Available online at www.sciencedirect.com
SCIENCE DIRECT
211
JNMU
Journal of Nanjing Medical University, 2008, 22(4):211-216
www.elsevier.com/locate/jnmu
Research Paper

Inhibiting NF-κB increases cholesterol efflux from THP-1 derived-foam cells treated with AngII via up-regulating the expression of ATP-binding cassette transporter A1☆

Kun Liu[1], Yanfu Wang[1], Zhijian Chen*, Yuhua Liao, Xiang Gao, Jian Chen
Hua Zhong University of Science and Technology, Union Hospital, Institute of Cardiology, Wuhan430022, Hubei Province, China
Received 18 March, 2008

Abstract

Objective: To study the role of nuclear factor-kappa B(NF-κB) in cholesterol efflux from THP-1 derived-foam cells treated with Angiotensin Ⅱ(Ang Ⅱ). **Methods**:Cultured THP-1 derived-foam cells were treated with Ang Ⅱ or preincubated with tosyl-phenylalanine chloromethyl-ketone(TPCK) NF-κB inhibitor. The levels of activated NF-κB in the cells were examined by sandwich ELISA. Cellular cholesterol content was studied by electron microscopy scanning and zymochemistry via fluorospectrophotometer and cholesterol efflux was detected by scintillation counting technique. ABCA1 mRNA and protein were quantified by RT-PCR and Western blotting. **Results**:Addition of TPCK to the cells before Ang Ⅱ stimulation attenuated the response of NF-κB p65 nuclear translocation induced by Ang Ⅱ and showed no peak in foam cells group and caused a reduction in cholesterol content and an increase in cholesterol efflux by 24.1%($P < 0.05$) and 41.1%($P < 0.05$) respectively, when compared with Ang Ⅱ group. In accordance, the ABCA1 mRNA and protein were increased by 30% and 19%($P < 0.05$) respectively, when compared with Ang Ⅱ group. **Conclusion**:Ang Ⅱ can down-regulate ABCA1 in THP-1 derived-foam cells via NF-κB, which leads to less cholesterol efflux and the increase of cholesterol content with the consequence of the promotion of atherosclerosis.

Key words: Angiotensin Ⅱ; nuclear factor- kappa B; ATP-binding cassette transporter A1; cholesterol efflux; atherosclerosis

INTRODUCTION

Atherosclerosis is the fundamental pathogenesis of ischemic diseases e.g. coronary artery disease(CAD), cerebral infarction and peripheral arteriosclerosis obliterans. Foam cells abundant with cholesterol contribute greatly to the cause of atherosclerotic plaques. The cholesterol efflux from foam cells, protects against the forming of foam cells[1]. The regulation of cholesterol efflux would benefit the treatment of atherosclerosis and arouses more interest[1-3].

ABCA1, an ATP-binding cassette transporter, predominantly promotes cellular phospholipid and cholesterol efflux[4-6]. A series of studies show that the gene mutation of ABCA1 is responsible for Tangier disease(TD) characterized by cholesterol ester accumulation in foam cells resulting in atherosclerosis[7-9]. Thus ABCA1 plays a key role in cholesterol efflux from foam cells.

Since atherosclerosis is now considered as a chronic inflammation disease[10], we and others have subsequently reported that angiotensin Ⅱ(Ang Ⅱ) as well as other proinflammatory cytokines can decrease the expression of ABCA1 in cultured foam cells[11-15]. Yet it remains to be elucidated whether angiotensin Ⅱ shares similarities in the signal pathway of the downregulation of ABCA1 by atherogenic cytokines such as IL-1β TNF-α in which activated NF-κB is implicated[14,15]. The present study was undertaken to investigate the

☆ This work was supported by the National Basic Research and Development Program of China(973 Program, No.2007CB512000) (Sub-Project, No.2007CB512005)
*Correspondence author.
E-mail address: drchenok@sohu.com.
[1]These authors contributed equally to this work.

effects of inhibiting NF-κB on the expression of ABCA1 in THP-1 derived-foam cells treated with Ang Ⅱ.

MATERIALS AND METHODS

Reagents

10%(v/v) fetal calf serum(Gibco); RPMI1640 medium(Sigma); Goat polyclonal antibody against the NF-κB carboxy terminus of p65(Santa Cruz); Rabbit polyclonal antibody against the amino terminus of NF-κB p65(Santa Cruz); goat anti-rabbit IgG-HRP(Vector); SuperScript preamplification system(Takara); Taq polymerase(Takara); phenylmethyl sulfonylfluoride (Sigma); nitrocellulose membrane(Invitrogen); rabbit monoclonal antibody to human ABCA1(Santa Cruz).

Laboratory apparatus

Microtip of the sonifier(Branson Instruments Inc.); HITACHI 650-60 Spectrophotometer; Model 450 microplate reader(BIORAD).

LDL isolation and oxidization

LDLs were purified from human plasma obtained from healthy volunteers by two step ultracentrifugation. Human lipoproteins was isolated from nonfrozen human plasma. Total plasma, including VLDL, IDL and any residual chylomicrons/remnants(CM), and LDL (d<1.063) fractions were isolated by differential ultracentrifugation flotation, using potassium bromide. The purity of isolated LDL was confirmed by lipoprotein electrophoresis on an agarose gel. The isolated LDL was oxidized with $CuCl_2$(10 umol/L) for 24 h at 37℃. Oxidized-LDL(ox-LDL) was measured by the thiobarbituric acid-reactive substances assay.

THP-1 cell culture

Human THP-1 cells, a human monocytic leukemia cell line, were purchased from the Cell Bank of Wuhan University(Hubei Province, China). Monocytes were cultured in RPMI1640 medium and supplemented with 10%(v/v) fetal calf serum in a humidified atmosphere of 5% CO_2 at 37℃. Cells were initially differentiated into macrophages by addition of phorbol 12-myristate 13-acetate(PMA, 160 ng/ ml) for 72 h and then macrophages were transformed into foam cells by incubation with ox- LDL(50 μg/ml) in medium for 48 h. In our experiment, some cells were exposed to Ang Ⅱ (10^{-5} mM) as Ang Ⅱ group, some were preincubated with tosyl-phenylalanine chloromethyl-ketone(TPCK,10 μM) as TPCK group and the remainder treated without AngII or TPCK as a control group. Before assay, all these cells were incubated for an additional 12h in serum-free media containing 0.2% BSA/RPMI 1640 and apolipoprotein A-I (apoA-I, 10 μg/ml).

Cellular cholesterol content and cholesterol efflux analysis

After the cells were harvested and fixed, single foam cell was identified and the cellular cholesterol content was roughly observed by electron microscopy scanning. The cellular total cholesterol content was precisely measured by zymochemistry. Briefly, the cells were sonified using the microtip of the sonifier and the fluorescence of the sonicates reacted with assay solution for 60 min at 37℃(measured by HITACHI 650-60 Spectrophotometer, excitation, 325 nm; emission, 415 nm) to determine cholesterol content. Scintillation counting technique was applied in the measurement of the variance of cholesterol efflux by detecting the media and cellular[^{3}H] cholesterol (0.37×10^6 Bq/ml) after addition of apoA-I(10 μg/ml) for 12 h. The cholesterol efflux was expressed as the percentage effluxed(media counts/(media counts+cellular counts) × 100).

Sandwich ELISA for activated NF-κB

Cultured foam cells were treated for not more than 4h with PBS, Ang Ⅱ (1×10^{-5}mM) or TPCK(10 μM) for 2h before Ang Ⅱ (1×10^{-5}mM). The stimulation was stopped respectively at 0m, 30 min, 1 h, 2 h and 4 h and the cells were harvested. Nuclear proteins were extracted from the cells. NF-κB was measured by Sandwich ELISA method. Goat polyclonal antibody against the NF-κB carboxy terminus of p65(3 mg/ml, 100 ml/well) was coated overnight at 4℃ on microtiter plates. Then the plates were blocked with 1 % BSA overnight at 4℃. 100 ml of dilutions of nuclear proteins in a ratio of 1: 500 were added to the plates at 37℃ for 2 h. Rabbit polyclonal antibody against the amino terminus of NF-κB p65 was added in each well and incubated at 37℃ for 2 h. Then the plates were incubated with goat anti-rabbit IgG-HRP diluted 1 :1000 (100 ml/well) at 37℃ for 2 h. The plates were washed, and OPD 2 mM with 0.006% H_2O_2 in citric acid-phosphate buffer (100 ml/ well) was added. Then the plates were covered and incubated at 37℃ for 1 h. The reaction was stopped by adding citric acid 2M(50 ml/well). The optical density at 450 nm was read with a Model 450 microplate reader. Assays were done in triplicate wells.

RT-PCR for ABCA1

Oligonucleotide primers for ABCA1 were synthesized by Bio Basic Inc. according to the following sequences:5'-ACAACCAAACCTCACACTACTG-3' and 5'-ATAGATCCCATTACAGACAGCG-3'. The cDNA of ABCA1 was obtained by reverse transcription from the total RNA(2 μg) extracted from the cultured cells in a SuperScript preamplification system. The cDNA was amplified by polymerase chain reaction with primers as described for 38 cycles using the Taq polymerase to detect the gene expression of ABCA1.

Western-blotting analysis for ABCA1

Cell monolayers were lysed in a mixture of 2% Triton X-100, 1% protease inhibitor phenylmethyl sulfonylfluoride and 5 mM Tris-HCl buffer(pH 8.5). The cell lysate was homogenized on ice. Equal amounts of protein(60 μg) were separated on 6% bis-tris sodium dodecylsulfate-polyacrylamide(SDS-PAGE) gels and electrophoretically transferred to a nitrocellulose membrane and blocked in 5% skimmed milk for 2h at room temperature. Membranes were incubated with a primary rabbit monoclonal antibody to human ABCA1(1:200) for overnight at 4℃, and then with a secondary goat anti-rabbit IgG antibody(1:5000) conjugated to horseradish peroxidase for 1h at 37℃. ABCA1was visualized by enhanced chemiluminescence(ECL).

Statistical analysis

All values were presented as mean ± SD. Statistical analysis was performed with one-way ANOVA using SPSS 12.0 software. $P < 0.05$ was considered statistically significant.

RESULTS

Levels of activated NF-κB

In this experiment, the optical density at 450 nm represented the levels of activated NF-κB. The time course of activated NF-κB was observed. Ang Ⅱ stimulation caused a rapid increase of NF-κB p65 nuclear translocation and the peak of NF-κB p65 was 30-60 min in foam cells, but the addition of TPCK to the cells for 2 h before AngII stimulation attenuated the response of NF-κB p65 nuclear translocation induced by Ang Ⅱ and showed no peak in foam cells group(Fig. 1).

Inhibiting NF-κB lessened the formation of THP-1 derived-foam cells treated with Ang Ⅱ

The observation was made by electronic microscope that cellular lipid droplets appeared after macrophages transformed into foam cells. As shown in Fig. 2, cellular lipid droplets in foam cells preincubated with TPCK before Ang Ⅱ were less than those in foam cells only treated with Ang Ⅱ.

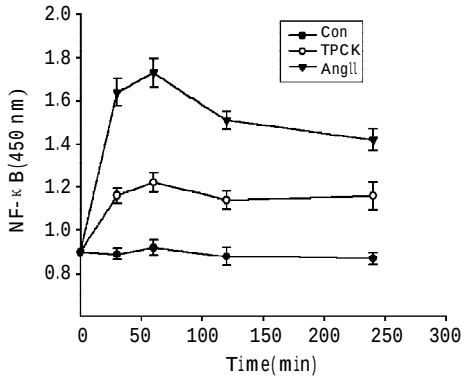
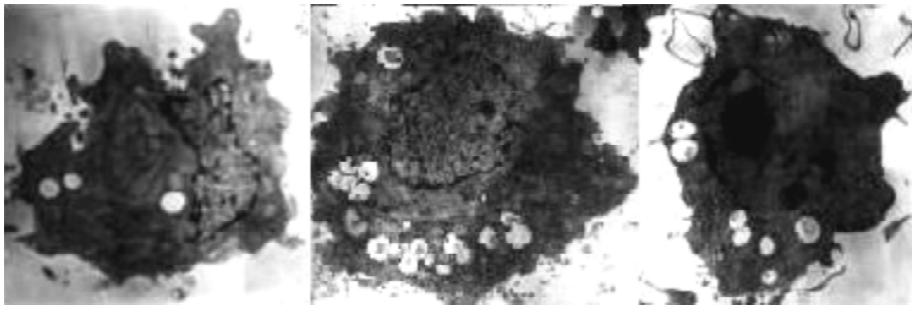


Fig. 1 NF-κB level curve in different groups



A:Foam cell group; B:Ang Ⅱ group; C: TPCK group

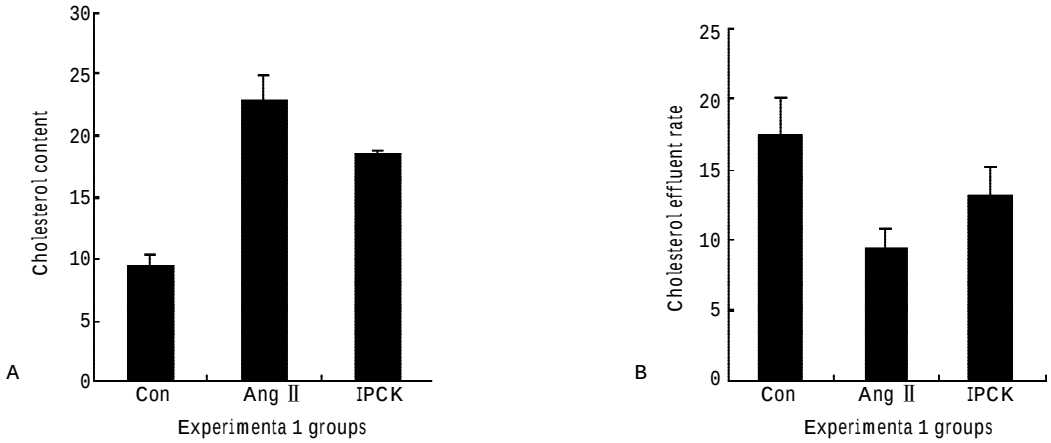
Fig. 2 Different experimental groups(× 80000)

Inhibiting NF-κB reduced cholesterol content and promoted cholesterol efflux from foam cells treated with Ang Ⅱ

To examine whether inhibiting NF-κB would have effects on cholesterol efflux from foam cells induced by Ang Ⅱ, cholesterol content and cholesterol effluent rate were observed. Treatment of THP-1 derived foam cell with Ang Ⅱ resulted in an increase in cholesterol content by 140.5%(22.03 ± 2.70 *vs.* control 9.16 ± 0.42,$P < 0.05$) and a reduction in cholesterol efflux by 46.4%(5.03 ± 0.47)% *vs.* control(13.14 ± 1.95)%,$P < 0.05$). In contrast, addition of TPCK before Ang Ⅱ caused a reduction in cholesterol content by 24.1% (16.72 ± 0.56 *vs.* Ang Ⅱ group 22.03 ± 2.70,$P < 0.05$) and an increase in cholesterol efflux by 41.1% (9.31 ± 1.40)% versus Ang Ⅱ group(5.03 ± 0.47)%, $P < 0.05$) (Fig. 3).

Inhibiting NF-κB up-regulated the expression of ABCA1 of foam cells treated with Ang Ⅱ

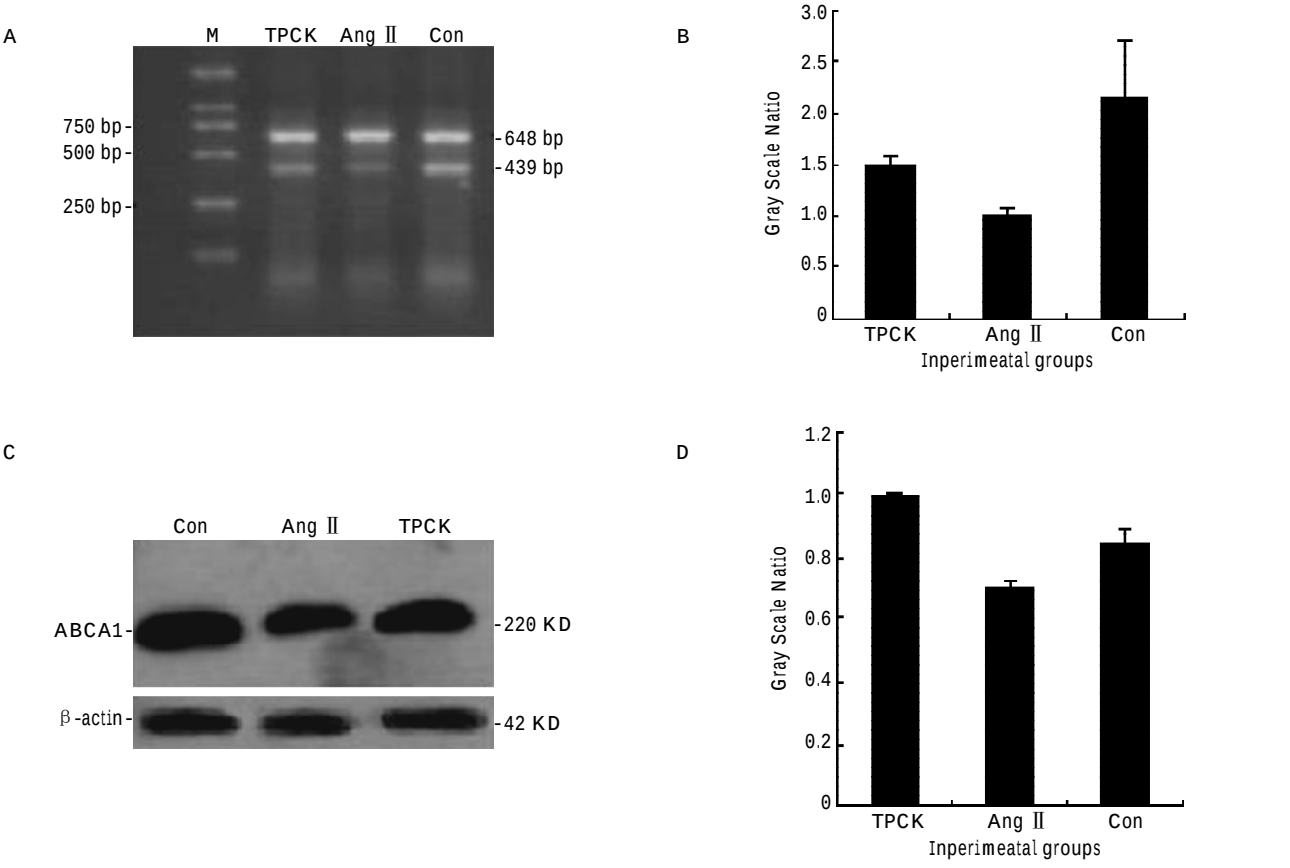
To confirm the effects of inhibiting NF-κB on cholesterol efflux from foam cells induced by AngII were correlated with the expression of ABCA1, ABCA1 mRNA and protein were quantified by RT-PCR and Western blotting. The expression of ABCA1 mRNA



A. Cholesterol content in different groups, Ang Ⅱ group vs. con group $P < 0.05$; TPCK group vs. Ang Ⅱ group $P < 0.05$; B:Cholesterol effluent rate in different groups, all $P < 0.05$ vs. Ang Ⅱ group.

Fig. 3 Cholesterol content and effluent rate in different groups($\bar{x} \pm s$, $n = 3$)

and protein reduced 41% and 30.4%($P < 0.05$) by Ang Ⅱ, as compared with control group. Addition of TPCK before Ang Ⅱ partially reversed these responses, the ABCA1 mRNA and protein were increased by 30% and 19%($P < 0.05$), respectively, as compared with Ang Ⅱ group(Fig. 4).



A:RT-PCR assay of mRNA expression of ABCA1, M: marker, TPCK: TPCK group, Ang Ⅱ: Angiotension Ⅱ group, Con:control group, GAPDH (MW 648 bp), ABCA1 mRNA(MW 439 bp); B:Gray scale ratio of ABCA1 mRNA expression product in different groups; TPCK group vs. Ang Ⅱ group, $P < 0.05$,Con group vs.Ang Ⅱ group, $P < 0.05$; Con group vs. TPCK group, $P < 0.05$; C:Western Blot electrophoregram of ABCA1(MW 220 KD); D:Laying gray scale value of the expression of ABCA1 protein in control group was 100%, 30.4% decreased in Ang Ⅱ group compared with control group; in TPCK group, 19% increased vs. Ang Ⅱ group, both $^{*}P < 0.05$ vs. Ang Ⅱ group.

Fig. 4 Expression of ABCA1 in different foam cell groups of THP-1($\bar{x} \pm s$, $n = 3$)

DISCUSSION

Emerging evidence demonstrates that the rennin-angiotensin system(RAS) is involved in the pathogenesis of atherosclerosis. HOPE(Heart Outcomes Prevention Evaluation) study indicates that the morbidity and mortality of CAD patients with normal blood pressure administered with angiotensin-converting enzyme inhibitor(ACE-Ⅰ) was remarkably lower than those without ACE-Ⅰ[16]. Correspondingly in animal models with atherosclerosis, ACE-I or ARB could attenuate atherosclerotic lesions[17,18]. In human coronary atherosclerotic plaques, AngⅡ, AT1 receptor, and ACE were expressed at strategic sites and RAS might contribute to inflammatory processes within the vascular wall and to the development of acute coronary syndromes[19]. These investigations reveal that AngⅡ also acts as a proinflammatory molecule powerfully promotes atherosclerosis.

AngⅡ-induced vascular damage are mediated by the activation of transcriptional factors as well as redox signaling systems and production of endogenous growth factors[20]. AS a key nuclear transcriptional factor is involved in atherosclerosis, NF-κB, which plays an important role in the regulation of a variety of gene involved in the inflammatory response(and has been linked to the onset of atherosclerosis after its activation)[21,22] can be activated by AngⅡ. In vascular smooth muscles, AngⅡ activates NF-κB via the nuclear translocation of p65, one NF-κB dimer, to promote vascular lesions[23,24]. To our knowledge, our study first showed that AngII activated NF-κB in foam cells and inhibiting NF-κB in foam cells induced by AngII could markedly cause an increase in cholesterol efflux and a reduction in cholesterol content.

Our previous study has demonstrated that AngⅡ can decrease the expression of ABCA1 in cultured foam cells[12], a receptor of apolipoprotein A-Ⅰ (apoA-Ⅰ). The interaction between ABCA1 and apoA-Ⅰ helps cholesterol efflux from foam cells and high density lipoprotein (HDL)-mediated reverse cholesterol transport. In transgenic mice, ABCA1 overexpression resulted in a significant increase in cholesterol efflux and marked elevation in HDL-cholesterol levels, which greatly lessened atherosclerotic lesions[25,26]. So ABCA1 and its relations have promising potentials for atherosclerotic therapy. ABCA1 was down-regulated by an inhibitor of NF-κB, TPCK, in RAW cells treated with lipopolysaccharide[27]. However, it was up-regulated by the inhibition of NF-κB via the overexpression of IkBa, one upstream regulator, in foam cells[28]. The results presented in this study indicated that inhibiting NF-κB in THP-1 derived-foam cells partially and markedly suppressed the downregulation of ABCA1 by AngII, which demonstrated that AngⅡ could down-regulate ABCA1 in foam cells via NF-κB. As reported previously, some atherogenic cytokines such as IFN-γ IL-1β TNF-α also down-regulated ABCA1 in cultured foam cells via NF-κB[13-15]. Based on that, it is our opinion that AngⅡ as well as other proinflammation cytokines can down-regulate ABCA1 in foam cells via NF-κB. Therefore it may be pivotal and beneficial in regulating NF-κB to increase the expression of ABCA1, which in turn leads to the promotion of cholesterol efflux from foam cells.

Referrences

[1] Pennings M, Meurs I, Dan Y, Out R, Hoekstra M, Vanberkel T, *et al*. Regulation of cholesterol homeostasis in macrophages and consequences for atherosclerotic lesion development. *FEBS Lett* 2006; 580: 5588-96.

[2] Wang X, Rader DJ. Molecular regulation of macrophage reverse cholesterol transport. *Curr Opin Cardiol* 2007; 22: 368-72.

[3] Williams KJ, Feig JE, Fisher EA. Cellular and molecular mechanisms for rapid regression of atherosclerosis: from bench top to potentially achievable clinical goal. *Curr Opin Lipidol* 2007; 18: 443-50.

[4] Oram JF, Lawn RM. ABCA1: The gatekeeper for eliminating excess tissue cholesterol. J *Lipid Res* 2001; 42: 1173-9.

[5] Schmitz G, Langmann T. Structure, function, and regulation of the ABC1 gene product. *Curr Opin Lipidol* 2001; 12: 129-40.

[6] Santamarina-Fojo S, Peterson K, Knapper C, Qiu Y, Freeman L, Cheng JF, *et al*. Complete genomic sequence of the human ABCA1: analysis of the human and mouse ATP-binding cassatte A promoter. *Proc Natl Acad Sci* 2000;97: 7987-92

[7] Bodzioch M, Orsó E, Klucken J, Langmann T, Böttcher A, Diederich W, *et al*. The gene encoding ATP binding cassette transporter 1 is mutated in Tangier disease. *Nat Genet* 1999; 22: 347-51.

[8] Mott S, Yu L, Marcil M, Boucher B, Rondeau C, Genest J. Decreased cellular cholesterol efflux is a common cause of familial hypoalphalipoproteinemia: role of the ABCA1 gene mutations. *Atherosclerosis* 2000; 152:457-68.

[9] Oram JF. Tangier disease and ABCA1. *Biochim Biophys Acta* 2000; 1529: 321-30.

[10] Libby P. Inflammation in atherosclerosis. *Nature* 2002; 420:868-74.

[11] Takata Y, Chu V, Collins AR, Lyon CJ, Wang W, Blaschke F, *et al*. Transcriptional repression of ATP-binding cassette transporter A1 gene in macrophages: a novel atherosclerotic effect of angiotensin Ⅱ. *Circ Res* 2005; 97: e88-96.

[12] Wang Y, Chen Z, Liao Y, Mei C, Peng H, Wang M, *et al*. Angiotensin Ⅱ increases the cholesterol content of foam cells via down-regulating the expression of ATP-binding cassette transporter A1. *Biochem Biophys Res Commun* 2007; 353:650-4.

[13] Alfaro Leon ML, Evans GF, Farmen MW, Zuckerman SH. Post-transcriptional regulation of macrophage ABCA1, an early response gene to IFN-γ. *Biochem Biophys Res Commun* 2005; 333:596-602.

[14] Chen M, Li W, Wang N, Zhu Y, Wang X. ROS and NF-κB but not LXR mediate interleukin-1β signaling for the downregulation of ATP-binding cassette transporter A1. *Am J Physiol Cell Physiol* 2007; 292:C1 493- 501.

[15] Mei C, Chen Z, Liao Y, Wang Y, Peng H, Chen Y.Interleukin-10 inhibits the downregulation of ATP binding cassette transporter A1 by tumour necrosis factor-alpha in THP-1 macrophage-derived foam cells. *Cell Biol Int* 2007;31:1456-61.

[16] Yusuf S, Sleight P, Pogue J, Bosch J, Davies R, Dagenais G. Effects of an angiotensin-converting enzyme inhibitor, ramipril, on cardiovascular events in high risk patients: the Heart Outcomes Prevention Evaluation Study investigators. *N Engl J Med* 2000; l342:145-53.

[17] Ferrario CM. Use of angiotensin Ⅱ receptor blockers in animal models of atherosclerosis. *Am J Hypertens* 2002; 15: S 9-13.

[18] Claridge MW, Hobbs SD, Quick CR, Day NE, Bradbury AW, Wilmink AB. ACE inhibitors increase type Ⅲ collagen synthesis: a potential explanation for reduction in acute vascular events by ACE inhibitors. *Eur J Vasc Endovasc Surg* 2004; 28:67-70.

[19] Schieffer B, Schieffer E, Hilfiker-Kleiner D, Hilfiker A, Kovanen PT, Kaartinen M, *et al*. Expression of angiotensin Ⅱ and interleukin 6 in human coronary atherosclerotic plaques: potential implications for inflammation and plaque instability. *Circulation* 2000;101:1372-8.

[20] Ruiz-Ortega M, Lorenzo O, Rupérez M, Esteban V, Suzuki Y, Mezzano S, *et al*. Role of the renin-angiotensin system in vasucular diseases: expanding the filed. *Hypertension* 2001;38:1382-7.

[21] de Winther MP, Kanters E, Kraal G, Hofker MH. Nuclear factor-κB signaling in atherogenesis. *Arterioscler Thromb Vasc Biol* 2005; 25:904-14.

[22] Van Oostrom AJ, van Wijk J, Cabezas MC. Lipaemia, inflammation and atherosclerosis: novel opportunities in the understanding and treatment of atherosclerosis. *Drugs* 2004; 64:19-41.

[23] Ruiz-Ortega M, Lorenzo O, Rupérez M, Suzuki Y, Egido J. Angiotensin Ⅱ activates nuclear transcription factor-κB in aorta of normal rats and in vascular smooth muscle cells of AT1 konckout mice. *Nephrol Dial Transplant* 2001;16[suppl 1]: 27-33.

[24] Zhang L, Cheng J, Ma Y, Thomas W, Zhang J, Du J. Dual pathways for nuclear factor-κB activation by angiotensin Ⅱ in vascular smooth muscle phosphorylation of p65 by IκB kinase and ribosomal Kinase. *Circ Res* 2005;97:975-82.

[25] Singaraja RR, Bocher V, James ER, Clee SM, Zhang LH, Leavitt BR, *et al*. Human ABCA1 BAC transgenic mice show increased high density lipoprotein cholesterol and apoA-Ⅰ dependent efflux stimulated by an internal promoter containing liver X receptor response element in intron1. *J Biol Chem* 2001;276: 33969-79.

[26] Vaisman BL, Lambert G, Amar M, Joyce C, Ito T, Shamburek RD, *et al*. ABCA 1 overexpression leads to hyperalpha lipoproteinemia and increased biliary cholesterol excretion in transgenic mice. *J Clin Res* 2001;108: 303-9.

[27] Baranova I, Vishnyakova T, Bocharov A, Chen Z, Remaley AT, Stonik J, *et al*. Lipopolysaccharide down-regulates both scavenger receptor B1 and ATP binding cassette transporter A1 in RAW cells. *Infection and Immunity* 2002;70:2995-3003.

[28] Ferreira V, van Dijk KW, Groen AK, Vos RM, van der Kaa J, Gijbels MJ, *et al*. Macrophage-specific inhibition of NF-κB activation reduces foam-cell formation. *Atherosclerosis* 2007;192:283-90.