Original Article
D-4F alleviates renal lipid abnormalities and increases the protein levels of renal LXRβ in LDL receptor-null mice fed a western diet

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Abstract: *Objectives:* Lipid abnormalities appear to contribute to both the initiation and progression of renal injury, regardless of etiology. D-4F contains a class A amphipathic helix with a polar face and a nonpolar face that allows it to bind lipids similar to apoA-I. We designed the present study to test whether D-4F treatment could alleviate lipid abnormalities in the kidneys and determine the role of liver X receptors (LXRs) in D-4F treatment. *Methods:* Female LDL receptor-null (LDLR$^{-/-}$) mice 3-4 weeks old were administered a Western diet (21% fat and 0.3% cholesterol) for 18 weeks. Then, all the mice were randomly assigned to 3 groups for treatment with D-4F given in drinking water-a high-dose group (0.5 mg/ml), a low-dose group (0.3 mg/ml), and a control group (just drinking water)-for 6 weeks before examination. Hematoxylin-eosin (HE), periodic acid-Schiff (PAS), and Masson trichrome staining were used to evaluate renal histology. Lipid levels were determined by automatic biochemical analyzer and an enzyme-linked immunosorbent assay. Western blotting analysis was used to determine the expression of the LXR protein levels. *Results:* D-4F treatment displayed, after HE staining, a significant decrease in the glomerular tuft-to-Bowman's capsule diameter ratio and the number of cells per glomerulus. The cells also showed decreased PAS staining and Masson trichrome staining. The plasma levels of low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), total cholesterol (TC), and triglyceride (TG) in the mice treated with D-4F did not show any significant changes. However, the addition of D-4F to the drinking water decreased the levels of LDL-C, TC and TG and increased the level of HDL-C in the kidney. The protein levels of LXRβ were significantly increased in the high-dose group, and the protein levels of LXRα did not change significantly after D-4F treatment. *Conclusions:* Our study suggests that D-4F could alleviate renal lipid accumulation, improve lipid metabolism and prevent renal histological deterioration. These protective effects might be associated with LXRβ in the kidney instead of the plasma lipid levels.

Keywords: Lipid abnormalities, renal disease, apoA-I mimetic peptide, D-4F, liver X receptors

Introduction

There has been a longstanding recognition of the potential relationship between lipoproteins and renal disease [1-4]. Numerous animal studies have examined the role of lipid abnormalities in renal disease. These studies demonstrated that lipid abnormalities appear to contribute to both the initiation and progression of renal injury, regardless of etiology [4]. LDL receptor-null (LDLR$^{-/-}$) C57BL/6J mice fed a high-fat/high-cholesterol diet develop severe hyperlipidemia and serve as models for lipid abnormalities as the initiators of renal disease [5].

D-4F, an 18-amino acid mimetic peptide of apoA-I, contains a class A amphipathic helix with a polar and a nonpolar face that allows it to bind lipids similar to apoA-I [6-8]. We previously reported that when administered in drinking water for 6 weeks, D-4F increased cardiac high-density lipoprotein cholesterol (HDL-C) levels and decreased cardiac low-density lipoprotein cholesterol (LDL-C), total cholesterol (TC), and triglyceride (TG) levels in LDLR$^{-/-}$ C57BL/6J mice fed a Western diet (WD) [8]. These results suggested that D-4F treatment could alleviate lipid abnormalities in cardiac tissue, but whether it could exert the same protective effect in renal tissue is not completely understood. Taking into

consideration that liver X receptors (LXRs) are activated when cells accumulate excess cholesterol and that the expression of LXRα at protein levels is significantly increased after D-4F treatment [8-10], we designed the present study to test whether D-4F treatment could alleviate lipid abnormalities in the kidneys and determine the role of LXRs in D-4F treatment.

Materials and methods

Materials

D-4F with the primary sequence Ac-DWFK-AFYDKVAEKFKEAF-NH2 was synthesized by the Shanghai Ketai Bio-Technique Co Ltd. (Shanghai, China). The purity of synthetic peptides (typically > 98%) was established by analytical high-performance liquid chromatography and confirmed by mass spectral analysis. Sodium pentobarbitone was purchased from Merck KGaA (Darmstadt, Germany). Enzyme-linked immunosorbent assay (ELISA) kits were obtained from Abcam (Cambridge, United Kingdom). A bicinchoninic acid (BCA) protein assay kit was purchased from Pierce Biotechnology (Rockford, IL, USA). Protease inhibitor cocktails were purchased from Roche Diagnostics GmbH (Mannheim, Germany). Antibodies were all purchased from Abcam (Cambridge, United Kingdom).

Animals and experimental protocol

Female LDLR$^{-/-}$ C57BL/6J mice were provided by the Model Animal Research Center of Nanjing University and all the mice were cared for in accordance with the standards for laboratory animals established by the People's Republic of China (GB14925-2001). The WD (21% fat and 0.3% cholesterol, Beijing Keaoxieli Co., Ltd. (Beijing, China)) was administered to the mice when they were 3-4 weeks old, and the treatment continued for 18 weeks. Then, all the mice were randomly assigned to 3 groups for treatment with D-4F given in drinking water-the high-dose group (0.5 mg/ml), the low-dose group (0.3 mg/ml), and the control group (just drinking water)-for 6 weeks before examination. Each group contained 10 mice. To ensure the efficacy of D-4F, the drinking water was changed every day. Each mouse consumed approximately 2.5 ml of water per day, and there was no significant difference in water or food consumption among the groups. All the mice were continued on the WD. After the experimental period, the mice were anesthetized by an intraperitoneal injection of sodium pentobarbitone (50 mg/kg). Blood samples were collected for measurements of plasma biochemical indexes. All the kidneys were rapidly excised, immersed in an ice-cold saline solution, and blotted dry. After removing any excess connective tissue, the kidneys were fixed in 10% neutral-buffered formalin and embedded in paraffin. The remaining kidneys were stored at -80°C after liquid nitrogen freezing.

Biochemical analysis

The plasma levels of LDL-C, HDL-C, TC and TG were determined by an automatic biochemical analyzer (Roche 6000; Roche Diagnostics, Indianapolis, IN). The renal levels of LDL-C, HDLC, TC and TG were determined by ELISA using a commercial kit.

Histological studies of kidney

Transverse sections of the kidneys were fixed in 10% buffered formalin and embedded in paraffin. Two-micron-thick paraffin sections were prepared for histological examination with hematoxylin-eosin (HE), periodic acid-Schiff (PAS) and Masson trichrome staining. Semiquantitative evaluation was performed at ten non-overlapping vision fields that were randomly selected under an Olympus microscope (Olympus BX51T-PHD-J11, Japan) and an image analysis program (Image-Pro Plus 3.0, Media Cybernetics, Rockville, MD, USA).

Western analysis

Protein was extracted from the renal tissue using 200 μL of ice-cold lysis buffer (pH 7.4) (100 mmol/L NaCl, 50 mmol/L HEPES, 5 mmol/L EDTA, 1% Triton X-100, protease inhibitor cocktail) in the presence of phosphatase inhibitors (50 mmol/L sodium fluoride, 10 mmol/L sodium pyrophosphate, 1 mmol/L sodium orthovanadate, 1 nmol/L microcystin). The protein concentrations were determined with the BCA protein assay kit. The lysates (10-30 μg of protein) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. The membranes were then blocked in 5% nonfat milk powder in phosphate-buffered saline for 1 hour at room temperature followed by an overnight incubation

Table 1. Effect of D-4F on WD-induced Weight Gain in LDLR$^{-/-}$ mice (n=10/group)

	0.5 mg/ml D-4F	0.3 mg/ml D-4F	Control
Body weight, g	21.51±0.60[a,b]	23.00±0.46[a]	24.79±0.66

Results are expressed as the mean ± SEM. [a]P<0.05, compared with the control group. [b]P<0.05, compared with the 0.3 mg/ml D-4F group. Each group contained 10 mice.
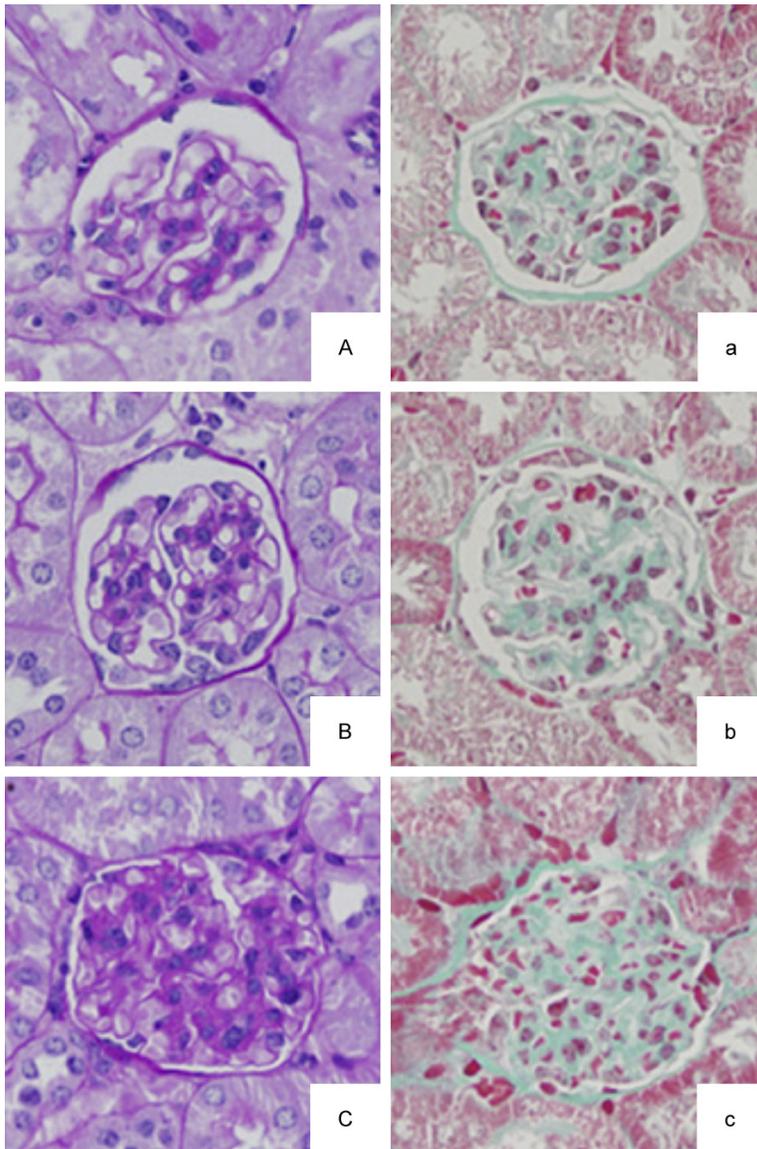
Figure 1. The sections were stained with periodic acid-Schiff (PAS) staining (A-C) or Masson trichrome staining (a-c) and visualized at 400× magnifications. (A and a) are from the high-dose group (0.5 mg/ml D-4F), (B and b) are from the low-dose group (0.3 mg/ml D-4F), and (C and c) are from the control group.

with anti-LXRα (1:500) and anti-LXRβ (1:1000). Then, the membranes were incubated for 1 hour with horseradish peroxidase-labeled anti-

IgG (1:1000) at 37°C. The antigen-antibody complexes were revealed by an electrochemiluminescence detection system (Santa Cruz Biotechnology, Santa Cruz, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control to normalize the data. The results were expressed as fold increases compared with the control.

Statistical analysis

Continuous variables were expressed as the mean values ± SEM. The differences between the groups were compared by Student's *t* test for phenotypes with equal variance and by adjusted Student's *t* test for those with unequal variance. All analyses were conducted using the SPSS software for Windows, version 13.0 (SPSS, Chicago, IL). Statistical significance was set at P<0.05.

Results

The effect of D-4F on WD-induced weight gain in LDLR$^{-/-}$ Mice

Compared with the control group, body weight was significantly decreased, with a trend toward dose-responsivity, in the WD-fed mice treated with D-4F (**Table 1**).

The effect of D-4F on renal histology in LDLR$^{-/-}$ mice

To evaluate the effect of D-4F on renal histology, we performed HE, PAS and Masson trichrome staining on renal paraffin sections. Compared with those in the control group, the renal paraffin sections of the mice with D-4F treatment displayed, after HE staining, a significant decrease in the glomerular tuft-to-

Table 2. Effect of D-4F in LDLR^{-/-} mice (n=10/group)

	0.5 mg/ml D-4F	0.3 mg/ml D-4F	Control
Glomerular tuft/Bowman's capsule diameter ratio, %	0.58±0.03[a,b]	0.69±0.03[a]	0.77±0.02
Number of cells per glomerulus	28.60±1.03[a,b]	37.70±1.16[a]	57.80±2.30
ECM protein content, %	14.48±0.76[a,b]	17.85±0.79[a]	20.20±0.51
Collagen deposition and fibrosis areas, %	23.83±4.08[a,b]	27.03±5.04[a]	32.28±4.17

Results are expressed as the mean ± SEM. [a]P<0.05, compared with the control group. [b]P<0.05, compared with the 0.3 mg/ml D-4F group. Each group contained 10 mice. A semiquantitative evaluation was performed at ten nonoverlapping vision fields that were randomly selected per mouse. Abbreviations: extracellular matrix (ECM).

Table 3. Effect of D-4F on plasma and renal lipid levels in LDLR^{-/-} mice (n=10/group)

Lipid levels (mmol/mg)		0.5 mg/ml D-4F	0.3 mg/ml D-4F	Control
Plasma	LDL-C, nmol/mg	3.25±0.59	3.76±1.09	3.77±0.66
	HDL-C, µg/mg	2.88±0.43	2.73±0.24	2.66±0.27
	TC, nmol/mg	1.27±0.95	1.33±0.49	1.34±0.46
	TG, nmol/mg	5.80±0.90	6.43±0.78	6.49±2.34
Renal	LDL-C, nmol/mg	27.51±3.67[a,b]	37.93±3.42[a]	45.73±2.32
	HDL-C, µg/mg	44.76±4.44[a,b]	32.77±3.56[a]	25.80±3.61
	TC, nmol/mg	29.30±3.59[a,b]	40.30±2.33[a]	45.51±4.85
	TG, nmol/mg	8.18±1.14[a,b]	10.53±0.77[a]	12.56±1.00

Results are expressed as the mean ± SEM. [a]P<0.05, compared with the control group. [b]P<0.05, compared with the 0.3 mg/ml D-4F group. Every group contained 10 mice. Abbreviations: low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), total cholesterol (TC), and triglyceride (TG).

Bowman's capsule diameter ratio and the number of cells per glomerulus. They also showed decreased PAS staining, which is a hallmark of mesangial expansion with decreased extracellular matrix protein content (**Figure 1**). Furthermore, Masson trichrome staining showed that collagen deposition and fibrotic areas were significantly decreased in the D-4F treatment groups (**Figure 1**). All these changes showed a trend toward dose-responsivity (**Table 2**).

The effect of D-4F on plasma and renal lipids in LDLR^{-/-} mice

To investigate a possible relationship between renal histological changes and lipid levels, we measured the lipid levels in both the plasmas and kidneys. Compared with the levels in the control group, the plasma levels of LDL-C, HDL-C, TC and TG in mice treated with D-4F did not show any significant changes. However, the addition of the D-4F to the drinking water decreased the levels of LDL-C, TC and TG, and increased the level of HDL-C in the kidneys, and these changes showed a trend toward dose-responsivity (**Table 3**).

The effect of D-4F on the expression of LXRs in LDLR^{-/-} mice

Western blotting analysis was performed to investigate the change of LXRs after D-4F treatment. Compared with the control group, the protein levels of LXRβ increased in the high-dose group and the protein levels of LXRα did not change significantly after D-4F treatment (**Figure 2**).

Discussion

The purpose of our current study was to investigate the role of D-4F in renal histology and lipid metabolism changes in LDLR^{-/-} mice and then further explore the possible underlying mechanism. We examined 1) whether there were altered renal histology and lipid metabolisms in LDLR^{-/-} mice treated with D-4F and 2) whether LXRs were involved in D-4F induced changes of renal histology and lipid metabolism.

The C57BL/6J mice that were fed a high-fat diet (HFD) for 12 weeks developed metabolic syndrome, including obesity and hyperglycemia, and had an increase in renal lipid accumulation, which correlated well with the increased renal triglyceride and cholesterol content [11-14]. Previous studies have also demonstrated that the kidneys of metabolic syndrome patients have lipid accumulation that plays an important role in the pathogenesis of renal disease [15-17]. Buga and colleagues subsequently found that these changes could be prevented partly by adding D-4F to drinking water (0.3 mg/ml D-4F) for 7 weeks. Compared with the LDLR^{-/-} mice on a HFD without D-4F treatment, the renal total triglyceride levels were sig-
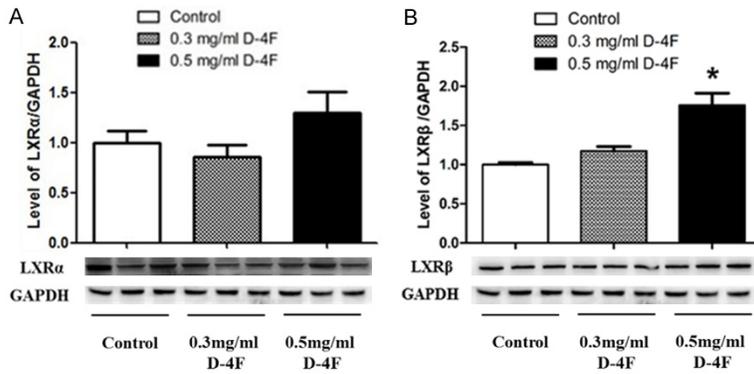
Figure 2. Western blotting analysis of LXRα (A) and LXRβ (B) proteins in the kidneys of the D-4F treatment and control groups. Every group contained 6 mice. GAPDH was included as a loading control. The quantitative data represent the band intensity and are given as the means ± SEM. *P<0.05, compared with the control group.

tended to exert their influence at the local levels, such as in the renal and cardiac tissues, and were not strong enough to influence circulating lipoprotein cholesterol levels [8].

Under HFD conditions, the C57BL/6J mice developed metabolic syndrome as well as kidney specific histological changes, including increased renal expression of vascular endothelial growth factors, plasminogen activator inhibitor-1, type IV collagen, and fibronectin. The subsequent development of glomerulosclerosis and proteinuria were also observed by Jiang and colleagues [14]. In the study by Buga and colleagues, the addition of D-4F in drinking water prevented the increase in the glomerular tuft-to-Bowman's capsule diameter ratio, the number of cells per glomerulus, and mesangial cells that is observed in LDLR-/- mice on a WD without D-4F treatment [18]. We confirmed these findings in our study. Furthermore, with the use of Masson trichrome staining, we also found that collagen deposition and fibrotic areas were significantly decreased in the D-4F treatment groups. Since we added two different doses of D-4F in the drinking water, all these changes showed a trend toward dose-responsivity and the higher dose of D-4F in drinking water seemed to exert a more obvious effect. Therefore, we suggested that through alleviating lipid accumulation and structure, D-4F treatment could delay or even reverse the process of renal disease induced by a HFD to a certain extent in the LDLR-/- mice.

nificantly decreased in the LDLR-/- mice on a HFD treated with D-4F. However, they did not see a change in renal total cholesterol levels [18]. Similar to their findings, we confirmed that the addition of D-4F to the drinking water decreased the renal total triglyceride levels. However, the difference was that, in our study, the renal total cholesterol levels also decreased after D-4F treatment. This effect was more obvious in the high-dose group (0.5 mg/ml D-4F). This might be partly because the mice in Buga's study were slightly older at the start of the study (7 versus 3 weeks) and were maintained on the high-fat diet for less than half as long as in our study (7 versus 18 weeks). Additionally, we also observed decreased LDL-C levels, as well as increased HDL-C levels. These altered lipid levels induced by the D-4F treatment were the same as with our previous work in cardiac tissue and are associated with alleviative lipid accumulation and improved cardiac function [8].

The effects of D-4F treatment on the plasma lipid levels in our study were the same as some other earlier studies [19]. Although D-4F treatment affected lipid accumulation in renal tissue, the plasma levels of LDL-C, HDL-C, TC and TG in the D-4F-treated mice were not influenced. The underlying mechanisms need to be further explored. Some studies assumed that the ability of D-4F to exert its positive effect might be independent of changes in circulating lipoprotein cholesterol levels [18, 19]. Aside from this, we speculated that the doses of 0.3 mg/ml and 0.5 mg/ml of D-4F in drinking water

LXRs, which belong to the nuclear receptor superfamily, are activated when cells accumulate excess cholesterol. After ligand binding, LXRs form heterodimers with retinoid X receptors and then regulate the transcription of genes involved in lipid/glucose metabolism and inflammation. LXRα is highly expressed in the liver, intestine, kidney, adipose tissue and macrophages, while LXRβ is ubiquitously expressed [20-23]. LXRs exert distinct effects on cholesterol and lipid metabolism in discrete cell types. The key biological effects of LXRs include the promotion of cellular cholesterol efflux from

macrophages and foam cells, an improvement in cholesterol flux from the periphery to the liver, the inhibition of inflammation, and the prevention of cell death [24]. We previously suggested that the level of cardiac tissue HDL-C was increased after D-4F treatment and that the protein expressions of LXRα but not LXRβ were consistently elevated as well. In the present study, the level of renal tissue HDL-C was also increased, but the protein expressions of LXRα did not rise. In contrast, the protein expressions of LXRβ increased in the higher D-4F dose treatment group. These results imply that D-4F might exert protective effects in different ways between different organs.

Conclusion

In conclusion, our study suggests that D-4F could alleviate renal lipid accumulation, improve lipid metabolism, and prevent renal histological deterioration. These protective effects might be associated with LXRβ in the kidneys instead of in the plasma lipid levels.

Acknowledgements

Disclosure of conflict of interest

None.

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