Fibroblast growth factor 21 enhances cholesterol efflux in THP-1 macrophage-derived foam cells

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Abstract. Fibroblast growth factor 21 (FGF21) is a novel metabolic regulator. The present study aimed to investigate the effect of FGF21 on cholesterol efflux and the expression of ATP binding cassette (ABC) A1 and G1 in human THP-1 macrophage-derived foam cells. Furthermore, the present study aimed to investigate the role of the liver X receptor (LXR) α in this process. A model of oxidized low-density lipoprotein-induced foam cells from human THP-1 cells was established. The effect of FGF21 on cholesterol efflux was analyzed using a liquid scintillation counter. The expression of ABCA1 and ABCG1 was determined using quantitative polymerase chain reaction and western blot analyses. FGF21 was found to enhance apolipoprotein A1- and high-density lipoprotein-mediated cholesterol efflux. FGF21 was also observed to increase the mRNA and protein expression of ABCA1 and ABCG1. Furthermore, LXRα-short interfering RNA attenuated the stimulatory effects induced by FGF21. These findings suggest that FGF21 may have a protective effect against atherosclerosis by enhancing cholesterol efflux through the induction of LXRα-dependent ABCA1 and ABCG1 expression.

Introduction

Atherosclerosis, one of the leading causes of morbidity and mortality worldwide, is a chronic inflammatory disease and a disorder of lipid metabolism (1). The accumulation of excess cholesterol has been recognized as a crucial event in the development of atherosclerosis (2); therefore, preventing or reversing cholesterol accumulation may be effective protective strategies against atherosclerosis. A growing body of evidence suggests that high density lipoprotein (HDL) has an important role in the removal of cholesterol from atherosclerotic plaques and the transport of the excess cholesterol back to the liver for its subsequent elimination as bile acids and neutral steroids. This process is termed reverse cholesterol transport (RCT) and is one of the major protective mechanisms against the development of atherosclerosis (3-5).

Cholesterol efflux from macrophage-derived foam cells is an initial and key step in RCT (6), and serves as an integrated measure of HDL quantity and quality (7). This cholesterol efflux involves numerous genes, including ATP-binding cassette (ABC) A1 and G1 (8). ABCA1 is a member of the ABC superfamily and is the defective gene in Tangier disease. ABCA1 has been reported to have an important role in the prevention of atherosclerosis through facilitating cholesterol efflux from macrophages to lipid-poor apolipoproteinA-I (apoA-I), and decreasing cholesterol accumulation in macrophages (9). Similar to ABCA1, ABCG1 is capable of promoting cholesterol efflux from macrophages to mature HDL particles, but not to apoA-I (10).

Liver X receptor (LXR) α, a member of the nuclear hormone receptor superfamily, has a crucial role in cholesterol metabolism (11). Upon activation, LXRα induces numerous genes, which are involved in cholesterol efflux, absorption, transport and excretion. ABCA1 and ABCG1 have been identified as direct targets of LXRα (12).

Fibroblast growth factor (FGF)21 is a member of the FGF superfamily and is predominantly secreted by the liver and adipose tissue. FGF21 has gained attention for its multiple metabolic functions (13). FGF21 was first identified to stimulate glucose uptake in mouse 3T3-L1 adipocytes, which was observed to be additive and independent of insulin (14). FGF21 has also been shown to improve function and survival of pancreatic β-cells (15) and to decrease glucagon secretion (16), which together enhance insulin sensitivity. In addition, FGF21 has been found to increase energy expenditure in mice with free access to food (17) and promote weight loss in diabetic...
Materials and methods

Materials. Recombinant Human FGF-21 was obtained from Shanghai Jinan Co., Ltd. (Shanghai, China). Phorbol-12-myristate-13-acetate (PMA), bovine serum albumin (BSA) and horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Gibco-BRL (Carlsbad, CA, USA). The total RNA extraction reagent RNAiso Plus, a PrimeScript™ RT reagent kit and a SYBR® Green PCR kit were purchased from Takara Bio, Inc. (Shiga, Japan). Reagents for western blot analysis were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Ox-LDL was obtained from Beijing Xiesheng Engineering Co., Ltd. (Beijing, China). LXRα-short interfering (si)RNA was synthesized by Shanghai Genechem Co., Ltd (Shanghai, China). ABCA1, ABCG1 and LXRα specific antibodies were purchased from Wuhan Boster Biological Engineering Co., Ltd (Wuhan, China). All other chemicals were of the highest grade available from commercial sources.

Cell culture. Human THP-1 cells were cultured at a density of 1x10⁶ cells/well in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in a 5% CO₂ incubator. TPH-1 monocytes were treated with 160 nmol/l PMA for 24 h to facilitate the differentiation of monocytes into macrophages. Following PMA treatment, macrophages were incubated with 80 mg/l ox-LDL in serum-free RPMI-1640 medium containing 0.3% BSA for 24 h in order to generate foam cells.

Measurement of cellular cholesterol efflux. Human macrophages were cultured as indicated. Macrophages were then labeled with 0.3 µCi/ml [3H]cholesterol in serum-free RPMI-1640 medium containing 0.2% BSA for 24 h. After 24 h, cells were washed with phosphate-buffered saline (PBS) and incubated in serum-free RPMI-1640 medium containing 0.2% BSA in the absence or presence of 10, 50 or 100 ng/ml FGF21 for 24 h. Cells were then washed with PBS and incubated in RPMI-1640 medium containing 0.2% BSA with 10 µg/ml apoA-I or 50 µg/ml HDL for 24 h. The efflux medium was obtained at the doses designated and centrifuged at 500 x g for 3 min. to remove any floating cells. Cell monolayers were washed twice with PBS and cellular lipids were extracted with isopropanol. Media and cell-associated [3H]cholesterol were then measured using a liquid scintillation counting method (21). Cholesterol efflux was expressed as the percentage of counts in the medium relative to the total count in the medium and cells combined.

Quantitative polymerase chain reaction (qPCR). Total RNA was extracted using the RNAiso Plus reagent (Takara Bio, Inc., Shiga, Japan) in accordance with the manufacturer's instructions. The PCR primer sequences used were as follows: Forward: 5'-CTGGTTCTATGCAGGCTAGGCT-3' and reverse: 5'-CTGGTGTTCCAGCCTCAGTTG-3' for ABCA1; forward: 5'-CTGGTGTTCCAGCCTCAGTTG-3' and reverse: 5'-CTGGTGTTCCAGCCTCAGTTG-3' for ABCG1; forward: 5'-CATCCTGGTCCTTCTCTGTA-3' and reverse: 5'-GAGTCAACGGATTTGGTCGT-3' for GAPDH. qPCR was performed using SYBR® Premix Ex Taq™ II in a Bio-Rad LightCycler with an iQ3.1 Realtime PCR system (Bio-Rad, Hercules, CA, USA). GAPDH was used as an internal control. Quantitative measurements were analyzed using the ΔΔCt method.

Western blot analysis. Cells were washed twice with PBS and harvested in lysis buffer (10 mM HEPES, pH 7.9; 10 mM KCl, 1.5 mM MgCl₂, 0.5% Nonidet P-40, 1 µg/ml leupeptin, 10 µg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride) for western blot analysis. Nuclei were pelleted at 5000 x g for 5 min at 4°C and the resulting supernatant was used as the cytosolic fraction. Nuclei were resuspended in lysis buffer, sheared for 15 sec using a microprobe sonicator (Hanghai Success Ultrasonic Equipment Co., Ltd., Hangzhou, China) and incubated on ice for 5 min. Subsequent to centrifugation at 12,000 x g for 5 min at 4°C, the supernatant was collected as nuclear extracts. Cellular lysates or nuclear protein extracts (40 µg) were separated using 10% SDS-PAGE and transferred onto Immobilon®-P membranes (Millipore, Billerica, MA, USA). Membranes were blocked in 5% skimmed milk in Tris-buffered saline with Tween-20 (0.1% Tween-20, pH 7.4) and incubated with human antibodies against ABCA1, ABCG1 and LXRα. Membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit polyclonal antibody (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China). GAPDH was used as an internal control. Proteins were visualized using an enhanced chemiluminescence detection system (Amersham International, Buckinghamshire, UK).

Transfection for LXRα silencing. siRNA specific to human LXRα and a nonsilencing control siRNA were synthesized by the Biology Engineering Corporation (Shanghai, China). Cells were transfected with each siRNA as described previously (22,23). Annealed oligos for shRNA expression were cloned into the pSilencer™2.1-U6 vector.
linearized with BamHI and HindIII. The oligonucleotide sequences used to construct siRNA were as follows: forward 5'-ACTGAAGCGGCAAGAGGAGTTCAAGAGACTCCTCTTGCCGCTTCAGTTTTTTT-3' and reverse 5'-AATTAAAAAAAACTGAAGCGGCAAGAGGAGTCTCTTGAACTCCTCTTGCCGCTTCAGTGGCC -3'.

The products were transformed into competent Escherichia coli DH5α cells (Beijing TransGen Biotech Co., Ltd., Beijing, China). and cultured on Luria-Bertani plates with 100 µg/ml ampicillin. Ampicillin-resistant colonies were selected using restriction digestion and verified using DNA sequencing. For transfection with siRNA, foam cells were plated in 96-well plates at a density of 1x10⁶ cells/well. After 24 h, cells were transfected with LXRα siRNA in OptiMEM® with 5 µg/ml Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA). After 48 h of transfection, western blot analysis revealed that LXRα siRNA suppressed the protein expression of LXRα by 80%, compared with the cells transfected with the control siRNA (Fig. 1).

Statistical analysis. All experiments were performed at least three times. Data are presented as the mean ± standard deviation. Results were analyzed by one-way analysis of variance and Student's t-tests, using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

FGF21 enhances apoA-I- and HDL-mediated cholesterol efflux from THP-1 macrophage-derived foam cells. The effect of FGF21 on cholesterol efflux in THP-1 macrophage-derived foam cells was analyzed using a liquid scintillation counter with apoA-I or HDL as a cholesterol acceptor. A dose course study was performed. Treatment with FGF21 at 50 and 100 ng/ml was found to enhance apoA-I-mediated cholesterol efflux compared with the control group (P<0.05), and
FGF21 at 100 ng/ml was found to enhance HDL-mediated cholesterol efflux compared with the control group (P<0.05).

**FGF21 upregulates the expression of ABCA1 and ABCG1 in THP-1 macrophage-derived foam cells.** ABCA1 and ABCG1 have been identified to have a key role in cholesterol efflux and foam cell formation in macrophages. In order to determine whether FGF21 enhances cholesterol efflux through modifying ABCA1 and ABCG1 expression, the expression of ABCA1 and ABCG1 was assessed using qPCR and western blot analyses in FGF21-treated THP-1 macrophage-derived foam cells. At 50 and 100 ng/ml, FGF21 was observed to significantly upregulate the mRNA expression of ABCA1 and ABCG1 (Fig. 3A). Similar results were observed for ABCA1 and ABCG1 protein expression (Fig. 3B).

**LXRα mediates the FGF21-induced upregulation of ABCA1 and ABCG1 in THP-1 macrophage-derived foam cells.** It is well established that LXRα activation induces the expression of ABCA1 and ABCG1. The following experiments were performed in order to examine the role of LXRα in FGF21-treated macrophages. qPCR and western blot analyses...
were performed to assess whether FGF21 alters LXRα expression. FGF21 was found to induce an increase in LXRα expression at the mRNA (Fig. 4A) and protein (Fig. 4B) levels. LXRα expression was then silenced using siRNA to examine the role of LXRα in the FGF21-induced upregulation of ABCA1 and ABCG1. The upregulation of ABCA1 and ABCG1 mRNA expression induced by FGF21 was observed to be attenuated in the LXRα-siRNA-treated foam cells compared with those solely treated with FGF21 (Fig. 4C). Similar results were obtained in the upregulation of ABCA1 and ABCG1 protein expression (Fig. 4D). FGF21-induced apoA-I- and HDL-mediated cholesterol efflux was also attenuated in foam cells treated with LXRα-siRNA compared with those solely treated with FGF21 (Fig. 4E and F). These findings suggest that the FGF21-induced activation of LXRα may be responsible for the effect of FGF21 on cholesterol efflux and ABCA1 and ABCG1 expression.

Discussion

To the best of our knowledge, the present study is the first study to demonstrate that macrophages are a target of FGF21, and preliminary data suggest that FGF21 may have a protective effect against atherosclerosis. FGF21 was found to enhance apoA-I- and HDL-mediated cholesterol efflux in THP-1 macrophage-derived foam cells. FGF21 was also observed to upregulate the mRNA and protein expression of ABCA1 and ABCG1. Furthermore, LXRα was found to be involved in facilitating these FGF21-induced effects.

A growing body of evidence has shown that FGF21 has numerous benefits on glucose and lipid metabolism and insulin sensitivity (13). Therefore, FGF21 may be beneficial for the treatment of metabolic diseases, including non-alcoholic fatty liver disease, type 2 diabetes and dyslipidemia, suggesting that FGF21 may have the potential to prevent the development of atherosclerosis. Thus, the present study aimed to investigate whether FGF21 has the capacity to reverse cholesterol transport, a process that is particularly important in atherosclerosis and the regression of atherosclerotic plaques.

Despite being a small fraction of the overall cholesterol efflux mediated by the RCT pathway, the efflux of cholesterol from macrophages has a significant inverse association with the risk of coronary artery disease, particularly atherosclerosis, independent of HDL cholesterol levels (6,7). In the present study, FGF21-treated foam cells were found to exhibit a dose-dependent increase in apoA-I- and HDL-mediated cholesterol efflux.

Cholesterol efflux involves several important transporters and mechanisms (6). ABCA1 and ABCG1 are two of the most important proteins involved in mediating cholesterol efflux from macrophages to extracellular acceptors, including apoA-I and HDL. Therefore, ABCA1 and ABCG1 have important roles in the formation and metabolism of HDL (24). It is well established that extracellular apoA-I is a determinant of ABCA1-dependent cholesterol efflux. Moreover, HDL contributes to ABCG1-dependent cholesterol efflux (6). In the present study, FGF21 was found to enhance apoA-I- and HDL-mediated cholesterol efflux; thus, the effect of FGF21 on ABCA1 and ABCG1 was also investigated. In FGF21-treated cells, the increase in apoA-I- and HDL-mediated cholesterol efflux was observed to be consistent with an upregulation of ABCA1 and ABCG1 mRNA and protein expression. Therefore, FGF21-mediated upregulation of ABCA1 and ABCG1 may have anti-atherosclerotic effects, through enhancing cholesterol efflux.

Numerous studies have revealed that LXRα has a significant role in lipid metabolism and that increases in LXRα in lipid-loaded macrophages upregulate ABCA1 and ABCG1 (9,12,22,23). Therefore, the present study investigated the effect of FGF21 on the expression of LXRα. The FGF21-induced upregulation of ABCA1 and ABCG1 expression was concurrent with an increase in LXRα expression. LXRα-siRNA was then used to determine the role of LXRα in the FGF21-induced upregulation of ABCA1 and ABCG1. Knockdown of LXRα using siRNA was found to attenuate the FGF21-mediated upregulation of ABCA1 and ABCG1. Furthermore, the increase in cholesterol efflux induced by FGF21 was attenuated with the LXRα-siRNA. These results suggest that LXRα has a key role in conferring the therapeutic benefits of FGF21 in improving lipid metabolism. Despite the unique pathway identified in the present study, the specific mechanism by which FGF21 affects cholesterol efflux requires further investigation.

The inverse correlation between plasma HDL levels and the risk of atherosclerosis has been extensively investigated (25,26). However, a recent study suggests that analysis of HDL function may be more beneficial than an assessment of HDL plasma levels (27). Although HDL has numerous beneficial functions, the protective effect of HDL against atherosclerosis is largely attributed to its capacity to promote cholesterol efflux from macrophage foam cells (28). FGF21-treatment has been reported to increase HDL plasma levels in diabetic monkeys (20). Notably, the present study found that FGF21 promoted RCT through enhancing cholesterol efflux, which is the most important function of HDL. This suggests that FGF21 may have the potential to protect against atherosclerosis.

In conclusion, the present study demonstrated that FGF21 increases apoA-I- and HDL-mediated cholesterol efflux in ox-LDL-stimulated macrophage foam cells, potentially through LXRα-dependent upregulation of ABCA1 and ABCG1. This suggests a direct association between FGF21 treatment and inhibition of atherosclerosis. While these findings support a promising role for FGF21 in cholesterol efflux, its regulation, function and clinical application require further investigation in order to establish its specific pathophysiological role in atherosclerosis.

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