Original Article
Regulation of macrophage cholesterol efflux and liver X receptor α activation by nicotine

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Abstract: Objective: This study aims to investigate the characteristics of liver X receptor α (LXRα) and its target gene expression, as well as cholesterol efflux in human macrophages treated by nicotine. Methods: Human monocyte-derived macrophages were collected. Before apoA-l-mediated human monocyte-derived macrophage cholesterol efflux, and mRNA expression of LXRα, and some of its target genes being detected, the macrophages were induced with or without nicotine. Results: Pre-incubation of Human monocyte-derived macrophages with nicotine, cholesterol efflux was suppressed to apolipoprotein AI. Nicotine also inhibited LXRαand some of its target genes mRNA expression involved cholesterol metabolism, and facilitated some inflammatory genes expression. Conclusion: The changed function of cholesterol efflux and some genes expression may be the pathogenetic cause, and LXR activity of macrophage may offer potential therapeutic benefit in the treatment of atherosclerosis. Thus nicotine can regulate foam cell formation by inhibiting LXR pathway.

Keywords: Nicotine, cholesterol efflux, human monocyte-derived macrophage, liver X receptor

Introduction

Early atherosclerotic lesion was the aggregation of macrophage rich in cholesterol under vascular endothelium. Macrophages changed into foam cells in the artery wall under various complicated mechanisms, thus started the atherosclerotic lesion. In the internal macrophage, liver X receptor (LXR) and the signal induced by LXR were important molecular basis for its lipid metabolism, and was also an important link of atherosclerosis induced by macrophages [1].

Nicotine was considered to be an important component in tobacco which causes atherosclerosis. However, the mechanism was still poorly understood. Lau et al [2] found that in LDLR^{-/-} model, local lesions in arterial wall increased significantly after treatment by nicotine, the expression of nuclear factor κB (NF-κB) upregulated, which suggested the enhanced inflammation. So nicotine induced atherosclerosis may be related with the inflammatory effect. In this study, we explored the effect of nicotine on LXR signal pathway and macrophage cholesterol efflux.

Materials and methods

Isolation of monocytes from peripheral blood of normal human volunteers

Human monocytes were obtained from peripheral blood of normal human volunteers through Ficoll density gradient centrifugation. The monocytes were diluted in RPMI 1640 medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) and seeded in 6-well plates at a density of 1×10^7 cells/mL. The monocytes were incubated at 37°C with 5% CO_2 for 2 hours. Adherent monocytes with the purity of more than 90% and the viability of more than 95% could then be harvested for further experimentation.

Transformation of macrophages

According to reference [3], the monocytes at a density of 3.0×10^6/mL were cultured in 6-well plates with RPMI 1640 medium containing 10% FBS, 100 u/mL penicillin and 100 µg/mL streptomycin and 3.2×10^{-7} mol/L phorbol 12-myri-

Table 1. Primers Used for RT-PCR

Gene	Primers (5'-3')	Product (bp)
LXRα	F: CTTCTGGAGACATCTCGGAGGT	410
	R: CTGATAGCAATGAGCAAGGCAA	
ABCA1	F: ATAAGCCCTCTATACATAAATGCC	470
	R: ACAGCGTAAAGTGCTTGGAATG	
SREBP2	F: ATAGGTGGCAGGGCAGAAAC	300
	R: AATCAAGACGCTACAGCAACTCA	
CETP	F: AAGATGCCCAAGATCTCCTG	445
	R: AAGCTCTGGAGGAAATCCAC	
PLTP	F: CTGCGAGAGGTGATTGAGAAGA	330
	R: CAGGCTATGAATGTGGGAAAAG	
apoE	F: GCGGATGGAGGAGATGGG	304
	R: AGGCAGGAGGCACGGGGT	
MMP-9	F: CTTCCAGTACCGAGAGAAAGCC	200
	R: CAAAGGTGAGAAGAGAGGGCC	
MIP-1α	F: CGCCTGCTGCTTCAGCTACAC	200
	R: TGTGGAGGTCACACGCATGTT	
GAPDH	F: CCCATGTTCGTCATGGGTGT	140
	R: TGGTCATGAGTCCTTCCACGATA	

ABCA1: ATP binding cassette A1; SREBP2: sterol regulatory element binding protein; PLTP: phospholipid transfer protein; CETP: cholesterol ester transfer protein.

Table 2. Effect of nicotine on apoA-I mediated cholesterol efflux in macrophages (%, $\overline{x} \pm s$, n=20)

	Group	0 (ng/mL)	100 (ng/mL)
Without	TO-901317	15.21±1.32	10.33±1.14▲
With	TO-901317	27.74±2.59△	21.91±1.57△, ▲

△$P<0.01$, *vs.* groups without TO-901317; ▲$P<0.05$, *vs.* groups without nicotine.

state 13-acetate (PMA, Alexis Biochemicals, Lausen, Switzerland) at 37°C with 5% CO_2 for 24 hours.

Determination of cholesterol efflux

The cholesterol efflux was determined as previously described [4]. The cells at a density of 3.0×10^6/mL were cultured in 6-well plates with RPMI 1640 medium containing 10% FBS, 100 u/mL penicillin and 100 μg/mL streptomycin and 0.2 μCi/L [3H] cholesterol (40-60 Ci/mmol; NEN, Boston, MA) for 48 h. Then they were washed with PBS and re-cultured in 6-well plates at a density of 3.0×10^6/mL. They were divided into four groups: 0 ng/ml nicotine with 2 μmol/L of TO-901317 group, 0 ng/ml nicotine without 2 μmol/L TO-901317 group,

100 ng/ml nicotine with 2 μmol/L TO-901317 group and 100 ng/ml nicotine without 2 μmol/L TO-901317 group. After culture for 24 h, the cells were washed with PBS and cultured with RPMI 1640 medium containing 10 μg/L apoA-I (Academy Bio-medical, Houston, TX) without FBS for 12 h. [3H] Cholesterol was determined in the efflux media and cells by scintillation counting. Cholesterol efflux (%) was calculated as CPM (counts/min) in the efflux media divided by total CPM (media plus cells) and multiplied by 100%.

Reverse transcription polymerase chain reactions (RT-PCR)

Total RNA was extracted from cells of each group by using RNAeasy kits from Qiagen (Valencia, CA). The TaqMan one-step RT-PCR Master Mix Reagent Kit was used to determine relative expression levels of mRNA genes using the ABI Prism 7700 sequence detection system (Applied Biosystems) according to the manufacturer's instructions. Primer sequences used in this study were shown in **Table 1**. PCR reaction conditions were 94°C, 5 min; 35 cycles of 94°C, 1 min; 55°C, 1 min and 72°C, 1 min; then 72°C, 10 min. The expression levels were normalized using GAPDH as control.

Data analysis

SPSS 16.0 statistical software was used for statistical analysis. Measurement data are presented as mean ± standard deviation (SD). Differences between two groups were evaluated by analysis of variance (ANOVA) and t-test. $P<0.05$ was considered statistical significance.

Results

Effects of nicotine on cholesterol efflux in macrophages

In this study, we found that cholesterol efflux mediated by apoA-I in macrophages obtained from human peripheral blood was decreased under 100 ng/mL nicotine. LXR activated by TO-901317 in macrophages promoted cholesterol efflux but 100 ng/mL nicotine could inhibit it (**Table 2**).

Effects of nicotine on the expression of LXRα and its downstream genes

The expression levels of LXRα, ATP binding cassette A1 (ABCA1), sterol regulatory element
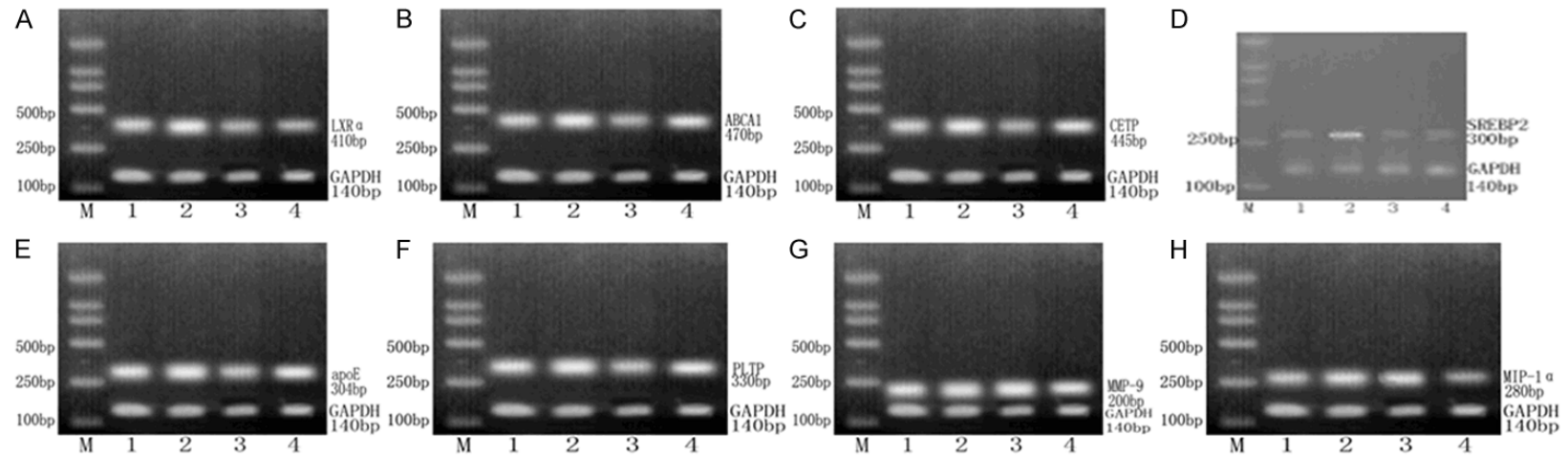
Figure 1. Gel electrophoresis map for PCR results of LXRα and its target genes in macrophage. M: DNA marker; 1: without TO-901317 and nicotine; 2: with TO-901317 and without nicotine; 3: nicotine 100 ng/ml without TO-901317; 4: nicotine 100 ng/ml with TO-901317; A: LXRα; B: ABCA1; C: CETP; D: SREBP2; E: apoE; F: PLTP; G: MMP-9; H: MIP-1α.

Table 3. Effect of nicotine on LXRα and its target gene expression in macrophages (%, $\bar{x} \pm s$, n=20)

Genes	0 ng/mL nicotine without TO-901317	0 ng/mL nicotine with TO-901317	100 ng/mL nicotine without TO-901317	100 ng/mL nicotine with TO-901317
LXRα	29.33±2.18	41.27±2.58*	18.12±3.29△	20.36±2.74★
ABCA1	32.78±2.43	48.69±3.34*	17.48±2.52△	29.37±1.78*,★
SREBP2	18.63±2.31	34.12±2.75*	10.73±2.45▲	23.26±1.94*,★
CETP	28.09±1.57	43.57±2.73*	13.75±1.32△	25.48±2.37*,★
PLTP	36.85±2.54	51.23±2.71*	21.93±2.47△	32.04±2.84*,★
apoE	34.49±2.59	52.78±3.81*	16.47±2.35△	28.14±2.73*,★
MMP-9	29.31±2.54	20.19±2.19*	41.38±2.14▲	30.63±2.23*,★
MIP-1α	21.18±1.78	34.07±2.75*	40.92±2.79△	19.14±2.04*,★

*$P<0.01$, *vs.* group without TO-901317 between similar saturation of nicotine; △$P<0.01$, ▲$P<0.05$ *vs.* group 0 ng/mL nicotine without TO-901317; ★$P<0.01$ *vs.* group 0 ng/mL nicotine without TO-901317.

binding protein (SREBP2), phospholipid transfer protein (PLTP), apoE and cholesterol ester transfer protein (CETP) were down-regulated under nicotine; the expression levels of ABCA1, SREBP2, PLTP, apoE and CETP were up-regulated under TO-901317. Nicotine could increase the expression levels of MMP-9 and MIP-1α while TO-901317 could decrease them (**Figure 1**; **Table 3**).

Discussion

The characteristics of atherosclerotic lesions are the changes of lipid metabolism in arterial wall resulting in chronic inflammation of vascular wall. Circulating monocytes entered the vascular endothelial space and differentiated into macrophages. These macrophages accumulated cholesteryl ester and transformed to foam cells when oxidized low density lipoprotein (oxLDL) existed which lead to the formation of atherosclerosis.

LXR belongs to the nuclear receptor superfamily of ligand activated transcription factor which including LXRα (NR1H3), LXR and retinoic acid receptor (RXR) heteromers. Some metabolites of cholesterol were their endogenous activation ligand, the synthetic GW3965 and TO901317 were their specific activators. LXR regulated the expression of target gene by directly binding the LXR response element (LXRE). It promoted or inhibited the expression of target gene through interaction with co-stimulatory factor or co-repressor. Recent studies showed that many genes involved in lipid metabolism balance, inflammatory and immune responses were regulated by LXR [1]. In macrophages, LXR regulated the balance of macrophage lipid

metabolism after activating by endogenous ligand. At the same time, the activation of LXR can inhibit the expression of some proinflammatory genes [6]. In this study we found that in macrophages cultured in vitro the activated LXR could up-regulate the expression of genes involved in lipid metabolism such as ABCA1, SREBP2, PLTP, apoE and CETP, it also could promote macrophage cholesterol efflux. On the contrary, the expression of genes involved in inflammatory reaction such as MMP-9 and MIP-1α were down-regulated. When the signal regulating cholesterol balance failed in macrophages it could lead to the formation of foam cells [7]. The activation of inflammatory cytokines in macrophages was the direct cause of the formation of foam cells and artery atheromatous lesions.

It is widely recognized by people that smoking is a risk factor of atherosclerosis. Nicotine is the main harmful component. In general, the physiological roles of nicotine were mediated by binding with its nicotinic acetylcholine (nACh) receptor. Heeschen reported that nicotine promoted the progress of atherosclerotic plaque lesions which leading to cardiac ischemic events occurred [8]. Aicher found that nicotine activated antigen-presenting cells in lesions and mainly activated several kinases, such as ERK1/2, p38MAPK and Akt. The activation of these cell kinases caused antigen presenting cells to activate innate or acquired immune function and secreted a large number of inflammatory cytokines involved in the immune and inflammatory responses, and leaded to the progression of atherosclerosis eventually [9]. Lau [2] found that nicotine leaded to the atheroscle-

rosis depended on promoting the secretion of inflammatory cytokines in macrophages. The mechanism maybe nicotine directly induced the expression of iNOS and TNF-α in monocytes and macrophages through nACh receptors. NF-κB could be activated by activated macrophages and nicotine could activate VCAM-1, COX-2 and PDGFβ after stimulating the target genes of NF-κB. These bioactive substances played important roles in the infiltration of inflammatory cells, migration and proliferation of smooth muscle cells, promoted the progress of atherosclerotic plaque lesions [5]. There were few studies on the effect of nicotine on lipid metabolism. It was reported that nicotine could inhibit the activity of CETP and PLTP [10].

In conclusion, in this study we found that nicotine could inhibit the LXR signaling pathway, affect the cholesterol efflux of macrophages and promote inflammation through LXR pathway, which may be one aspect of smoking can cause atherosclerosis.

Acknowledgements

Disclosure of conflict of interest

None.

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References

[1] Levin N, Bischoff ED, Daige CL, Thomas D, Vu CT, Heyman RA, Tangirala RK and Schulman IG. Macrophage liver X receptor is required for antiatherogenic activity of LXR agonists. Arterioscler Thromb Vasc Biol 2005; 25: 135-142.

[2] Lau PP, Li L, Merched AJ, Zhang AL, Ko KW and Chan L. Nicotine induces proinflammatory responses in macrophages and the aorta leading to acceleration of atherosclerosis in low-density lipoprotein receptor (-/-) mice. Arterioscler Thromb Vasc Biol 2006; 26: 143-149.

[3] Suzuki S, Nishimaki-Mogami T, Tamehiro N, Inoue K, Arakawa R, Abe-Dohmae S, Tanaka AR, Ueda K and Yokoyama S. Verapamil increases the apolipoprotein-mediated release of cellular cholesterol by induction of ABCA1 expression via Liver X receptor-independent mechanism. Arterioscler Thromb Vasc Biol 2004; 24: 519-525.

[4] Lin GR and Bornfeldt KE. Cyclic AMP-specific phosphodiesterase 4 inhibitors promote ABC-A1 expression and cholesterol efflux. Biochem Biophys Res Commun 2002; 290: 663-669.

[5] Zhang SL, Day I and Ye S. Nicotine induced changes in gene expression by human coronary artery endothelial cells. Atherosclerosis 2001; 154: 277-283.

[6] Ricote M, Valledor AF and Glass CK. Decoding transcriptional programs regulated by PPARs and LXRs in the macrophage: effects on lipid homeostasis, inflammation, and atherosclerosis. Arterioscler Thromb Vasc Biol 2004; 24: 230-239.

[7] Von EA, Nofer JR and Assmann G. High density lipoproteins and arteriosclerosis. Role of cholesterol efflux and reverse cholesterol transport. Arterioscler Thromb Vasc Biol 2001; 21: 13-27.

[8] Heeschen C, Jang JJ, Weis M, Pathak A, Kaji S, Hu RS, Tsao PS, Johnson FL and Cooke JP. Nicotine stimulates angiogenesis and promotes tumor growth and atherosclerosis. Nat Med 2001; 7: 833-839.

[9] Aicher A, Heeschen C, Mohaupt M, Cooke JP, Zeiher AM and Dimmeler S. Nicotine strongly activates dendritic cell-mediated adaptive immunity: potential role for progression of atherosclerotic lesions. Circulation 2003; 107: 604-611.

[10] Zhu PT, Bo P and Sun Y. Study on the correlation between Chinese medical syndrome types and serum levels of PLTP and CETP in coronary heart disease patients. Zhongguo Zhong Xi Yi Jie He Za Zhi 2011; 31: 749-752.