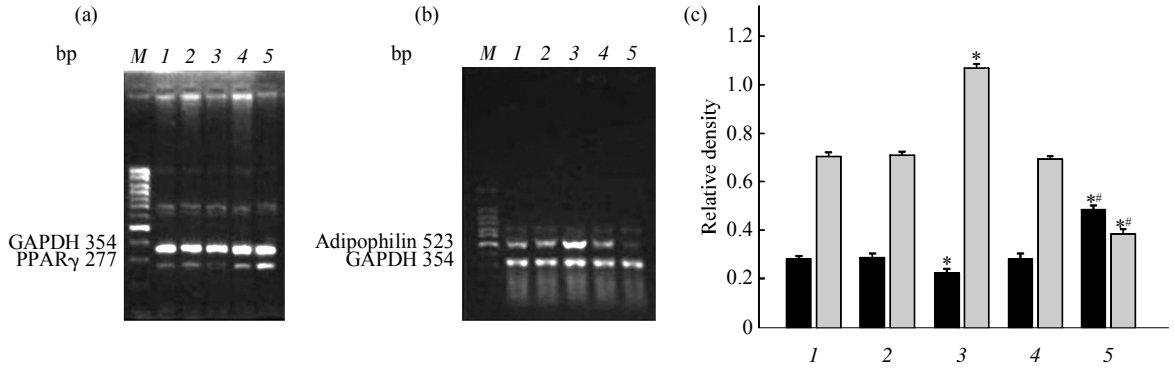

Fig. 2 The expression of mRNA of PPARγ and adipophilin in cells infected with retrovirus

Total RNA was extracted with TRIzol. For RT-PCR analysis, the first strand cDNA synthesis containing 2 μg of total RNA was primed with oligo(dT) and RevertAid™ First Strand cDNA Kit. RT-PCR was performed using 2×Taq PCR master mix and specific primers for mouse PPARγ, adipophilin or GAPDH. (a, b) The products of mRNA of PPARγ and adipophilin by RT-PCR. (c) The statistic charts. Data were represented as the $\bar{x} \pm s$ of five separate experiments. $^*P < 0.05$ versus the group of normal RAW264.7 cells. The expression of mRNA of adipophilin was high in the cells infected with retrovirus of pQCXIP-HA-Adipophilin, and was silenced in cells infected with retrovirus of adipophilin siRNA, but the expression of PPARγ was low in the cells infected with retrovirus of pQCXIP-HA-Adipophilin and high in cells infected with adipophilin siRNA. $^{\#}P < 0.05$, pQCXIP-HA-Adipophilin compared with adipophilin siRNA(n=3). ■: PPARγ mRNA; ▨: Adipophilin mRNA. *M*: Marker; *1*: Control; *2*: Empty vector group; *3*: HA-Adipophilin group; *4*: pSuper-retro-scramble siRNA group; *5*: pSuper-retro-adipophilin siRNA group.

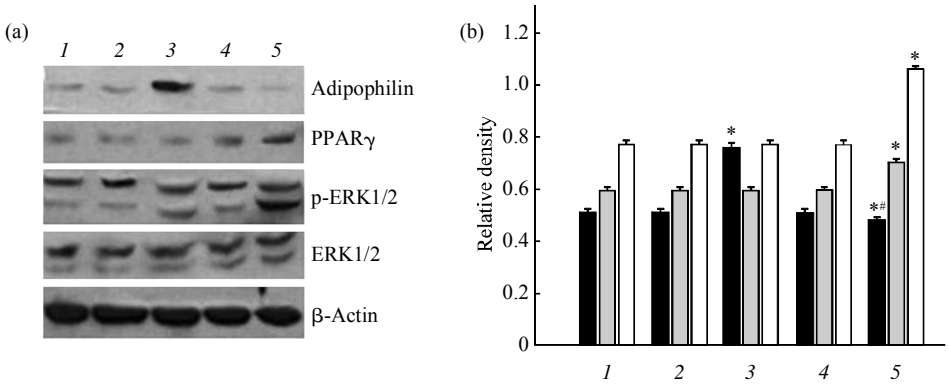

Fig. 3 The expression of proteins of PPARγ and adipophilin, and the activation of ERK1/2 in cells infected with retrovirus

Protein samples were immunoblotted with anti-adipophilin, anti-PPARγ, anti-p-ERK, anti-ERK1/2 or anti-β-actin antibodies. (a) The expression of proteins of PPARγ and adipophilin, and the phosphorylation of ERK1/2. (b) The statistic charts. Data were represented as the $\bar{x} \pm s$ of five separate experiments. $^*P < 0.05$ versus the group of normal RAW264.7 cells. The expression of protein of adipophilin was high in the cells infected with retrovirus of pQCXIP-HA-Adipophilin, and was silenced in cells infected with retrovirus of adipophilin siRNA, but the expression of PPARγ and the activation of ERK1/2 were high in the cells infected with retrovirus of adipophilin siRNA. $^{\#}P < 0.05$, pQCXIP-HA-Adipophilin compared with adipophilin siRNA(n=3). ■: Adipophilin; ▨: PPARγ; □: p-ERK1/2. *1*: Control; *2*: Empty vector group; *3*: Adipophilin group; *4*: Empty vector interference group; *5*: Adipophilin siRNA interference group.

2.2 Identificating the effects of adipophilin highexpression and siRNA on the expression of PPARγ and the activation of ERK1/2 and the intracellular lipid accumulation in cells

For detecting the effect of the highexpression of adipophilin in RAW264.7 cells on the expression of PPARγ and the activation of ERK1/2, we transfected RAW264.7 cells with recombinant retroviral vector pQCXIP-HA-Adipophilin encoding adipophilin and pSuper-retro-Adipophilin siRNA. In spite of the results indicated that there were 1.49 and 1.53-fold increase of adipophilin mRNA and protein expression respectively

in cells of adipophilin highexpression in comparison to cells transfected with the empty vector and the control (Figure 2, Figure 3), the expression of PPARγ mRNA decreased. And these changes were inversed by adipophilin siRNA. Next, we studied the impact of adipophilin highexpression and siRNA on lipid accumulation in RAW264.7 cells. As showed in Figure 4 and Table 2, there was not apparent effect on lipid accumulation when transfected RAW264.7 cells with recombinant retroviral vectors pQCXIP-HA-Adipophilin and pSuper-retro-adipophilin siRNA.

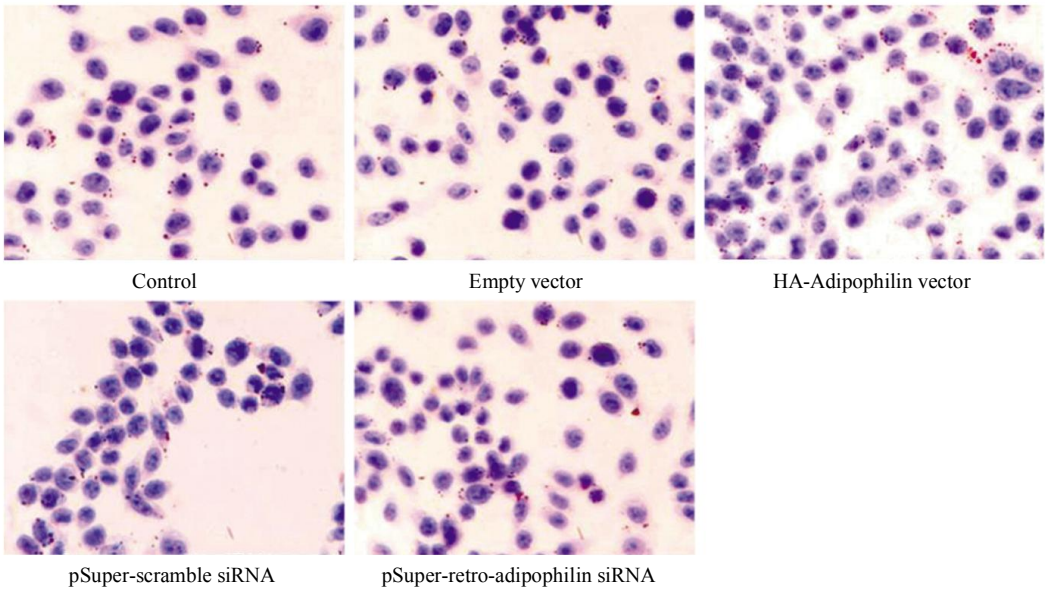

Fig. 4 The intracellular lipids of normal cells and of cells infected with retrovirus (×100)
The cells were stained with Oil Red O. The accumulation of intracellular lipids were similar in all groups.

Table 2 Effects of adipophilin mRNA highexpression and siRNA on the intracellular lipid accumulation in RAW264.7 cells ($\bar{x} \pm s$, $n = 3$)

Group	TC (mg/g)	FC (mg/g)	CE/TC (%)
Control	155 ± 25	132 ± 29	14.9 ± 2.4
24 h Ox-LDL	260 ± 24*	169 ± 28*	35.1 ± 2.1*
HA-Adipophilin	165 ± 29$^{*,\#}$	139 ± 13$^{\#}$	15.6 ± 1.9$^{\#}$
HA-Adipophilin+24 h Ox-LDL	305 ± 31$^{*,\#}$	185 ± 28$^{*,\#}$	39.5 ± 2.7$^{*,\#}$
Adipophilin siRNA	149 ± 21$^{\#}$	128 ± 23$^{\#}$	14.2 ± 2.1$^{\#}$
Adipophilin siRNA+24 h Ox-LDL	201 ± 23$^{*,\#,**}$	149 ± 21$^{*,\#,**}$	25.8 ± 2.2$^{*,\#,**}$

$^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus 24 h; $^{**}P < 0.05$ HA-Adipophilin+24 h Ox-LDL versus adipophilin siRNA+24 h Ox-LDL.

2.3 Adipophilin highexpression decreased the expression of PPARγ and the activation of ERK1/2 in RAW264.7 cells by exposure to Ox-LDL, but further increased the accumulation of intracellular lipids, and these changes were inversed by adipophilin siRNA

To further investigate the function of adipophilin in RAW264.7 cells when stimulated with Ox-LDL, we assessed the impact of adipophilin highexpression and siRNA on the expression of PPARγ and the activation of ERK1/2 and on lipids contents in cells loaded with 50 mg/L Ox-LDL, as a source of exogenous lipids, for 24 h. As shown in Figure 5 and 6, in the case of Dutch Fat, the expression of proteins of PPARγ and adipophilin were in opposition, that is when the cells loaded with 50 mg/L Ox-LDL, although the expression

of adipophilin protein increased more in the cells infected with pQCXIP-HA-Adipophilin retrovirus than that in the cells without retrovirus infection, there was not an obvious increase in the expression of PPARγ protein and also in the activation of ERK1/2, which were higher in the cells infected with retrovirus of adipophilin siRNA. The change of activation of ERK1/2 in cells infected with retrovirus was likely to the expression of PPARγ protein. The accumulation of intracellular lipids increased too, in the cells infected with pQCXIP-HA-Adipophilin retrovirus than the cells without retrovirus infection (Figure 7, Table 2). And adipophilin siRNA can decrease the accumulation of intracellular lipids in spite of incubation with 50 mg/L Ox-LDL for 24 h compared with the cells without retrovirus infection (Figure 7, Table 2).

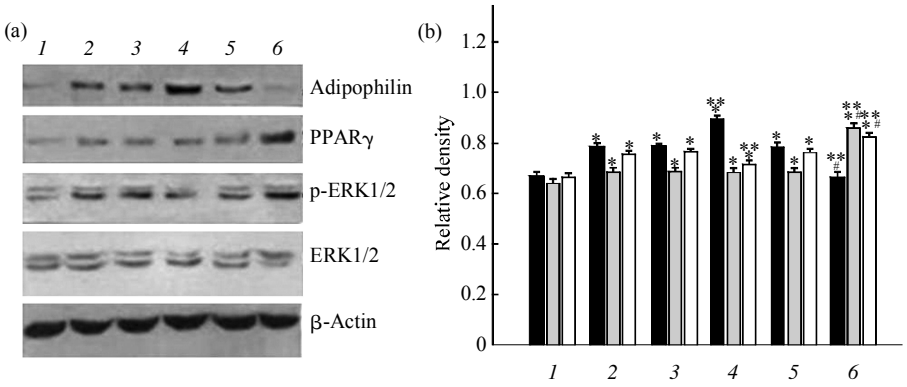

Fig. 5　In the case of Dutch Fat, the expression of proteins of PPARγ and adipophilin, and the activation of ERK1/2 in cells infected with retrovirus

Protein samples were immunoblotted with anti-adipophilin, anti-PPARγ, anti-p-ERK, anti-ERK1/2 or anti-β-actin antibodies. (a) The expression of proteins of PPARγ and adipophilin, and the phosphorylation of ERK1/2. (b) The statistic charts. Data were represented as the $\bar{x} \pm s$ of six separate experiments. $^{*}P < 0.05$ versus the control. $^{**}P < 0.05$ versus the group of normal RAW264.7 cells incubated with Ox-LDL for 24 h. In the case of Dutch Fat, the expression of adipophilin was higher in the cells which infected with retrovirus of pQCXIP-HA-Adipophilin, and was silenced in cells that infected with retrovirus of adipophilin siRNA, but the expression of PPARγ and the activation of ERK1/2 were higher in the cells infected with retrovirus of adipophilin siRNA. $^{\#}P < 0.05$, pQCXIP-HA-Adipophilin compared with adipophilin siRNA (n=3). ■: Adipophilin; ▨: PPARγ; □: p-ERK1/2. *1*: Control; *2*: 24 h Ox-LDL incubation group; *3*: Empty vector group incubated with Ox-LDL for 24 h; *4*: Overexpression adipophilin group incubated with Ox-LDL for 24 h; *5*: Empty vector interference group incubated with Ox-LDL for 24 h; *6*: Adipophilin siRNA interference group incubated with Ox-LDL for 24 h.

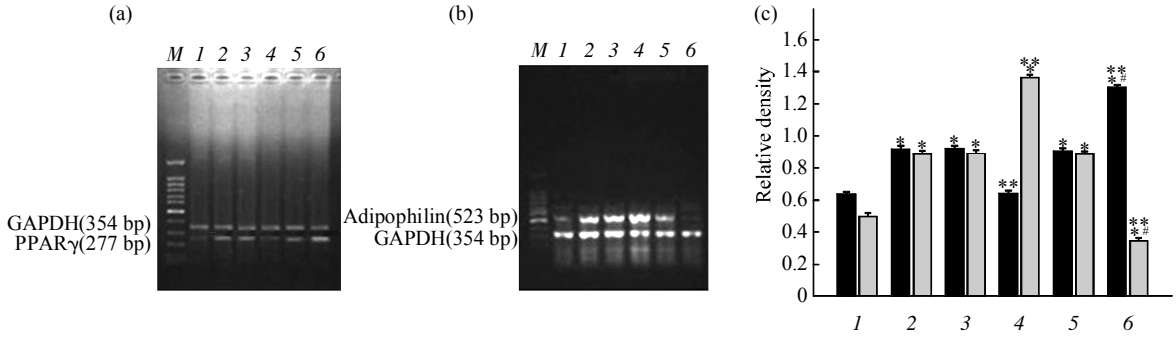

Fig. 6　In the case of Dutch Fat, the expression of mRNA of PPARγ and adipophilin in cells infected with retrovirus.

Total RNA was extracted with TRIzol. For RT-PCR analysis, the first strand cDNA synthesis containing 2 μg of total RNA was primed with oligo(dT) and RevertAid^{TM} First Strand cDNA Kit. PCRs were performed using 2×Taq PCR master mix and specific primers for mouse PPARγ, adipophilin or GAPDH. (a, b) The expression of mRNA of PPARγ and adipophilin. (c) The statistic charts. Data were represented the $\bar{x} \pm s$ of six separate experiments. $^{*}P < 0.05$ versus the control. $^{**}P < 0.05$ versus the group of normal RAW264.7 cells incubated with Ox-LDL for 24 h. In the case of Dutch Fat, the expression of adipophilin was higher in the cells infected with retrovirus of pQCXIP-HA-Adipophilin, and was suppressed in cells infected with retrovirus of adipophilin siRNA, but the expression of PPARγ was low in the cells infected with retrovirus of pQCXIP-HA-Adipophilin. $^{\#}P < 0.05$, pQCXIP-HA-Adipophilin compared with adipophilin siRNA (n=3). ■: PPARγ mRNA; ▨: Adipophilin mRNA. *M*: Marker; *1*: Control; *2*: 24 h Ox-LDL incubation group; *3*: Empty vector group incubated with Ox-LDL for 24 h; *4*: Adipophilin group incubated with Ox-LDL for 24 h; *5*: Empty vector interference group incubated with Ox-LDL for 24 h; *6*: Adipophilin siRNA interference group incubated with Ox-LDL for 24 h.

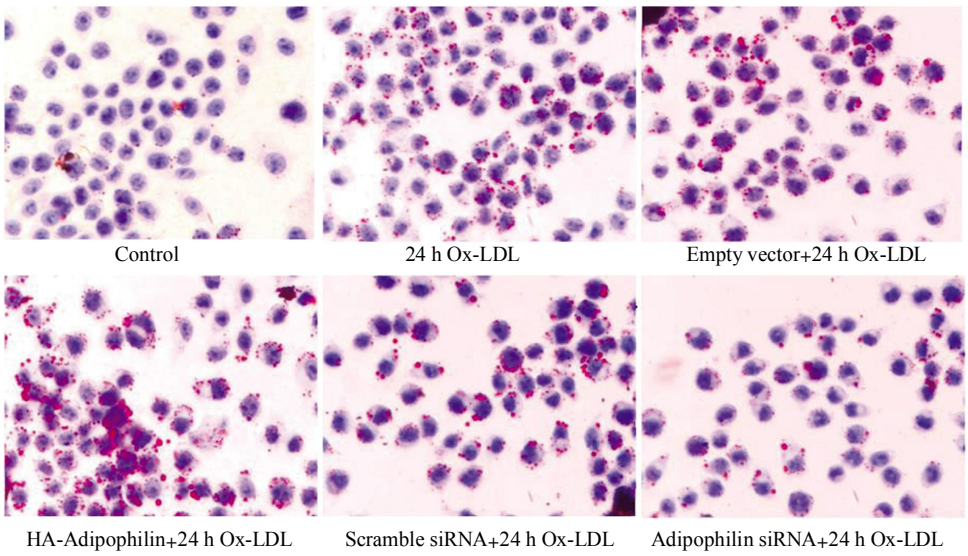

Fig. 7 In the case of Dutch Fat, the intracellular lipids accumulation of control and of cells infected with retrovirus (×100)
The cells were stained with Oil Red O. In the case of Dutch Fat, the amount of intracellular lipid droplets were increased in the cells infected with retrovirus of pQCXIP-HA-Adipophilin, and were reduced in cells that infected with retrovirus of adipophilin siRNA, but there was no difference in the cells infected with retrovirus of pQCXIP and pSuper-scramble siRNA and normal cells of 24 h Ox-LDL.

3 Discussion

Macrophage uptake of Ox-LDL, by accumulating excessive amounts of lipids, mainly CE, is thought to play a central role in foam cell formation and the pathogenesis of atherosclerosis. Thus, elimination of accumulated CE from macrophage foam cells represents a promising therapeutic approach to prevent atherosclerotic lesions. Indeed, different strategies have been reported to diminish cellular CE content, such as the inhibition of the activity of acyl-CoA: cholesterol acyltransferase 1 (ACAT1), a membrane-bound protein presented in macrophages and implicated in foam cell formation[15] because utilizing long-chain fatty acyl-CoA and cholesterol as the substrates to form cholesteryl esters, and the increase in expression of ABCA1, a transporter implicated in the efflux of cellular FC[16]. But few data exist on the effect of adipophilin on lipid storage in the cells and its relevance to atherosclerosis.

Adipophilin, a member of the family of lipid droplet-associated proteins, was first characterized as an mRNA induced early during adipocyte differentiation[1]. Brasaemle's [2] study about the expression pattern of adipophilin and perilipin in adipocytes showed that the specific localization of adipophilin to lipid droplets suggesting that adipophilin plays a role in the management of neutral lipid stores. Recently, adipophilin was detected in atherosclerotic lesions by *in situ* hybridization. Its expression increases during differentiation of monocytes into macrophages and incubation of macrophages with Ox-LDL. Furthermore, its overexpression in THP-1 macrophages enhances lipid accumulation[4, 17–18]. Shiffman's [19] study showed that adipophilin, the only gene up-regulated at 2.5 h, is a known marker for adipocyte differentiation, where it is found in association with lipid droplets[2]. Furthermore, by day 4 there were 121 up-regulated genes, and the expression of adipophilin was the highest up to 14.12-fold[19]. Its early induction potentially makes it a good marker of foam cell formation. Adipophilin also reduced the lipid droplet association of adipose triglyceride lipase and slows triacylglycerol turnover[20]. Adipophilin may represent an actively contributing manifestation of atherosclerosis. However, the molecular mechanisms of its involvement in atherogenesis are not fully understood. Whether it merely serves to stabilize intracellular lipid droplets or plays a regulatory role in foam cell activity warrants further investigation. And studies of the regulation of

adipophilin function in macrophages may provide a therapeutic opportunity for the treatment of atherosclerosis.

To elucidate the potential role of adipophilin in the formation of foam cells, our present study evaluated the expression of adipophilin in RAW264.7 cells infected with retrovirus stimulated with or without Ox-LDL and further certified the pathway by which it enhanced accumulation of lipids in macrophages.

In this article, we showed that the expression of protein of adipophilin was high in RAW264.7 cells infected with retrovirus of pQCXIP-HA-Adipophilin, and was silenced in cells infected with retrovirus of adipophilin siRNA. Surprisingly, for the first time to our knowledge, the expression of PPARγ and the activity of ERK1/2 were greatly enhanced in siRNA adipophilin transfected cells whether they were loaded with Ox-LDL or not(Figure 2, 3, 5, 6). Ox-LDL was recently demonstrated to activate macrophage gene expression through a member of the nuclear hormone receptor family, PPARγ [14, 19].

PPARs are known to regulate fat cell development, lipid and glucose metabolism, and they are implicated in metabolic disorders, such as hypertriglyceridemia and diabetes, which can lead to atherosclerosis. PPARγ also expressed in macrophage-derived foam cells of human atherosclerotic lesions [21] and PPARγ ligands such as fatty acids are components of Ox-LDL [22]. PPARγ has been shown to drive adipocyte differentiation [23] and promote Ox-LDL uptake through transcriptional induction of scavenger receptor CD36[6], a gene highly expressed in foam cells within human atherosclerotic aorta [24]. And Ox-LDL upregulated CD36 and PPARγ levels dose- and time-dependently[25]. Suppressed the scavenger recept CD36 by flavonols or Picomolar IL-10, for example, due to reduced PPARγ protein expression [26-27]. Because CD36 is significantly correlated with adipophilin in human carotid lesions [28]. And adipophilin has been reported to promote uptake of Ox-LDL by inducing the scavenger receptor CD36, *via* activation of PPARγ in macrophages [6, 19]. It was found that pioglitazone, a PPARγ agonist, increased adipophilin expression in macrophages[4].

All of the studies showed that PPARγ was closely related to foam cells formation and atherosclerosis. And some other studies were similar to our ones which presented that Ox-LDL-induced upregulation of adipophilin may be a consequence of the stimulation of PPARγ. One potential explanation is that Ox-LDL, an exogenous source of free cholesterol, can be internalized and, as a consequence, free cholesterol could induce PPARγ expression in macrophages as reported previously [29]. Two major oxidized lipid components of Ox-LDL, 9-hydroxyoctadecadienoic acid (9-HODE) and 13-hydroxyoctadecadienoic acid (13-HODE), were identified as endogenous activators and ligands of PPARγ [6], suggested that the Ox-LDL particle itself may be a source of endogenous PPARγ ligand. So the expression of PPARγ increased in the case of Dutch Fat. Moreover, it is possible that the highexpression of adipophilin may be a feedback for the expression of PPARγ, which was suppressed and increased in cells infected with retrovirus of pQCXIP-HA-Adipophilin and adipophilin siRNA respectively with Ox-LDL treatment in spite of Ox-LDL induced the expression of PPARγ. That was the different effect of endogenous and exogenous factor. That is adipophilin may be a regulation of the expression of PPARγ as a feedback without incubation of Ox-LDL which increased the expression of PPARγ. Take our previous studies [14] to consideration, these results established a link between adipophilin and the PPARγ signaling pathway showed that PPARγ may be a key regulator of foam cell gene expression and be an important regulator of adipophilin gene expression during atherogenesis, which revealed a novel signaling pathway controlling lipid metabolism in RAW264.7 cells.

Moreover, the results, when the cells were incubated with 50 mg/L Ox-LDL and then stained with Oil Red O or measured the lipids content in cells by HPLC, showed that the quantity of intracytoplasm lipids in cells infected with retrovirus of pQCXIP-HA-Adipophilin were much more than the control and pQCXIP and pSuper-scramble siRNA transfection cells. We also report highexpression of adipophilin can enhance accumulation of intracellular lipid droplets in RAW264.7 cells compared with normal cells in the case of Dutch Fat (Figure 7), and also enhance the accumulation of TG and CE. In contrast, the cholesterol ester in siRNA adipophilin transfected cells significant decreased (Figure 7, Table 2). One possible explanation about the significant increase in cholesterol ester in highexpression of adipophilin is that adipophilin may be localized around cytosolic lipid droplets in the macrophages protecting them from

the activity of cholesterol esterases such as hormone-sensitive lipase and thereby decreasing the availability of FC for efflux.

The phosphorylation of ERK1/2 also decreased and increased in cells infected with retrovirus of pQCXIP-HA-Adipophilin and adipophilin siRNA respectively with Ox-LDL treatment, which was similar to the expression of PPARγ. Our results were similar to others' that showed ERK1/2 phosphorylation was strongly upregulated by Ox-LDL in human mesangial cells or vascular endothelial cells[30-31]. There was study showed that Ox-LDL modulates PPARγ activity through phosphorylation, which is mediated by MAPK activation, facilitated foam cell formation [32]. But the change of adipophilin may also affect the phosphorylation of ERK1/2, like the effcet on the expression of PPARγ.

In the present study, we identified the induced expression of adipophilin mRNA in macrophages stimulated with Ox-LDL through ERK1/2-PPARγ signal pathway, which suggested that a potential role of these genes product in foam cell formation. Our data indicated that macrophage adipophilin expression is a consequence of lipid accumulation and contributes to further accumulation of lipids by inhibiting cellular cholesterol efflux. It is worthy to note that, in addition to adipophilin and perilipin [33-34], which expressed in lipid loaded macrophages [35], several other proteins have been identified on the surface of lipid droplets and in macrophages, such as caveolin [36-39] and it may play similar roles in lipid mobilization in specific cell types. For example, perilipin-deficient mice, which are resistant to diet-induced obesity, possess adipocytes with smaller lipid droplets and a higher rate of lipolysis in adipocytes compared with wild-type mice [40]. Thus, the generation of adipophilin-deficient mice or adipophilin knock-in mice would contribute to further understanding of the role of adipophilin in atherosclerosis.

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高表达和敲减 adipophilin 对细胞内 ERK1/2 活性、PPARγ 表达和细胞内脂质蓄积的影响 *

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摘要 本课题组以前的研究表明，adipophilin 通过 ERK1/2-PPARγ 信号转导通路促进细胞内的脂质蓄积．为了研究高表达和敲减 adipophilin 是否影响 RAW264.7 细胞内 ERK1/2 的活性、PPARγ 的表达以及细胞内的脂质蓄积，从而进一步证实这一通路，阐明 adipophilin 促进泡沫细胞形成的机制．重组 pQCXIP-HA-Adipophilin 和 pSuper-retro-adipophilin siRNA 逆转录病毒载体经酶切检测证实，并用 Sofast™ 介导转染到包装细胞 PA317 中，经培养后释放逆转录病毒．将收集的逆转录病毒感染 RAW264.7 细胞，经嘌呤霉素筛选后获得稳定高表达和敲减 adipophilin 的细胞系．用 50 mg/L 的氧化低密度脂蛋白处理细胞 24 h 后，用油红 O 染色法和高效液相色谱法测定细胞内的脂质蓄积情况，用半定量 RT-PCR 和蛋白质印迹分别检测 adipophilin 和 PPARγ 的 mRNA 和蛋白质的表达，用蛋白质印迹对与动脉粥样硬化发病有关的 ERK1/2 及其磷酸化进行检测．酶切结果表明，pQCXIP-HA-Adipophilin 和 pSuper-retro-adipophilin siRNA 重组逆转录病毒载体构建成功．在荷脂情况下，pQCXIP-HA-Adipophilin 转染的细胞能明显增加细胞内的脂质蓄积，但使 PPARγ 的表达和 ERK1/2 的磷酸化下调，这些作用可被 adipophilin siRNA 逆转．结果表明，adipophilin 与泡沫细胞的形成有关，adipophilin 可能是通过 ERK1/2-PPARγ 途径促进细胞内的脂质蓄积．

关键词 adipophilin，细胞外信号调节激酶 1/2，过氧化物酶体增殖物激活受体 γ，逆转录病毒
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