Adipophilin Antisense Oligonucleotides Decreased ACAT Activity *

YUAN Zhong-Hua **, YANG Yong-Zong, YIN Wei-Dong, YI Guang-Hui, WAN Zai-Yang
(*Institute of Cardiovascular Disease, Nanhua University, Hengyang* 421001, China)

Abstract Based on the finding of adipophilin expression with the cellular cholesteryl ester increasing, the aim of study was to investigate the active site of adipophilin in cellular cholesteryl metabolism. Mice peritoneal macrophages were incubated with 80 mg/L OxLDL or 80 mg/L OxLDL plus 1 mmol/L adipophilin antisense oligonucleotides respectively. At various time points, the incubated cell samples were observed with adipophilin immunofluoresence staining, flow cytometric analysis and cellular cholesterol analysis. The results shown, the antisense oligonucleotides treated cells contained significantly lower cholesteryl ester (19.9 ± 1.9) mg/g for 72 h. It was also shown by Oil Red O staining that the experimental group cells had less lipid droplets. During 96 h, expression of adipophilin increased in both groups. At 12 h, expression of adipophilin in OxLDL incubated cells increased quicker than that of the cells in antisense treated cells. At 96 h, the level of adipophilin expression in untreated control was significantly higher than that of antisense treated cells. The amount of OxLDL taking up by the cells was also observed with Ox-r[CL-^{3}H] LDL. During the observation, the amount of Ox-r[CL-^{3}H] LDL taking up increased gradually in both groups, however, from 24 h the taking up amount in antisense treated cells was less than that of untreated control. There was a statistical difference between the two groups from 24 h to 96 h. The activity of ACAT in the two groups was observed as well. From 6 h to 48 h, the relative ACAT activity increased in both groups. The relative ACAT activity kept unchanged from 48 h to 96 h in the two groups. At 48 h, the relative ACAT activity in antisense group was significantly lower than that of control group. The activity of ACAT was related to adipophilin expression but was not a linear relationship. This meant, at least partly, expression of adipophilin was related to cellular taking up of lipoproteins and to ACAT activity. Therefore, it is indicated that adipophilin has a role in metabolism of cellular lipid droplets and adipophilin is associated with ACAT.

Key words adipophilin, ACAT, macrophage, cholesterol

Deposition of excessive amounts of cholesteryl ester in macrophages of the vascular wall leading to foam cell formation is a key event in atherogenesis[1]. The microsomal enzyme acyl-CoA: cholesterol acyltransferase (ACAT) catalyzes the esterification of cellular cholesterol with fatty acids to form cholesteryl ester, which can then be stored in cytosolic droplets[2]. Therefore, inhibiting ACAT has received considerable interest as a possible therapy for atherosclerosis. Adipophilin, a marker of lipid accumulation initially described in adipocytes, was recently shown to be induced in macrophage foam cells[3]. We found that the expression of adipophilin increased in cultured macrophage as the cells accumulated more cholesteryl ester in previous study[4]. The early induction of adipophilin in macrophages by oxidized low density lipoprotein (Ox-LDL) potentially makes it a good marker of foam cell formation[5]. However, whether adipophilin merely serves to stabilize intracellular lipid droplets, or plays a regulatory role in lipid accumulation of the cell needs to be further investigated. To further elucidate the potential role of adipophilin in the molecular mechanism of atherogenesis, we used adipophilin antisense oligodeoxynucleotides in culture of mouse peritoneum macrophages to block the expression of adipophilin, and then to observe the effect of adipophilin antisense oligodeoxynucleotides on lipid accumulation and ACAT activity in the cultured macrophages.

1 Materials and methods

1.1 Materials

^{3}H-cholesterol, ^{14}C-oleic acid, and ^{3}H-cholesterol oleic ester were synthesized in Institute of Isotopes, China Atomic Sciences Academy. Epics Alfra flow cytometer is made by Beckman-Coulter, USA.

1.2 Cell culture and treatment

Macrophages isolated from C57BL/6J mice according to the method previously described by Brown *et al.*[6] were grown in RPMI1640 medium containing 10% fetal serum (*v/v*), penicillin (100 U/ml) and streptomycin (100 mg/L).

The cultured cells were treated as the following: the untreated group, cultured in the RPMI1640 medium containing 10% FCS; the control group, in the RPMI1640 medium containing 80 mg/L OxLDL or Ox-r[CL-^{3}H] LDL; the antisense group, in the RPMI1640 medium containing 80 mg/L OxLDL or Ox-r[CL-

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** Corresponding author.
Tel: 86-734-8281311, Fax: 86-734-8282232
E-mail: yzh5555@163.com
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^{3}H] LDL plus 10 mmol/L antisense-adipophilin oligonucleotides (ASODNs), or plus sense-adipophilin oligonucleotides (the sense group), or missense-adipophilin oligonucleotides (the missense group). The treatment time points of the cells were 0, 6 h, 12 h, 24 h, 48 h, 72 h and 96 h.

1.3 Cellular cholesterol measurement

The cells were harvested from culture dishes with the aid of a rubber policeman and transferred to a conical graduated centrifuge tube. The cells were washed three times by suspending them in phosphate-buffered saline (PBS), centrifuging at 800 *g* for 5 min, and aspirating the fluid. An appropriate volume of isopropyl alcohol (0.5 L for every 1 g protein) was then added to the pellet and the sample was sonicated with a microprobe. After centrifugation for 15 min at 800 *g* the clear supernatant was decanted and an aliquot was taken for cholesterol and cholesteryl ester determination by spectrophotofluorometer with axenon lamp (Shanghai analytic instrument factory. λ_{ex} = 325 nm, λ_{em} = 415 nm. Slits = 2 mm), as described previously[7]. The residue was dissolved in 0.1 mol/L sodium hydroxide and an aliquot was taken for protein determination by the method of Lowry *et al.*[8]

1.4 Oil red O staining

A modified oil red O staining was used to stain lipids in the cultured cells. Briefly, cells were fixed (1 min) using 50% isopropanol and then stained with the working solution of oil red O for 10 min, rinsed with deionized water for 3 times (1 min/time), and counterstained using Mayer's haematoxylin for 60 s to visualize nuclei.

1.5 Lipoprotein isolation, modification and characterization

Human LDL was isolated from normolipemic plasma by density gradient ultracentrifugation. The LDL was dialyzed against 6 liters of PBS containing 0.3 mmol/L EDTA, and the protein concentration was measured as described by Lowry *et al.*[8] For LDL oxidation, the LDL was dialyzed against 4 liters of PBS to remove EDTA, adjusted to 100 mg/L of LDL protein in PBS, and incubated with 10 μmol/L $CuSO_4$ for 18 h at 37℃. The extent of oxidation was assessed by measuring the thiobarbituric acid-reactive substances. To prevent further oxidation, butylated hydroxytoluene and EDTA were then added at 20 μmol/L and 0.1 mmol/L, respectively. The OxLDL was concentrated by centrifugation in CF50A membrane cones to approximately 1 g/L.

Oxidation of LDL was performed as the following: PBS (pH 7.4) was oxygenated at 2 L/min for 10 min, and $CuSO_4$ was added to a final concentration of 10 μmol/L for every 300 μg of LDL. Copper and BHT were removed from the OxLDL preparations after termination of the oxidation reaction by overnight dialysis against 4 L of a 0.15 mol/L concentration of sodium chloride containing 300 μmol of EDTA (pH 8.0) per liter. After dialysis, the OxLDL preparations were filtered through a 0.22-μm-pore-size filter to sterilize and remove aggregates and stored at 4℃. The final protein concentration in each preparation was determined by a method of Lowry[8].

1.6 Preparation of reconstituted LDL and reconstituted OxLDL

Reconstituted LDL (referred to as r[CL-^{3}H] LDL) was prepared from native LDL by the method of Romero *et al.*[9]. After removal of free ^{3}H by extensive dialysis against PBS, the LDL was oxidized as described above, the reconstituted OxLDL referred to as Ox-r[CL-^{3}H] LDL.

1.7 Assay the radioactivity of cellular Ox-r[CL-^{3}H] LDL

The cells were harvested from culture bottles with the aid of a rubber policeman and transferred to a conical graduated centrifuge tube. The cells were washed three times by suspending them in PBS, centrifuging at 800 *g* for 5 min, and aspirating the fluid. An appropriate volume of isopropyl alcohol (0.5 L/g) was then added to the pellet and the sample was sonicated with a microprobe. After centrifugation for 15 min at 800 *g* the clear supernatant was decanted to scintillation bottles. 3 ml scintillation solution (dimethylbenzene with 0.5% 2,5-diphenyloxazole and 0.03% 1,4-bis-2-(5-phenyloxazolyl)-benzene) was added to each bottles. The radioactivity (Ci/min) was determined by the scintillation counting.

The residue was dissolved in 0.1 mol/L sodium hydroxide and an aliquot was taken for protein determination by the method of Lowry[8].

1.8 Assessing the expression of adipophilin with flow cytometry

Cultured macrophages were harvested, washed, and stained with a saturating concentration of the monoclonal adipophilin antibody followed by incubation with a FITC-labeled second antibody. Flow cytometric analyses were performed on a Epics Alfra flow cytometer.

1.9 ACAT activity assay

Cell homogenates were prepared and assayed for ACAT activity as described by Cadigan *et al.*[10]

1.10 Statistical analysis

All values are expressed as ($\bar{x} \pm s$). The significance of the difference in mean values between different treated cells was evaluated using the unpaired Student's test. All statistics were performed with the aid of a software statistical package (SPSS 10.0). Statistical significance was set at $P < 0.05$.

2 Results

2.1 Effects of adipophilin antisense on cellular cholesterol and lipid droplet

After 72 h incubation with 80 mg/L OxLDL, cellular cholesterol ester of the cultured preritoneum macrophages increased significantly (Table 1), from (2.5 ± 2.2) mg/g to (46.6 ± 3.4) mg/g. Whereas, the cells incubated with 80 mg/L OxLDL plus 1 mmol/L adipophilin ASODNs for 72 h contained significantly lower cholesterol ester, (19.9 ± 1.9) mg/g. The cellular cholesterol of the cells cultured with OxLDL-sense or OxLDL-missense were almost unchanged.

Table 1 Changes of cellular cholesterol

Group	Cellular cholesterol/(mg·g^{-1})		
	Free	Total	Ester
untreated	40.1 ± 3.1	42.6 ± 2.0	2.5 ± 2.2
control	51.2 ± 1.7	97.8 ± 3.9	46.6 ± 3.4
antisense	45.5 ± 2.1	65.4 ± 1.7 *	19.9 ± 1.9 *
sense	43.6 ± 1.1	89.2 ± 2.8	45.6 ± 1.8
missense	47.1 ± 3.3	90.5 ± 1.6	43.4 ± 2.9

* $P < 0.05$ vs control. $n = 10$.

Neutral lipids in cells appeared red with oil red O staining, and the nucleolus appeared blue when counterstained with hematoxylin. The cells cultured without OxLDL contained no lipid droplets (Figure 1a). The cells incubated with OxLDL alone contained the most red lipid droplets (Figure 1b). The cells incubated with oxLDL-antisense had fewer lipid droplets (Figure 1c).

2.2 Ox-r[CL-^{3}H]LDL uptake of the cells

The cells were incubated in 80 mg/L Ox-r[CL-^{3}H] LDL with or without 1 mmol/L adipophilin ASODNs for 0, 6 h, 12 h, 24 h, 48 h and 96 h. Figure 2 shows that Ox-r[CL-^{3}H] LDL uptake of the cells incubated with adipophilin ASODNs (the antisense group) was significantly lower than that of the cells incubated without adipophilin ASODNs (the control group) at 24 h, 48 h and 96 h.

2.3 Expression of adipophilin

Figure 3 shows that expression of adipophilin in the control group was gradually higher than in the cells in the antisense group, at 96 h, the difference was significant. In the experiment, the increase in expression of adipophilin was earlier than the increase in Ox-r[CL-^{3}H] LDL uptake. Therefore, we speculated that expression of adipophilin might have caused more Ox-r[CL-^{3}H] LDL uptake.

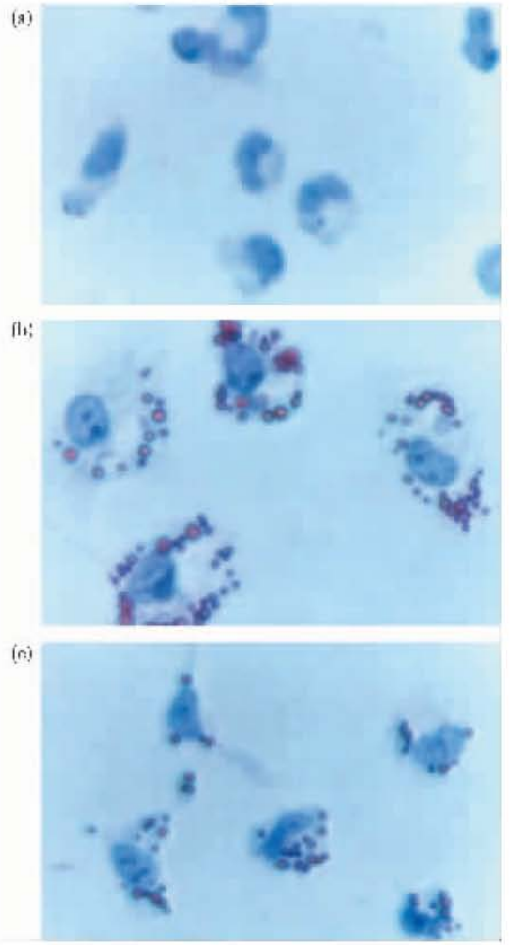

Fig. 1 Microphotos of mouse peritoneum macrophages stained with oil red O

Neutral lipids in cells appeared red with oil red O staining, and the nucleolus appeared blue counterstained with hematoxylin. (a) The cells cultured without OxLDL contained no lipid droplet. (b) The cells incubated with OxLDL alone showed the most red lipid droplets. (c) The cells incubated with OxLDL-adipophilin ASODNs had less lipid droplets (×400).

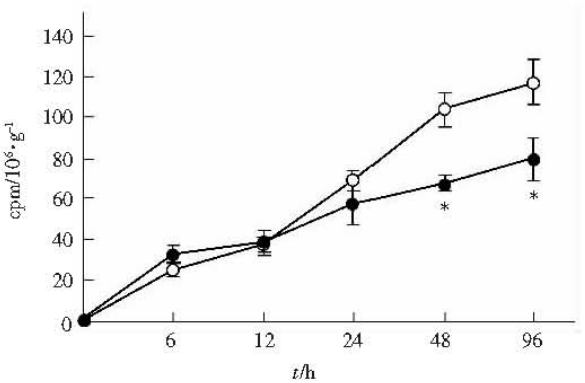

Fig. 2 Time course of Ox-r[CL-^{3}H] LDL uptaking of the cells

○—○: Control; ●—●: Antisen. * Compared with the control group, $P < 0.05$.

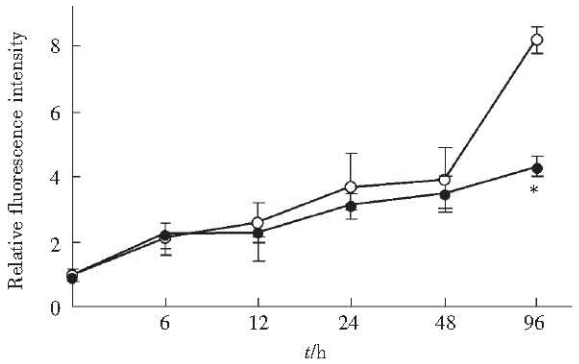

Fig. 3 Time course of the relative fluorescence intensity of adipophilin protein

○—○: Control; ●—●: Antisense. * Compared with the control group, $P < 0.05$.

2.4 ACAT activity

Figure 4 shows ACAT activity in different treated cells. From 6 h to 48 h, the ACAT relative activity increased. At 48 h, the difference was significant, with antisense being lower than that of without antisense.

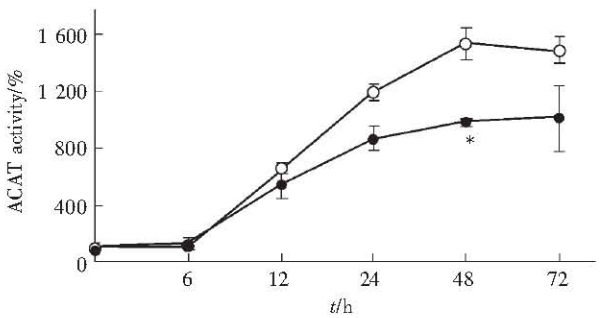

Fig. 4 Time course of the relative ACAT activity

○—○: Control; ●—●: Antisense. * Compared with the control group, $P < 0.05$.

2.5 The relationship between expression of adipophilin and ACAT activity

Figure 5 and Figure 6 showed the relationship between expression of adipophilin and ACAT activity in different treated cells groups. In untreated, $r^2 = 0.6176$, in treated $r^2 = 0.8212$, the relationship was not linear.

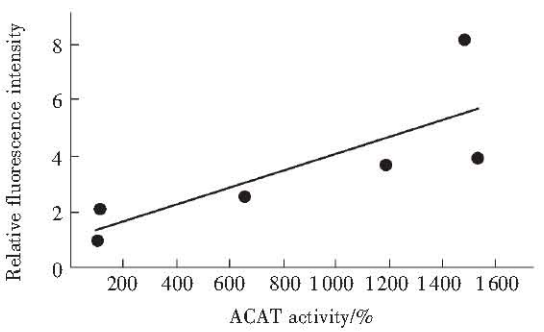

Fig. 5 The correlation between the relative ACAT activity and the relative fluorescence intensity of adipophilin protein in the control group

$y = 0.003x + 1.0429$, $r^2 = 0.6176$.

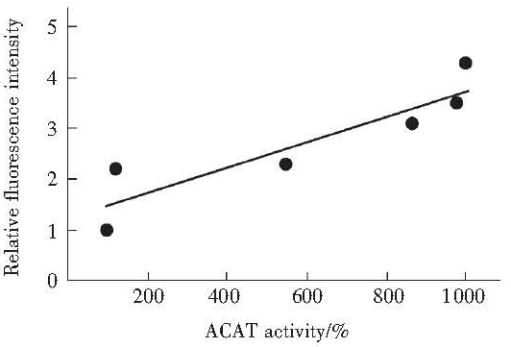

Fig. 6 The correlation between the relative ACAT activity and the relative fluorescence intensity of adipophilin protein in the antisense group

$y = 0.0025x + 1.2126$, $r^2 = 0.8212$.

3 Discussion

Cholesterol, cholesterol metabolites, and immediate biosynthetic precursors of cholesterol play essential roles in cellular membrane physiology, dietary nutrient absorption, reproductive biology, stress responses, salt and water balance, and calcium metabolism. However, an excess of cholesterol is a major culprit for atherosclerosis. The cells of most organs and tissues satisfy their requirements for membrane cholesterol via endogenous cholesterol biosynthesis[1]. Many cell types, however, have acquired mechanisms to internalize exogenous sources of cholesterol, usually in the form of plasma-derived lipoproteins[2]. Examples include steroid-synthesizing cells, hepatocytes, and macrophages and smooth muscle cells in atherosclerotic lesions, often referred to as foam cells.

The microsomal enzyme acyl-CoA: cholesterol acyltransferase catalyzes the esterification of cellular cholesterol with fatty acids to form cholesterol esters. ACAT activity is found in many tissues, including macrophages, the adrenal glands, and the liver. In macrophages, ACAT is thought to participate in foam cell formation and thereby to contribute to atherosclerotic lesion development. Disruption of the gene for ACAT in mice resulted in decreased cholesterol esterification in ACAT-deficient fibroblasts and adrenal membranes, and markedly reduced cholesterol ester levels in adrenal glands and peritoneal macrophages; the latter finding will be useful in testing the role of ACAT and macrophage foam cell formation in atherosclerosis[2]. In the current study, the cells

took up more Ox-r[CL-^{3}H] LDL as ACAT activity of the cells increased (Figure 2 and Figure 4).

What happened with respect to adipophilin in the current study? During 96 h experiment, the expression of adipophilin had been increasing, and Ox-r[CL-^{3}H] LDL take up had been increasing as well. We could not identified what was the cause, what was the consequence. However, by comparing the untreated and antisense treated cells, we speculated that it could be the change of adipophilin expression influenced the Ox-r[CL-^{3}H] LDL take up by the cells, because we observed that the change of adipophilin expression occurred in advance of the increase of the Ox-r[CL-^{3}H] LDL take up by the cells. This would implicated that the function of adipophilin is not just to maintain the structure of the lipid droplet, but it is at least associated with cells' function of taking up exogenous lipoproteins. Now that adipophilin could be involved in intracellular cholesterol flow, then we further checked its relationship with ACAT. As Figure 5 and Figure 6 showed, adipophilin was positively related with the activity of ACAT. But this relationship was linear.

Chen *et al.*[11] also reported similar results indicating that adipophilin may be an important protein in the regulation of lipid droplet metabolism in lipid-laden macrophages and that this regulation may be mediated by PKC activity. Buechler *et al.*[12] found that enzymatically modified low-density lipoprotein (E-LDL) induces rapid foam cell formation in monocytes and upregulates adipophilin mRNA and protein within 2h of incubation. When the THP-1 macrophages incubated with acetylated LDL, Larigauderie *et al.*[13] found that triglycerides and cholesteryl esters were increased in macrophages overexpressing adipophilin by 40% and 67%, respectively, whereas their accumulation was reduced when endogenous cellular adipophilin was depleted using siRNA approach. This rapid induction of adipophilin is accompanied by a significant increase of free fatty acids in monocytes incubated with E-LDL. Adipophilin facilitates the uptake of free fatty acids, and they demonstrated that free fatty acids increase is related to the early upregulation of adipophilin expression in blood monocytes. Fatty acids are ligands for peroxisome proliferator-activated receptor-γ(PPARγ), and the upregulation of adipophilin mRNA by PPARγ agonists like 15d-PGJ2 and ciglitazone indicates that PPARγ may mediate the induction of adipophilin expression in human blood monocytes. Taken together, we think that the function of adipophilin and its regulation could be miscellaneous.

In summary, in the current study we demonstrated that the ACAT activity and the cellular taking up of exogenous lipids can be decreased by inhibiting adipophilin expression with antisense technique.

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反义 adipophilin 寡核苷酸降低 ACAT 活性*

袁中华** 杨永宗 尹卫东 易光辉 万载阳

（南华大学心血管病研究所，衡阳 421001）

摘要 已经发现，随着细胞内胆固醇酯的积聚，adipophilin 的表达明显增加. 在此基础上，为了寻找 adipophilin 在细胞内胆固醇代谢的作用点. 小鼠腹腔巨噬细胞与 80 mg/L OxLDL 或 80 mg/L OxLDL 加 1 mmol/L adipophilin 反义寡核苷酸共孵育，在不同的时间点取样，使用免疫荧光染色，流式细胞仪分析和细胞内胆固醇测定. 结果显示，72 h 后，反义寡核苷酸处理组细胞内胆固醇酯显著下降到（19.9 ± 1.9）mg/g，油红 O 染色显示，该组细胞质内红色脂滴明显减少. 96 h 的观察期间内，两组细胞 adipophilin 蛋白的表达量都增加. 从 12 h 开始，未用反义寡核苷酸处理组 adipophilin 蛋白的表达量开始大于反义寡核苷酸处理组. 在 96 h 时间点，统计学处理，未用反义寡核苷酸处理组明显高于反义寡核苷酸处理组，差别有显著性. 使用 Ox-r［CL-^{3}H］LDL 观察两组细胞摄入 OxLDL 的量，实验发现，两组细胞摄入 Ox-r［CL-^{3}H］LDL 的量逐渐增加. 但是，反义寡核苷酸处理组摄入 Ox-r［CL-^{3}H］LDL 的量从 24 h 后明显低于未用反义寡核苷酸处理组，在 48 h 和 96 h 时间点，统计学处理，两组差别有显著性. 观察 ACAT 的活性发现，ACAT 相对活性从 6 h 到 48 h 表现为增加，但从 48 h 到 96 h，ACAT 相对活性趋于稳定. 在 48 h 时间点，两组比较差别有显著性，反义寡核苷酸处理组的活性明显低于未用反义寡核苷酸处理组. 相关分析发现，ACAT 的活性与 adipophilin 蛋白的表达量有一定的联系，但不是直线相关. 结果表明，adipophilin 表达的高低与细胞摄入外源性脂蛋白，与 ACAT 的活性有一定的联系. 提示 adipophilin 与脂滴的代谢功能密切相关，且 ACAT 有可能是其潜在的位点.

关键词 adipophilin，ACAT，巨噬细胞，胆固醇

学科分类号 R361.3

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* 湖南省教育厅课题资助项目（01C191）.

** 通讯联系人.

Tel：0734-8281311，Fax：0734-8282232，E-mail：yzh5555@163.com

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