Abstract. – OBJECTIVES: ATP-binding cassette transporter A1 (ABCA1) plays a crucial role in reverse cholesterol transport and anti-atherosclerosis. Cyclic AMP (cAMP) could increase the ABCA1 expression. Angiotensin (Ang)-(1-7) can protect endothelial cells, inhibit smooth muscle cell growth, ameliorate inflammation and exert anti-atherosclerotic effects. In this study, we attempted to clarify the effect of Ang-(1-7) on expression of ABCA1, and explored the role of cAMP in the regulation of ABCA1 in RAW 264.7 macrophages.

MATERIALS AND METHODS: RAW 264.7 macrophages were cultured. Then the macrophages were incubated with different concentration Ang-(1-7) or 10 mM MDL respectively, or 10 mM adenylate cyclase inhibitor MDL-12330A (MDL) plus 1000 nM Ang-(1-7) for 24 h. The expression of ABCA1 was examined by real-time quantitative PCR and western blot analyses. cAMP expression was measured by Enzyme-Linked Immuno Sorbent Assay. Cellar cholesterol efflux from RAW 264.7 macrophages was analyzed using liquid scintillation counting assays. The cellular total cholesterol and free cholesterol were performed to determine by High Performance Liquid Chromatography assays.

RESULTS: Our results showed that Ang-(1-7) increased ABCA1 expression at both the mRNA and protein levels in a dose-dependent manner. Consequently, the increase in cholesterol efflux was consistent with an ABCA1 expression increase. The cAMP expression was up-regulated by Ang-(1-7). When being treated with MDL and Ang-(1-7), the ABCA1 expression, cellular cholesterol efflux and cholesterol content were partially reversed by MDL.

CONCLUSIONS: Ang-(1-7) could increase ABCA1 expression partially due to the cAMP pathway.

Key Words: Angiotensin-(1-7), ATP-binding cassette transporter A1, Cyclic AMP, Reverse cholesterol transport.

Introduction

It has been well known that foam cells formation when monocyte-derived macrophages swallowed atherogenic lipoproteins is one of the hallmarks of early atherosclerosis. Thus, it is becoming important in atherosclerosis therapy to find proper ways to prevent the formation of foam cells. There is increasing evidence to suggest that high density lipoprotein (HDL) can protect against atherosclerosis due to reverse cholesterol transport (RCT), a process that carries excess cholesterol from foam cells back to liver to remove from human bodies. The RCT pathway is currently considered to be the only mechanism by which the human body clears away excess cholesterol. Previous research showed that the RCT could be promoted by various kinds of mechanisms (e.g. targeted receptor-mediated process, passive aqueous diffusion). Among all of them, ATP-binding cassette (ABC) A1 which is member of ATP-binding cassette transporter superfamily was thought to be able to mediate the transfer of cellular cholesterol to lipid-poor apolipoprotein A-I (apoA-I), so it is the rate-limiting step in RCT. Cyclic AMP (cAMP) produced by adenylate cyclase (AC) as an intracellular second messenger regulates important cellular functions. Recent research showed that cAMP
plays a crucial role in increasing ABCA1 expression and regulating cellular lipid efflux\(^{8,9}\).

Ang-(1-7) which is a new member of renin-angiotensin system (RAS) can potentially help to protect endothelial cells, inhibit smooth muscle cell growth, ameliorate inflammation and exert anti-atherosclerotic effects\(^{10-14}\). Recently, we also found that Ang-(1-7) could ameliorate Ang II-induced apoptosis of human umbilical vein endothelial cells by suppressing lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1)\(^{15}\). However, whether Ang-(1-7) could affect the ABCA1 expression and ABCA1-dependent cholesterol efflux is still unknown, and whether cAMP pathway involves in this progress is unclear. Here in this study, we systematically discussed the effect of Ang-(1-7) on expression of ABCA1, and explored the role of cAMP in the regulation of ABCA1 in RAW 264.7 macrophages. Our results showed that Ang-(1-7) could increase the ABCA1 expression and cholesterol efflux through cAMP pathway to eventually promote RCT.

**Materials and Methods**

**Materials**

Murine RAW 264.7 macrophages line was purchased from Cell Bank of the Chinese Academy of Science (Shanghai, China). Ang-(1-7), apoA-I and adenylyl cyclase (AC) inhibitor MDL-12330A (MDL) were purchased from Sigma-Aldrich (St Louis, MO, USA); TRizol Reagent was from Invitrogen (Carlsbad, CA, USA), Rabbit anti-ABCA1 and β-actin-specific antibodies were obtained from Abcam (Cambridge, UK). BCA Protein Assay Kit was from Pierce Chemical (Rockford, IL, USA); cAMP enzyme immunoassay (EIA) kit was from Design Inc. (Ann Arbor, MI, USA).

**Cell Culture**

The RAW 264.7 macrophages were seeded in six-well plates at 1.0×10⁶ cells per well in Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% fetal bovine serum (FBS), 20 IU/mL penicillin, and 20 µg/mL streptomycin, and maintained at 37°C in an humidified atmosphere of 5% CO₂.

**RNA Isolation and Real-Time Quantitative PCR**

Total RNA from cells was extracted using TRizol reagent according to manufacturer protocol. The targeted genes and primer sequences are as follows: ABCA1: Forward primer 5’-AACAGTTTGTGCCCCTTTTGG-3’, Reverse primer 5’-AGTTCCAGCTGGGTACCTT-3’, β-actin: Forward primer 5’-CTTAGAAGCTTGGTGGA-3’, Reverse primer 5’-CTGCTAGCAGTCCCTGACCTG-3’. Real-time quantitative PCR, using SYBR Green detection chemistry, was performed on ABI 7300 Real-Time PCR System under the following conditions: 95°C denaturation for 2 minutes, followed by 35 cycles of 95°C for 30 seconds and 60°C for 30 seconds. RT-PCR was performed in a real-time PCR system (Applied Biosystems, Foster City, CA, USA). Quantitative measurements were determined using the △△Ct method and the expression of β-actin was used as the internal control.

**Western Blot Analyses**

The macrophages were harvested and protein extracts prepared as previously described\(^{16}\). They were, then, subjected to western blot analyses [10% SDS-polyacrylamide (SDS-PAGE); 30 μg protein per lane] using mouse anti-ABCA1 and β-actin-specific antibodies. Immunoreactivity was detected by the electrochemiluminescence (ECL) test.

**Cellular Cholesterol Efflux Experiments**

The cellular cholesterol efflux experiments were also performed as previously described\(^{17}\). The macrophages were cultured and labeled with 0.2 μCi/mL [3H] cholesterol for 72h. Cells were washed with phosphate-buffered saline (PBS) and incubated overnight in RPMI-1640 medium containing 0.1% (w/v) bovine serum albumin (BSA). Equilibrated [3H] cholesterol-labeled cells were washed with PBS and incubated in efflux medium containing RPMI-1640 medium and 0.1% BSA with 20 μg/mL human plasma apoA-I for 6h. Monolayers were washed twice in PBS, and cellular lipids were extracted with isopropanol. Medium and cell-associated [3H] cholesterol was measured by liquid scintillation counting. Percent efflux was calculated by the following equation: [total media counts / (total cellular counts + total media counts)] ×100%.

**High Performance Liquid Chromatography (HPLC) Assays**

HPLC analysis was conducted as previously described\(^{17}\). After washed with PBS for three times, cells were removed by scraping with a rubber and sonicated using an ultrasonic proces-
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ABC A1 is a key player in RCT and is important for regulating cellular cholesterol homeostasis. Because ABCA1 was up-regulated by Ang-(1-7), we next examined the effects of Ang-(1-7) on apoA-I specific cholesterol efflux and cholesterol content by liquid scintillation counting and HPLC in RAW 264.7 macrophages. As shown in Figure 1C and Table I, cholesterol efflux was increased while cellular cholesterol content was decreased when cells were treated with Ang-(1-7), suggesting that Ang-(1-7) increased ABCA1-mediated cholesterol efflux in RAW 264.7 macrophages.

cAMP is Involved in Ang-(1-7) up-regulation ABCA1 Expression in RAW 264.7 Macrophages

To confirm whether the cAMP expression can be affected by Ang-(1-7), ELISA was performed. The study revealed that Ang-(1-7) increased cAMP expression in a dose-dependent manner (Figure 2A).

In order to study whether Ang-(1-7) increases ABCA1 expression through cAMP pathway, we finally examined the effect of adenylyl cyclase (AC) inhibitor MDL on up-regulation of ABCA1 by Ang-(1-7). As shown in Figure 2B, 2C and 2D, treatment with MDL the expression of cAMP, ABCA1 mRNA and protein was significantly decreased. When together treatment with MDL and Ang-(1-7), the expression of cAMP and ABCA1 was decreased compared with Ang-(1-7) group, but still higher than control group. At the same time, cellular cholesterol efflux (Figure 2E) and cholesterol content (Table II) in cells treated by the combination of MDL and Ang-(1-7) were partially abolished compared with those treated by Ang-(1-7) alone.

Discussion

In the progression of atherosclerosis, a major factor is the differentiation of monocytes to macrophages that accumulate cholesterol to form foam cells in the vessel wall. HDL plays a key role in protecting against atherosclerosis mainly by RCT, a process that carries excess cholesterol from the arterial wall back to the liver for removal from the body. It has been demonstrated that ABCA1 plays key regulatory roles in RCT by mediating foam cells and other peripheral cellular free cholesterol efflux to apoA-I. So ABCA1 is an important target for the prevention and treatment of atherosclerosis.
Figure 1. The effects of Ang-(1-7) on ABCA1 expression and the cholesterol efflux. The RAW 264.7 macrophages were incubated with 10, 100 or 1000 nM Ang-(1-7) for 24h respectively. A, ABCA1 gene expression was measured by real-time quantitative PCR. B, ABCA1 protein expression was measured by western blot analyses. C, Cellular cholesterol efflux from RAW 264.7 macrophages was analyzed using liquid scintillation counting assays. Data are the mean ± SD of three independent experiments. *p < 0.05 compared with the control group.

Table I. The effect of Ang-(1-7) on cellular cholesterol content in RAW 264.7 macrophages.

<table>
<thead>
<tr>
<th>Ang-(1-7)</th>
<th>Control</th>
<th>10 nM</th>
<th>100 nM</th>
<th>1000 nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mg/dL)</td>
<td>421 ± 42</td>
<td>410 ± 36</td>
<td>298 ± 24*</td>
<td>223 ± 22*</td>
</tr>
<tr>
<td>FC (mg/dL)</td>
<td>166 ± 16</td>
<td>162 ± 15</td>
<td>122 ± 12*</td>
<td>98 ± 15*</td>
</tr>
<tr>
<td>CE (mg/dL)</td>
<td>255 ± 21</td>
<td>248 ± 22</td>
<td>176 ± 18*</td>
<td>125 ± 14*</td>
</tr>
<tr>
<td>CE/TC (%)</td>
<td>60.6</td>
<td>60.5</td>
<td>59.1</td>
<td>56.1</td>
</tr>
</tbody>
</table>

The RAW 264.7 macrophages were incubated with 10, 100 or 1000 nM Ang-(1-7) for 24h respectively. HPLC was performed to determine the cellular total cholesterol and free cholesterol. Data are the mean ± SD of three independent experiments. *p < 0.05 compared with the control group.
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Figure 2. cAMP is involved in Ang-(1-7) up-regulation of ABCA1 and ABCG1. A, The RAW 264.7 macrophages were incubated with 10, 100 or 1000 nM Ang-(1-7) for 24h respectively. cAMP expression was measured by ELISA. B-E, The RAW 264.7 macrophages were incubated with incubated with 10 mM MDL or 1000 nM Ang-(1-7) for 24h respectively, or 10 mM MDL plus 1000 nM Ang-(1-7) for 24h. B, cAMP expression was measured by ELISA. C, ABCA1 gene was measured by real-time quantitative PCR. D, ABCA1 protein expression was measured by western blot analyses. E, Cellular cholesterol efflux from RAW 264.7 macrophages was analyzed using liquid scintillation counting assays. Data are the mean ± SD of three independent experiments. *p < 0.05 compared with the control group; †p < 0.05 compared with the Ang-(1-7) group.
RAS which is a hormone system could help regulate blood pressure and fluid balance for human beings. As we all know, Ang II, one member of RAS, may be able to induce the formation of atherosclerosis. Ang-(1-7) which is a newly found member of RAS is an important biological role of angiotensin terminal active product family. Previous research showed that Ang-(1-7) which acted as an endogenous antagonist of Ang II could have regulation effects on cardiovascular system. Ang-(1-7) can help dilate blood vessels, lower blood pressure, and inhibit transference and proliferation of vascular smooth muscle cell via signaling pathways (e.g. Nitric Oxide/Cyclic GMP pathway, the calcineurin-NFAT (nuclear factor of activated T cells) signaling pathway, nuclear factor κB (NF-κB) signaling pathway)\textsuperscript{10-14}. Our previous study also found Ang-(1-7) can ameliorate Ang II-induced apoptosis of human umbilical vein endothelial cells by suppressing LOX-1\textsuperscript{15}. In this study, we systematically investigated how Ang-(1-7) affects the expression of ABCA1, and the related mechanism was also discussed.

In this work, we found that Ang-(1-7) treated cells exhibited a significant increase in ABCA1 expression at both mRNA and protein levels. Consequently, the increase in apoA-I-mediated cholesterol efflux was consistent with an ABCA1 expression increase in Ang-(1-7) treated cells. These findings suggest that Ang-(1-7) may have atheroprotective properties by increasing cholesterol efflux and reducing macrophage foam cell formation.

The second messenger cAMP is a crucial regulator of several important cellular functions, including the regulation of glycogen, sugar, and lipid metabolism. Its generation and degradation is respectively regulated by the AC from adenosine triphosphate and phosphodiesterase families of enzymes\textsuperscript{19,20}. Activation of cAMP signaling is induced by the binding of tracellular ligand to a G protein-coupled receptor. Previous reports suggested an important role of cAMP in increasing ABCA1 expression and cholesterol efflux\textsuperscript{8,9}. In our study, the data showed that the cAMP expression was up-regulated by Ang-(1-7). Then, in order to learn whether the regulation of Ang-(1-7) on ABCA1 is through cAMP pathway, we studied the effect of AC inhibitor MDL on the Ang-(1-7) regulation. Results showed that when being treated with MDL and Ang-(1-7), the ABCA1 expression was decreased compared with Ang-(1-7) group, however, it is still higher than control group. At the same time, cellular cholesterol efflux and cholesterol content were also partially reversed by MDL. The above results proved that the regulation of Ang-(1-7) on ABCA1 expression is partially via cAMP pathway.

### Conclusions

Our data indicate that Ang-(1-7) could increase ABCA1 expression and cholesterol efflux of RAW 264.7 macrophages partially due to the cAMP pathway. These findings provide a theoretical basis for the prevention and treatment of atherosclerosis.

### Acknowledgements

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### Conflict of Interest

The Authors declare that there are no conflicts of interest.
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References


