Adenylyl Cyclase 1 as a Major Isoform to Generate cAMP Signaling for ApoA-1-mediated Cholesterol Efflux Pathway

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Abstract

HDL apolipoprotein A-1-mediated cholesterol efflux pathway requires multiple cellular proteins and signal transduction pathways, including adenylyl cyclase (AC) / cAMP signaling. Due to the existence of multiple transmembrane AC isoforms, it was not known how many AC isoforms are expressed and which ones are essential for cholesterol efflux in macrophage foam cells. These questions were investigated in THP-1 macrophages in this study. QRT-PCR detected mRNAs for all nine transmembrane AC isoforms, but only the mRNA and protein of AC1 isoform were consistently upregulated by cholesterol loading and apoA-1. AC1 shRNA interference decreased AC1 mRNA and protein levels, resulting in reduction of apoA-1-mediated cAMP production and cholesterol efflux, while the intracellular cholesterol levels remained high. Confocal microscopy shows that apoA-1 promotes translocation of cholesterol and formation of cholesterol-apoA-1 complexes (protrusions) on the cholesterol-loaded macrophage surface. AC1 shRNA interfered macrophages showed no translocation of cholesterol to the cell surface. AC1 shRNA interference also disrupted cellular localization of an intracellular cholesterol indicator protein ADFP, and the expression as well as surface translocation of ABCA1. Together, our study showed that AC1 is a major isoform for apoA-1-activated cAMP signaling to promote cholesterol transport and exocytosis to the surface of THP-1 macrophage foam cells.

Key words: ApoA-1, Adenylyl cyclase 1, cAMP, Cholesterol efflux, Macrophage
Introduction

High-density lipoproteins (HDL) are one of the major cholesterol carriers in human plasma, and their levels are inversely correlated with the prevalence of atherosclerotic cardiovascular diseases (1). Every milligram increase of HDL may reduce the mortality rate of the cardiovascular disease by approximately 2 to 4 percent (2). The beneficial effect is in part attributed to the role of HDL apolipoproteins (mainly apoA-1) in promoting cholesterol removal from macrophage foam cells, leading to reduction of intracellular lipid accumulation and the risk of ischemic cardiovascular disease.

HDL apolipoprotein-mediated cholesterol removal pathway involves many cellular proteins, including cell surface binding proteins/receptors(3, 4), intracellular signal transduction(3, 5), vesicle transport(6, 7) and exocytotic components(6, 8). Of these, signal transduction is one of the most complex events, which includes the activation of adenylyl cyclase and cAMP generation(9, 10), activation of Cdc 42(11), G protein (12), Rac GTPase(13), Janus kinase 2 (JAK2)(14), c-Jun N-terminal kinases (JNKs) and p38 MAP kinase(11), protein kinase A (PKA)(10), protein kinase C (PKC)(15), phospholipase C and D (PLC and PLD) (16), as well as intracellular calcium release (17). How exactly these proteins and signal mediators facilitate cholesterol removal from cells remains ambiguous. We have investigated a hypothesis on whether apoA-1 promotes vesicle transport and
exocytosis of cholesterol, by activation of intracellular signal transduction through some of the proteins and kinases mentioned above(18).

Adenylyl cyclase is also known as adenylyl cyclase and adenylate cyclase (AC), and this enzyme catalyzes the conversion of adenosine triphosphate (ATP) to 3', 5'-cyclic AMP (cAMP), a universal second messenger that regulates diverse cellular functions including secretion and exocytosis (19). Structurally, AC consists of a 120-kDa catalytic subunit and a 50-kDa regulatory subunit, with a total of ten isoforms found in mammalian tissues. Isoforms 1 to 9 are localized on the cell membrane, and are called the transmembrane adenylyl cyclases, which are activated by G protein coupled receptors (GPCR) (19). Isoform 10 is a soluble adenylyl cyclase found in the cytosol, nucleus, mitochondria, and centriole (20). HDL and apoA-1 activate adenylyl cyclase and increase cAMP level, which results in PKA activation and cholesterol efflux(9, 10). PKA inhibitors, such as H89, significantly decrease HDL and apoA-1-mediated cholesterol efflux(9, 10, 20). These observations suggest the AC/cAMP/PKA signaling cascade is required for HDL apolipoprotein-mediated cholesterol efflux pathway. Although AC isoform expressions have been reported in macrophages from liver and lung (21), it has not been known how many AC isoforms are expressed in macrophage foam cells, the principal components in atherosclerotic lesions, and which AC isoform(s) is required for apoA-1 mediated cholesterol efflux in foam cells is unknown as well. We investigated these questions by using QRT-PCR screening, shRNA interference, biochemical analysis and confocal microscopy in
this study. Here we report that AC1 is a major isoform activated by apoA-1 to promote cholesterol efflux from THP-1 macrophages foam cells.
Materials and Methods

Cell culture and cholesterol loaded with ac-LDL
Human monocytic THP-1 was obtained from ATCC and maintained in RPMI 1640 media according to instructions from the source. To set up for experiments, the cells were plated in culture dishes or microscope cover slips, and induced by 160 nM PMA for 48 hours to differentiate into macrophages. The cells were then incubated with 50-µg/ml acLDL for 48 h to become cholesterol–loaded macrophage foam cells, while control cells were incubated without acLDL in the same media as described in our recent study (6).

Relative qRT-PCR analysis
Total RNA was extracted using Trizol reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer’s instructions. qRT-PCR was conducted in ABI 7500 Real-Time PCR system from Applied Biosystems (Life Technologies, Grand Island, NY, USA) with reagents obtained from TaKaRa Biotechnology Co., Ltd. (Dalian, China). Total RNA (300 ng) from each condition was used for the first strand synthesis. PCR cycles were performed with specific primers at the following conditions: 95°C for 30s, 95°C for 5s and 60°C for 34s with 40 cycles, 95°C for 15s and 60°C for 1 min and 95°C for 15s with 1 cycle. Primers for AC1, AC4 and GAPDH were listed in table 1 and the primers for the rest of AC isoforms used the sequences as described in the reference (22).

Construction shRNA lentiviral particles and transduction
The human adenylyl cyclase AC1, AC4 and scrambled shRNA oligonucleotides were synthesized by Genomeditech Co., Ltd (Shanghai, China) as listed in table
2. The synthetic shRNA fragments, with BamH I and EcoR I restriction sites at 5' and 3' end respectively, were cloned into pGMLV-SC1 RNAi lentiviral vectors. The shRNA lentiviral particles were generated and transduced into the THP-1 cells by using the lentiviral particles 30 optimal multiplicity of infection (MOI) as described in our recent study(6).

**Cellular cAMP and total cholesterol determination**

To determine cellular cAMP levels, macrophages foam cells were treated with or without 10-μg/ml apoA-1 for 10 minutes. The culture media were removed, and the cells were rapidly washed with PBS buffer. The resulting cells were lysed with 0.1M HCl and centrifuged to obtain supernatant fractions. The supernatant was assayed in duplicate using Cyclic AMP EIA Kit from Cayman Chemical Company (Ann Arbor, MI, USA) according to the manufacturer’s instructions. The protein concentration was determined in the same supernatants by Biorad protein assay kit. Final cAMP concentration was expressed as pmol/mg protein (mean ± SD of three separate experiments). To determine cellular cholesterol, the macrophage foam cells were treated with or without 10-μg/ml apoA-1 for 6 hours. The culture media were then removed, and the cells were washed three times with PBS. The resulting cells were lysed, and total cholesterol was assayed in duplicate with an assay kit (Applygen Technologies Inc. Beijing, China) according to the manufacturer’s instructions. The protein concentration was determined in the same lysate by Biorad protein assay kit. Total cellular cholesterol was expressed as nmol/ mg protein (mean± SD of three to four individual experiments).
Cholesterol efflux assay

Macrophages were simultaneously labeled and loaded in RPMI media containing 0.2 μCi [³H] cholesterol (10.5 Ci/mmol, Shanghai Atomic Institute, Chinese Academy of Sciences, Shanghai, China), 50 μg/ml acLDL and 2 mg/ml BSA for 48 hours(6). The resulting cells were subsequently subjected to equilibrate in RPMI media contained 2 mg/mL BSA for 24 hours. The cells were then incubated in a fresh RPMI medium with or without 10 μg/ml of apoA-1 for 6 hours. Levels of [³H] cholesterol were separately determined in the efflux media and the cells by using a scintillation counter. Cholesterol efflux (%) was calculated as the CPM in the efflux media, divided by the total count (media plus cells) and multiplied by 100%. Net apoA-1-mediated cholesterol efflux was obtained by subtracting efflux of control cells from that of the cells incubated with apoA-1.

Western blotting analysis

THP-1 macrophages-derived foam cells were treated as indicated in figure legends and total cellular protein was extracted with RIPA buffer (Beyotime Institute of Biotechnology, Beijing China), and equal amounts of protein were separated by 10% SDS-PAGE and transferred electrophoretically to polyvinylidene difluoride membranes (Millipore, Bedford, MA USA). Western blotting was carried with a standard protocol using rabbit polyclonal antibodies of AC1 andAC4 (both at 1:500 dilutions) from Abnova (Taipei, China), followed by the goat anti-rabbit IgG-HRP (1:1000 dilutions) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Protein bands were detected with ECL and were
quantified with Molecular Imager ChemiDoc XRS+ System (Bio-Rad, Wuhan, China).

**Confocal microscopy**

THP-1 macrophages either transduced by AC1 shRNA, scrambled lentivirus or controls were cultured on glass cover slips. The cells were loaded with or without 50 µg/ml acLDL for 48 hours, were treated with or without 10 µg/ml apoA-1 for 6 hours, then were washed for 5 minutes with PBS twice to remove unbound apoA-1. The cells were fixed in 4% paraformaldehyde in PBS for 20 minutes, and then washed three times with PBS for 10 min each to remove free fixative, before being labelled with different probes. For detection of the apoA-1 and cholesterol colocalization on the cell surface, cells were incubated in 5% BSA in PBS containing anti-apoA-1 antibody (1:400 dilutions from Abcam) at room temperature for 1 hour, and washed three times with PBS for 10 minutes each. The cells were subsequently incubated with goat anti-rabbit Alexa 568-conjugated secondary antibody (1:1000 dilutions from Abcam) and a cholesterol probe filipin III at 50 µg/mL (Cayman Chemical) for 1 hour at room temperature. Subsequently, the cells were washed three times with PBS for 10 minutes each. The cells in cover slips were mounted with ProLong® antifade reagents and cured at least for 48 hours before examination with confocal microscopy. For visualization of intracellular protein targets, the fixed cells in cover slips were simultaneously permeabilized and blocked in PBS containing 5% BSA, 0.1% NP 40 for 45 minutes as described in our recent study(6). The cells were incubated
either with a rabbit anti-ADFP or anti-ABCA1 antibody (1:100 dilutions, both from Abcam) at 4°C overnight, and then washed three times for 10 minutes each with PBS containing 5% BSA and 0.1% NP 40. The cells were incubated with goat anti-rabbit secondary antibody-conjugated with Alexa 568 (1:1000 dilutions, Abcam) for 1 h at room temperature, and then washed three times with PBS for 10 minutes each at room temperature. The cover slips containing the processed cells were mounted with ProLong® antifade reagents as mentioned above. All samples were examined with a confocal laser-scanning microscope with an excitation laser corresponding to optimal wavelength of the probes (Zeiss LSM 710, Germany). The images were acquired and processed with software Zen® (Zeiss, Germany). Fiji (Image J) was used to determine the corrected cell fluorescence according to the method by McCloy and Burgess (23, 24).

**Statistical analysis**

Statistical analysis was performed using one-way ANOVA with GraphPad Prism (GraphPad Software), followed by the Newman-Keuls multiple comparisons test. The level of significance was set at P < 0.05 for all results.
Results

AC1 as a major isoform upregulated by cholesterol and apoA-1. Real-time qRT-PCR expression profiling showed that mRNAs of all transmembrane adenylyl cyclases were detected in THP-1 macrophages. AC1, AC3 and AC4 mRNAs were significantly upregulated by cholesterol loading, and the mRNA levels of AC1 to AC4 were increased by apoA-1 (Fig. 1A). Of them, AC1 mRNA levels had the greatest response to ac-LDL cholesterol loading and apoA-1 stimulation. Western blotting confirmed that AC1 protein levels were also increased by cholesterol loading and apoA-1 (Fig. 1B). Thus, our next studies were focused on the characterization of AC1 in the cholesterol efflux process, since the protein levels of other isoforms were not consistently influenced by cholesterol loading and apoA-1.

Reduction of cAMP production and cholesterol efflux by AC1 shRNA interference. To establish functional relevance of the AC1 isoform with apoA-1 mediated cholesterol efflux, AC1 shRNA was used to interfere with AC1 expression, and then its effects on the cholesterol efflux process were determined. To begin, the mRNA and protein levels of the target were determined to ensure reduction of the isoform following AC1 shRNA interference in THP-1 macrophages. QRT-PCR showed that AC1 shRNA interference reduced AC1 mRNA by 75%, compared to control, and by more than 90% when compared to that of apoA-1 or scrambled shRNA interfered cells due to the higher level of AC1 in the two groups (Fig. 2A). The AC1 protein levels in the AC1 shRNA interfered
cells were reduced by 45% to 55% when compared to control, A-1-treated cells or scrambled shRNA control (Fig. 2B), suggesting that AC1 protein turnover was slower than that of AC1 mRNA. Functionally, both cAMP level and apoA-1-mediated cholesterol efflux were greatly reduced in the AC1 silenced macrophages after incubation with apoA-1, in comparison to those in the non-shRNA interfered macrophages and in scrambled shRNA interfered macrophages (Fig. 2C and 2D). In contrast, the intracellular cholesterol level was significantly higher in the AC1 shRNA interfered cells than that in non-shRNA treated cells or scrambled shRNA interfered cells (Fig. 2E). As an additional control, we investigated if AC4 shRNA interference could influence cAMP production and cholesterol efflux in THP-1 macrophages in response to apoA-1 stimulation, but no consistent effects on these two parameters were observed even though AC4 shRNA effectively reduced the levels of AC4 mRNA and protein (Fig. 3). Taken together, the results indicated that AC1 was the major isoform responsible for apoA-1-activated cAMP production and subsequent cholesterol efflux from cholesterol-loaded macrophages.

**Reduction of cholesterol translocation to the cell surface by AC1 shRNA interference.** Prior studies with immunogold electron microscopy and biochemical analysis show that apoA-1 binding to the cell membrane promotes cholesterol transport and secretion of lipids to form lipid protrusion complexes on the cell surface(6, 8, 25). However, these studies have not directly shown colocalization of cholesterol and apoA-1 in the lipid complexes on the cell surface.
Here we investigated if it was possible to observe colocalization of cholesterol and apoA-1 on the surface lipid protrusion complexes and if AC1 shRNA interference would affect the appearance of the complex, by using a laser scanning confocal microscopy. Filipin III, an antibiotic from *Streptomyces filipinensis*, was used as a cholesterol probe because it selectively binds to cholesterol and produces blue fluorescence upon excitation (26). Confocal images showed that ac-LDL-loaded macrophages had an increased level of blue fluorescence from filipin III, an indication of higher levels of intracellular cholesterol, in comparison to non-ac-LDL loaded macrophages which had lesser blue fluorescence (Fig. 4, control and Ac-LDL panels). The macrophages incubated with apoA-1 were observed with red fluorescence surrounding the cell surface. A majority of apoA-1 was located on the smooth cell membrane, and some were also located in the surface complexes consisting of red fluorescence from apoA-1 around the outer layer and blue coloration from cholesterol content within, as indicated by arrows (Fig. 4, apoA-1 panel). Overall levels of blue fluorescence from filipin III were reduced in these macrophages incubated with apoA-1, indicating that apoA-1 promoted cholesterol efflux and reduced amounts of the intracellular cholesterol in these cells (Fig. 4, apoA-1 panel). In the AC1 shRNA interfered macrophages, blue fluorescence from filipin remained high and apoA-1 appeared on the relatively smooth cell surface with few protrusion complexes (Fig. 4, AC1 shRNA +apoA-1 panel). Following incubation of the scrambled shRNA interfered cells with apoA-1, the intracellular cholesterol
(indicated by filipin fluorescence) decreased and the protrusion complexes were also observed, which were similar to those in non-shRNA interfered macrophages incubated with apoA-1 (Fig. 4, scrambled + apoA-1 panel). Thus, confocal microscopy was able to clearly demonstrate that apoA-1 promoted formation of the lipid complexes protrusion on the membrane surface of non-shRNA and scrambled shRNA interfered cells. Quantification of filipin fluorescence reflected changes in intracellular cholesterol as expected (Fig. 4, fluorescence intensity panel). These results were consistent with the idea that AC1 was the major isoform for apoA-1-activated production of the second messenger cAMP to promote cholesterol efflux to the cell surface.

**ADFP distribution in AC1 shRNA interfered cells.** Adipophilin (ADFP) is a lipid droplet coat protein located on the outermost monolayer of lipid droplets (27), and a specific marker of lipid accumulation in THP-1 macrophages and atherosclerotic lesions (28, 29). ADFP is also associated with cholesterol transport vesicles in the process of apoA-1-mediated cholesterol efflux(6). If ADFP is localized in the downstream of apoA-1 activated cAMP signaling pathway, we would expect to see the cellular distribution of ADFP changed, and potential alterations could be detected by confocal microscopy. As shown in the previous study (6), cholesterol loading increased ADFP level with punctate distribution in cytoplasm (Fig. 5, control and Ac-LDL panels). ADFP levels were dramatically reduced in apoA-1 incubated non-shRNA and scrambled interfered macrophages (Fig. 5, apoA-1 and scrambled + apoA-1 panels). In the AC1
shRNA silenced macrophages, ADFP level remained high and appeared in the cytoplasm and nucleus (Fig. 5, AC1 shRNA+ apoA-1 panel). Quantification of ADFP levels was also consistent with accumulation of the intracellular cholesterol (Fig. 5, fluorescence intensity panel). Unexpectedly, AC1 was found to be required for maintaining normal cellular distribution of ADFP.

**Effect of AC1 shRNA interference on ABCA1 levels and localizations.** ATP binding cassette protein A1 (ABCA1) is a critical component in apoA-1 mediated cholesterol efflux(30). Studies suggest that ABCA1 is also a component of cholesterol transport vesicles(6, 31). We showed here that cholesterol loading increased ABCA1 levels in cytoplasm, in comparison to the non-cholesterol loaded cells (Fig. 6, control and AC-LDL panels). Treatment with apoA-1 increased ABCA1 protein levels in the cytoplasm and cell surface in non-shRNA and scrambled shRNA interfered macrophages (Fig. 6, apoA-1 and scrambled shRNA + apoA-1 panels). AC1 shRNA interference reduced total and membrane ABCA1 (Fig. 6, AC1 shRNA+apoA-1 panel). In line with confocal images, quantification of ABCA1 levels showed that AC1 shRNA interference reduced ABCA1 levels (Fig.6, fluorescence intensity panel). Together, the results indicated that AC1 was also required for ABCA1 expression and cellular localization.
Discussion

Activation of ACs produces cAMP as a second messenger that regulates diverse intracellular signaling pathways of important cellular functions, including metabolism, gene expression, cell growth, apoptosis and secretion (32). This study shows for the first time that all 9 transmembrane AC isoforms are expressed in THP-1 foam cells, but only AC1 acts as a major signaling “switch” for apoA-1-mediated cholesterol efflux pathway in lipid-loaded macrophages. Since HDL apolipoprotein-mediated cholesterol efflux pathway plays a vital role in protecting against cardiovascular diseases, AC1 is a potential target that can be selectively activated to promote cholesterol clearance from foam cells, and reduce the risk of ischemic cardiovascular diseases should an AC1-specific agonist becomes available.

Findings from multiple approaches in this study support AC1 as the main isoform for apoA-1 mediated cholesterol efflux pathway in cholesterol-loading macrophage foam cells. We showed that the AC1 mRNA and protein levels are upregulated by both cholesterol and apoA-1. AC1 shRNA interference reduces apoA-1-stimulated cAMP production and cholesterol efflux from cells. As a result, the intracellular cholesterol levels remain higher, in comparison to non-shRNA and scrambled–shRNA transduced macrophages. In contrast, AC4 shRNA interference has no influence on apoA-1-mediated cAMP production, cholesterol efflux and intracellular cholesterol levels. We also observed formation of the cholesterol complex (protrusions) with apoA-1 on the cell membrane by confocal
microscopy with double labeling technique, as seen with immunogold electron microscopy (6, 8, 25). No cholesterol and apoA-1 complexes were observed on the cell surface in AC1 silenced macrophages. Taken together, we concluded that AC1 is the major isoform for cAMP production in the apoA-1-mediated cholesterol efflux pathway.

It is well known that cAMP signaling is compartmentalized by anchoring a specific adenylate cyclase and PKA in particular sites of cytoplasm through scaffold proteins, which results in activation of only a subset of downstream substrates in response to a specific receptor activation (33). In addition, compartmentalization of cAMP signaling is enforced by cyclic nucleotide phosphodiesterases (PDEs) that hydrolyses cAMP and limit its diffusion to nearby regions (34). PDE4 inhibitors rolipram and cilomilast potentiate apoA-1-mediated cholesterol efflux from THP-1 macrophages (35), suggesting that PDE4 is the isoform participating in compartmentalization of apoA-1-mediated AC1/cAMP signaling within THP-1 macrophages. These mechanisms explain why we only observe the formation of apoA-1 and cholesterol complexes in some areas of the cell surface, as apoA-1-triggered AC1/cAMP signaling is confined to a specific region to promote vesicle transport of cholesterol and exocytosis to the cell surface, resulting in the formation of lipid complexes with apoA-1 (18).

ADFP is a protein marker of cholesterol buildup in foam cells of atherosclerotic lesions (28, 29). ADFP levels, evaluated by qualitative and quantitative methods, remain high after AC1 shRNA interference, indicating intracellular cholesterol
accumulation, which is also consistent with the findings from direct measurement of the cellular cholesterol (Fig. 2E) and quantification of filipin fluorescence (Fig. 4, fluorescence intensity panel). Surprisingly, AC1 shRNA interference also completely changed the ADFP distribution pattern, from punctate appearance in cytoplasm to whole cell localization, including nuclear accumulation. This suggests that AC1 is also required for the cellular localization of ADFP, although its cellular functions remain to be determined in the future.

ABCA1 mutations are the cause of Tangier disease, a rare condition characterized by severe HDL deficiency, cholesterol accumulation in tissue macrophages and atherosclerosis, due to a defect in the apoA-1-mediated cholesterol efflux process (38-40). ABCA1 expression is upregulated by cAMP-increasing agents, including forskolin and prostaglandin (20, 35), cAMP-specific PDE inhibitors (20, 35), as well as cAMP analogs 8-bromo-cyclic AMP (20, 36, 37). Mouse cells have better responses to the mentioned agents than that of human cells, because the mouse ABCA1 genome has a strong cAMP-responsive enhancer located in the first intron, which increases ABCA1 expression in response to cAMP levels (38), whereas the human genome has a weak cAMP binding sequences at the −220 to −80 bp within the promoter region to increase ABCA1 expression (39). ABCA1 is phosphorylated by cAMP-activated PKA to modulate its activity(9, 10). In this study, we showed that apoA-1 increases ABCA1 expression in non-AC1 shRNA transduced cells and scrambled shRNA transduced macrophages. In the AC1 shRNA silenced macrophages, reduced
cAMP production would lead to decrease ABCA1 expression and apoA-1-mediated cholesterol efflux.

Functionally, several mechanisms have been proposed to explain how ABCA1 facilitates apoA-1-mediated cholesterol efflux from cells. ABCA1 is considered as an active transporter for cholesterol and phospholipids across the cell membrane since it shares a similar amino acid sequence with other ABC transporters (25, 30, 40). Along with others, we show that ABCA1 functions as a component of cholesterol transport vesicles (6, 31, 41). Phillips and colleagues propose that ABCA1 is a membrane lipid translocase that facilitates phospholipid and free cholesterol efflux to apoA-1 for HDL biogenesis (25, 42). Lipid raft is a membrane domain enriched with cholesterol, glycosphingolipid and signaling proteins, such as caveolin, a characteristic protein of caveolae that are considered a subset of the lipid raft (43, 44). Caveolin-1 modulates internalization and degradation of ABCA1, resulting in reduced cholesterol efflux (45, 46). Caveolin-1 knockout leads to higher levels of apoA-1-mediated cholesterol efflux in mouse embryonic fibroblasts (43, 44). In this context, it is worth determining if AC1/cAMP signaling could also affect the lipid raft and/or caveolin-1 expression to modulate apoA-1-mediated cholesterol efflux in macrophage foam cells.

In addition to adenylyl cyclase/cAMP/PKA signaling, HDL apolipoprotein-mediated cholesterol efflux pathway is known to involve many other signal proteins and kinases, as mentioned in the introduction. How does AC1/cAMP signaling affect other signaling proteins and kinases? Since both PKA and Epac
are immediate downstream effectors of cAMP, we are determining if agonists for PKA or Epac can replace apoA-1 in the promotion of cholesterol efflux from macrophage foam cells. Then, we would know if cAMP signaling transmits to a next step through PKA or Epac. This approach is similar to the previous studies using JNK activators anisomycin and hydrogen peroxide to substitute for apoA-1, to partly promote cholesterol efflux from cells(11). We will investigate the next target using a similar strategy to this study and so on, until the entire signaling pathway is outlined.

In summary, this study identified AC1 as the major enzyme for apoA-1-activated cAMP production in macrophage foam cells. The second messenger cAMP, generated by AC1, may activate its downstream effectors and promote the vesicle transport of cholesterol for exocytosis to the cell surface, forming cholesterol complexes that are then released to media as nascent HDL particles (18). This pathway would provide an effective mechanism to remove excess cellular lipids that are otherwise accumulated within the macrophages, and thus reduce the risk of cardiovascular diseases.
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### Table 1. QRT-PCR primer sequences

|   | Forward Primer Sequence | Reverse Primer Sequence |
|---|-------------------------|-------------------------|
| AC1 | 5' AGGATGAGAAACGAGAAGCAGGAG 3'  | 5' ACCCACGATGTCAGCAAAACAGGAT 3'  |
| AC4 | 5' CTTCCTCCTCTCTCCTCATCC 3'  | 5' GGCTATTCTCAGTCCTGGTCTG 3'  |
| GAPDH | 5' GTCTCCTCCTGACTTCAACAGCG 3'  | 5' ACCACCCCTGTGCTGCTGACCAA 3'  |
### Table 2. shRNA interference sequences

|    | Sequences                                                                 |
|----|--------------------------------------------------------------------------|
| AC1| 5'gatccGCTAGTATTCTGCATCTGTTCTCCTCGAGGAAGCAGATGCA
     | CAGAATACTAGCTTTTTTTg 3' (forward)                                        |
|    | 5'aattcAAAAAAAGCTAGTATTCTGCATCTGTTCTCCTCGAGGAAGCAGATGCAGAATACTAGCg 3' (reverse) |
| AC4| 5'gatccCCGGCCTACCTATCTGGTCATCGATCTCGAGATCGATG
     | ACCAGATAGGTAGGTTTTTT 3' (Forward)                                        |
|    | 5'aattcAAAAACCTACCTATCTGGTCATCGATCAAGGATCGATGAC
     | ACCAGATAGGTAGGTCGGCG 3' (Reverse)                                        |
| Scrambled| 5'gatccGTTCCTCCGAACGTGTCACGTTTTCAAGAGAAGCAGTGACAC
          | GTTCGGAGAACACTTTTTTACGCTg 3' (forward)                                   |
|    | 5'aattcACGCGTAAAAAGTTCTCCGAACGTGTCACGTTCTCTTTG
     | AAACGCTGACACGTTCGGAGAACg 3' (reverse)                                    |
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Figure 1. AC isoform expressions in THP-1 macrophages. THP-1 macrophages were prepared and total RNA was extracted for qRT-PCR as described in Materials and Methods. Relative mRNA levels for each
transmembrane AC isoform was shown (A). The symbols ** and *** indicate p<0.01 and P<0.001 respectively, compared to control. The symbol ## indicates p<0.01, compared to the ac-LDL loaded cells. AC1 protein levels determined by Western blotting under the same conditions, with GAPDH as a loading control (B). The symbol *** indicates p<0.001, compared to control and the symbol ## indicates p<0.01, compared to the ac-LDL loaded cells. The experiments were repeated four times. Labels: control(Con); ac-LDL (macrophages loaded with ac-LDL); and apoA-1 (macrophages loaded with ac-LDL and then incubated with apoA-1).
Figure 2. Effects of AC1 shRNA interference on cAMP levels and cholesterol efflux. All macrophages, except the control, were loaded with ac-LDL cholesterol and incubated with apoA-1 for the experiment. AC1 mRNA levels determined by qRT-PCR (A). The symbol *** represents P<0.001, compared to control. The symbol ### indicates P<0.001, compared to the A-1 and scrambled
shRNA groups. AC1 protein expression determined by Western blotting, with GAPDH as a loading control (B). The symbol ** represents \( P<0.01 \), compared to control. The symbol *** indicates \( P<0.001 \), compared to all. The cAMP levels in control and AC1 shRNA transduced cell groups (C). The symbol *** indicates \( P<0.001 \), compared to the A-1 and scrambled shRNA groups. No difference was seen between control and AC1 shRNA silenced cells. ApoA-1-mediated cholesterol efflux (D). The symbol *** represents \( P<0.001 \), compared to the A-1 and scrambled shRNA groups. No difference between control and AC1 shRNA silenced cells. The levels of intracellular cholesterol (E). The symbol ** indicates \( P<0.01 \), compared to the A-1 and scrambled shRNA groups. No difference between control and AC1 shRNA silenced cells. Labels: Con, A-1, AC1 and Scr represent control, apoA-1 incubation, AC1 shRNA and scrambled shRNA interfered macrophages, respectively. The experiments were repeated four times.
Figure 3. AC4 shRNA interference on cAMP production and cholesterol efflux. All macrophages, except the control, were loaded with ac-LDL cholesterol and incubated with apoA-1 for the experiment. AC4 mRNA levels (A). The symbol *** represents $P<0.001$, compared to control. The symbol #### represents $P<0.001$, compared to the A-1 and scrambled shRNA groups. AC4 protein
expression (B). GAPDH was used as loading control. The symbol *** represents P<0.001, compared to all other groups. The cAMP levels in AC4 shRNA transduced and other groups (C). The symbol *** indicates P<0.001, compared to control. No difference among the A-1, AC4 shRNA and scrambled shRNA groups. ApoA-1-mediated cholesterol efflux (D). Abbreviations: Con, A-1, AC4 and Scr represent control, apoA-1 treated alone, AC4 shRNA interference, and scrambled shRNA interfered cells, respectively. The experiments were repeated four times.
Figure 4. Localization of cholesterol and apoA-1 in macrophages observed by confocal microscopy. Macrophages were prepared in glass cover slips, and
all cells except the control were loaded with ac-LDL cholesterol for confocal microscopy study. Blue filipin fluorescence represented cellular cholesterol and red fluorescence indicated the apoA-1 binding to the cell surface. Control macrophage (Control panel). Macrophage loaded with ac-LDL cholesterol (Ac-LDL panel). Macrophage incubated with apoA-1(ApoA-1 panel). Red arrows indicated cholesterol and apoA-1 complexes (protrusions) on the cell surface in which cholesterol (blue fluorescence) was encircled by apoA-1 (red fluorescence). Macrophage with AC1 shRNA plus apoA-1 (AC1 shRNA + apoA-1). ApoA-1 was localized on relatively smooth cell surface without the cholesterol complex. Macrophage with scrambled shRNA plus apoA-1 (Scrambled shRNA + apoA-1). Cholesterol and apoA-1 complex were visible on the cell surface. Scale bar represented 5 µm in all images. The experiments were repeated three times and representative images were shown. Quantification of filipin fluorescence (fluorescence intensity panel). Each bar represents mean of the corrected cell filipin fluorescence from 80 to 100 cells from the three separate experiments. The symbol *** represents p< 0.001, compared to Control, ApoA-1 or Scrambled shRNA+ apoA-1.
Figure 5. AC1 shRNA interference on expression and cellular localizations of ADPF in macrophages observed with confocal microscopy. Macrophages,
except the control, were loaded with ac-LDL cholesterol for confocal microscopy study as in Figure 4. Red fluorescence represented ADFP while blue fluorescence indicated a nucleus stained by DAPI. Control macrophage (control panel). Macrophage loaded with ac-LDL cholesterol (Ac-LDL panel). Intense red fluorescence with punctate distribution in cytoplasm showed accumulation of intracellular cholesterol. Macrophage incubated with apoA-1 only (ApoA-1 panel). Macrophages with AC1 shRNA interference plus apoA-1 (AC1 shRNA + apoA-1). Macrophage with scrambled shRNA interference plus apoA-1 (Scrambled shRNA + apoA-1). Scale bar represented 5 µm in all images. The experiments were repeated three times and representative images were shown. Quantification of ADFP levels (fluorescence intensity panel). Each bar represents mean of the corrected cell fluorescence from 80 to 100 cells from three separate experiments. The symbol ** and *** represent p< 0.01 and p< 0.001 respectively, compared to Control, ApoA-1 or Scrambled shRNA+ apoA-1 group. The symbol ## represents P< 0.01, compared to Ac-LDL group.
Figure 6. Translocation of ABCA1 to the cell surface observed by confocal microscopy. Macrophages, except the control, were loaded with ac-LDL
cholesterol for confocal microscopy study as in Figures 4 and 5. Red fluorescence represents ABCA1 while blue fluorescence showed nucleus stained by DAPI. Control macrophage (Control panel). Macrophage loaded with ac-LDL (Ac-LDL panel). Macrophage incubated with apoA-1 only (ApoA-1 panel). Macrophages with AC1 shRNA interference and incubated with apoA-1 (AC1 shRNA + apoA-1 panel). Macrophage with scrambled shRNA interference and apoA-1 incubation (Scrambled shRNA + apoA-1 panel). Arrows indicated ABCA1 on the cell surface. Scale bar represented 5 µm in all images. The experiments were repeated three times and representative images were shown. Quantification of total cellular ABCA1 levels (Fluorescence intensity panel). Each bar represents mean of corrected cell fluorescence from 80 to 100 cells from three separate experiments. The symbol ** and *** represent p<0.01 and P<0.001 respectively, compared to Control, or AC1 shRNA+ apoA-1 group. The symbol ## represents P<0.01, compared to Ac-LDL group.