

**NO-1886 suppressing atherosclerosis in high-fat/ high-sucrose/
high-cholesterol fed Bama minipigs is related to upregulating
ATP-binding Cassette Transporter A1**

Abbreviated title: **NO-1886 suppresses atherosclerosis in minipigs**

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ABSTRACT

Objectives: It is widely believed that high-density lipoprotein cholesterol (HDL-C) functions to transport cholesterol from peripheral cells to the liver by reverse cholesterol transport (RCT), a pathway that may protect against atherosclerosis by clearing excess cholesterol from arterial cells. A cellular ATP-binding cassette transporter (ABC) called ABCA1 mediates the first step of RCT. NO-1886 has been proven to be highly effective in raising HDL-C and reducing atherosclerosis.

However, the mechanism of inhibiting atherosclerosis for NO-1886 is not fully understood. **Methods and results:** In this study, the effects of NO-1886 on ABCA1 were investigated in high-fat/ high-sucrose/ high-cholesterol fed Chinese Bama minipigs. Administration of NO-1886 (0.1g/kg body weight/day) in the diet for five months significantly reduced atherosclerosis lesions, and significantly increased plasma HDL-C and apolipoprotein A-I levels. The mRNA and protein levels of ABCA1 in the liver, retroperitoneal adipose tissue and aorta were increased by NO-1886 as well. Multivariate linear regression analysis showed that the levels of lipoprotein lipase (LPL) in plasma and the levels of ABCA1 in aorta were independently associated with the atherosclerotic lesion area. In addition, NO-1886 up-regulated Liver X Receptor α (LXR α) and affected expression of scavenger receptor-type B class 1 (SR-BI) in the liver. **Conclusion:** These results demonstrated that the mechanism of inhibiting atherosclerosis for NO-1886 is associated with its effect on ABCA1.

Supplementary key words: NO-1886; atherosclerosis; ABCA1; LXR α ; SR-BI;

minipig

Introduction

Tsutsumi et al (1) reported that a novel compound, NO-1886, possessed a potent lipoprotein lipase (LPL)-enhancing activity. Administration of NO-1886 increased LPL activity in the postheparin plasma, adipose tissue, and myocardium in rats, with a concomitant reduction in plasma triglyceride (TG) level and elevation of high density lipoprotein cholesterol (HDL-C) levels (2). LPL hydrolyzes chylomicron and very low density lipoprotein (VLDL) to remnants and low density lipoprotein (LDL); whereas cholesterol, apoproteins, and surface phospholipids are released from triglyceride-rich lipoproteins fuse with smaller HDL particles, forming mature cholesterol-rich HDL particles. A negative relationship has been observed between the HDL cholesterol concentration and the extent of atherosclerosis in many studies (3-5). One of explanations to this protective effect of HDL against atherosclerosis is RCT, i.e. the process by which cholesterol is removed from extrahepatic tissues and returned to the liver for conversion into bile acids and excretion into bile.

The first crucial step in the RCT pathway is the movement of excess cellular cholesterol and phospholipid from cell membranes to nascent HDL particles (6, 7). The ABCA1 plays a key role in this step. Subsequently, HDL particles act in conjunction with the cholesterol esterifying enzyme, lecithin: cholesterol acyltransferase. Cholesteryl ester accumulating in HDL then involves a number of different fates: uptake in the liver in HDL containing apolipoprotein by LDL receptors, selective uptake of HDL cholesteryl ester in the liver or other tissues involving scavenger receptor B I, or transfer to triglyceride-rich lipoproteins as a result of the

activity of cholesteryl ester transfer protein, with subsequent uptake of triglyceride-rich lipoprotein remnants in the liver (8).

NO-1886 has a role in reducing atherosclerosis. Since this compound elevates HDL-C level and HDL-C is atheroprotective because of its role in RCT, we suppose that the mechanism of inhibiting atherosclerosis for NO-1886 is related to, the gatekeeper of RCT pathway, ABCA1. So in the present study, we investigated the effects of NO-1886 on ABCA1 in high-fat/ high-sucrose/ high-cholesterol fed Chinese Bama minipigs.

Material and Methods

Material

NO-1886 ([4-(4-Bromo-2-cyano-phenylcarbonyl)-benzyl]-phosphonic acid diethyl ester, CAS 133208-93-2; Lot.No.C00C99) was synthesized in the New Drug Research Laboratory of Otsuka Pharmaceutical Factory, Inc (Tokushima, Japan). Sucrose was obtained from Liuzhou sugar Co. (Guangxi, China) and lard was obtained from Hengyang Meat Product Co. (Hunan, China). Cholesterol was obtained from Sigma (>99% pure; St. Louis, MO).

Animals and diets

Male Chinese Bama minipigs, 2 months of age, were obtained from the barrier unit at the Laboratory Animal Center of Chongqing Medical University (Chongqing, China). Animals were randomized into four groups with similar body weight (n=7 in normal diet fed group; n=7 in normal diet plus NO-1886 group; n=8 in high-fat/high-sucrose/high-cholesterol fed group; n=8 in high-fat/high-sucrose/high-cholesterol fed and supplemented NO-1886 group). The high-fat/high-sucrose/high-cholesterol diet used in this study was normal pig diet supplemented 10 % lard, 37% sucrose and 2% cholesterol which was similar to a “diabetogenic” and “atherogenic” diet (1, 9) (The composition of the diets is described in Table 1). Animals were housed in single pens under controlled conditions (temperature between 18°C and 22°C, relative air humidity 30-70%, with 4 air

changes per hour) and fed 3 times daily on a restricted schedule with normal control diet (CD), or normal diet plus NO-1886 (CD1886), or high-fat/high-sucrose/high-cholesterol diet (HFSCD), or high-fat/high-sucrose/high-cholesterol supplemented NO-1886 diet (HFSCD1886). All pigs received the same amount of food (4.0% body weight). The dose of NO-1886 administration for CD1886 and HFSCD1886 groups was 0.1g/kg body weight/day. The total study period was 5 months. Blood samples for plasma lipids and glucose inspection were obtained without sedation by pricking an ear vein with a lancet and collecting drops in a hematocrit tube at the end of each month after fasting overnight. The animals were sacrificed at the end of month 5. The liver, the retroperitoneal adipose tissue and the aorta were dissected free from adjacent tissues and frozen in liquid nitrogen. Institutional guidelines for animal care and use were followed.

Plasma measurement

Glucose, total cholesterol (TC), HDL-C, and TG were determined by commercially enzymatic methods (test kits, Shanghai Rongsheng Biotech Inc. Shanghai, China). Plasma free fatty acids (FFA) was measured by a colorimetric method (kits supplied by Nanjing Jianchen Biotech Inc. Nanjing, China). Apolipoprotein A-1 (apoA-1) was measured by immunoturbidimetry (test kits, Shanghai Rongsheng Biotech Inc. Shanghai, China). LPL activity in postheparin plasma was measured by an immunochemical method described previously (10). Then heparin (150 units/kg) was injected into the femoral vein, 10 min later blood was collected, the plasma was

separated by centrifugation at 1000G for 15 min, and the glycerol tri[^{14}C]oleate used as substrate and selective blocking of hepatic lipase activity with antiserum to rat hepatic lipase.

Morphological examination of atherosclerotic lesions

At the end of the experimental period, the animals were killed by phlebotomy under light anesthesia with sodium pentobarbital (30mg/kg, iv. Jilin Northern medicine Inc. china). Atherosclerotic lesions were analyzed by a method described previously (11). The aorta was dissected from the aortic valve to the iliac bifurcation and as much adventitia as possible was removed to prevent errors during Sudan IV staining of the vessel. The aorta was opened longitudinally and pinned flat on a Styrofoam surface. After overnight fixation in 10% formalin, the aorta was rinsed in 70% ethanol for 10 minutes and then stained with 0.5% Sudan IV in 35% ethanol-50% acetone for 20 minutes. Destaining was carried out for 20 minutes in 80% ethanol. Lipid deposition in the aorta was determined by morphological assessment of the percentage of lesion-covered aorta as visualized by Sudan IV staining of the region between the aortic root and bifurcation. Fatty streak lesions on enlarged photographs were traced on a digital tablet and atherosclerotic lesions were analyzed using image analysis software (NIH image). The aortic lesion area was calculated as a percentage as follows: atherosclerotic lesion area divided by the area of the aorta \times 100.

Biochemical analysis of the artery wall

Because all aortas were fixed and stained for fatty streak lesion determination, they were not suitable for chemical analysis; therefore, common carotid arteries were used for cholesterol and cholesteryl ester analyses to determine the effect of NO-1886 on arterial lipid deposition. It has been shown that fatty streak lesions in the common carotid artery develop similarly to those in the aorta. Therefore, thirty common carotid arteries were washed in ice-cold saline, blotted dry between sheets of filter paper, and weighed. Cholesterol and cholesteryl ester contents in the arteries were measured after they were homogenized in phosphate buffer and the lipid was extracted from the homogenates as described by Folch et al (12); quantitative gas-liquid chromatography was performed as described by Rapp et al (13). The samples were analyzed for free cholesterol before and after saponification, and the calculated difference represents the cholesteryl ester concentration. Cholesterol (Sigma Inc.) was used as standards for free-cholesterol determinations.

RNA preparation and real-time quantitative RT-PCR

Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed to determine the relative expression levels of the ABCA1 in the minipigs' tissues. Total RNA was extracted from liver, retroperitoneal adipose tissue and aorta by using RNA extraction Trizol reagent (Invitrogen). After contaminated genomic DNA was digested with DNase I (Roche Diagnostics), first standard cDNA was synthesized by a SuperScript™ preamplification system (Invitrogen) from 2 µg of the total RNA. PCR was performed by using primers (sense and antisense) for

cDNA 5'-GGG GTG GTG TTC TTC CTC ATT -3' and 5'- CAG GCT TCC GCT TCC TTC TAT -3' (for ABCA1) and 5'- CCT GTA CGC CAA CAC AGT GC -3' and 5'- ATA CTC CTG CTT GCT GAT CC -3' (for β -actin) (synthesized by Sangon Technology Co., Ltd.). Primers were validated by analysis of template titration and dissociation curves. Each reaction (50 μ l) contained 0.3 μ mmol/L primers, 25 μ l of 2x SYBR Green PCR master mix reagent and 2 μ l of template, and was amplified by 40 cycles of denaturation (94 °C and 30 s), annealing (60 °C and 30 s), and extension (72 °C and 30 s). The quantification of ABCA1 and β -actin mRNA was achieved in an ABI PRISM 7700 sequence detection system (Applied Biosystems) and analyzed using ABI PRISM sequence detector software (Version 1.6.3, Applied Biosystems). Transcript levels were normalized to the amount of β -actin transcript. Standard curves were generated for target genes and compared with β -actin using serial dilutions of mRNA, and they were found to be linear from 0.08 to 50 ng RNA in the reaction mixture. This range included effective concentrations used in the experiments. Melting curve analysis was performed to confirm production of a single product in these reactions. All analyses were performed in triplicate.

Protein isolation and western blotting

Protein was isolated from flash-frozen Minipig's liver, retroperitoneal adipose tissue and aorta samples as previously described (14). Total protein (10 to 50 μ g/lane) was electrophoresed and separated on a 6 to 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond-C, Amersham), which was soaked

in 5% nonfat dry milk in TBST (pH 7.6). Membranes were incubated overnight with a rabbit polyclonal antibody to human ABCA1 (Novus Biologicals) at a dilution of 1:500 on a rotating platform at 4°C or a rabbit polyclonal antibody to human LXR α (Novus Biologicals) at a dilution of 1:400 on a rotating platform at 4°C, and or a rabbit polyclonal antibody to human SR-BI (Novus Biologicals) at a dilution of 1:1000 on a rotating platform at 4°C. Subsequently, membranes were rinsed in TBST (pH 7.6) and incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibodies (Zymed Laboratories) diluted in TBST (1:2000) for 1 hour on a rotating platform at 4°C. Bands were visualized using a HRP developer, and background-subtracted signals were quantified on a laser densitometer (Bio-Rad). Blots were probed with mouse anti- β -actin monoclonal antibody (Sigma-Aldrich) to ensure equal protein loading. All protein levels were assessed by densitometry with β -actin used as a control.

Statistical analysis

Results are expressed as means \pm S.D.. Statistical analysis were performed using the MANOVA. Statistical significance was obtained when *P* values were less than 0.05. Multivariate linear regression analysis was used to evaluate the relationship among levels of ABCA1, SR-BI, LPL and lesion areas of the aorta in HFSCD fed minipigs. For nonquantitative data, results were representative from at least three independent experiments.

Results

Postheparin plasma LPL activity

NO-1886 supplementation caused a marked increase in postprandial postheparin plasma LPL activity (47.6 ± 5.4 nmol/ml/min in HFSCD1886 group vs. 31.9 ± 8.1 nmol/ml/min in the HFSCD group, $P < 0.01$; and 16.4 ± 4.2 nmol/ml/min in CD1886 group vs. 7.3 ± 1.7 nmol/ml/min in CD group, $P < 0.05$). NO-1886 supplementation also increased fasting plasma LPL activity in pigs both from HFSCD1886 group and CD1886 group, even though there is no statistical difference between HFSCD1886 and HFSCD, and between CD1886 and CD groups (Table 2).

Plasma TC

Plasma total cholesterol level was significantly increased in the HFSCD and HFSCD1886 groups than the control groups by the 1st month of feeding and remained this way for the duration. NO-1886 also slightly raised plasma TC when normal diet was added the drug in the process of experiment. No overall difference in plasma TC could be detected between the HFSCD group and HFSCD1886 group. In the 1st and 2nd month, administration of NO-1886 in HFSCD group caused an increase of plasma TC, even higher than HFSCD group. We consider that this was because of an increase of plasma HDL-C level, which is significantly raised by NO-1886 (Table 3).

Plasma HDL-C and apoA-I

HDL cholesterol levels in HFSCD fed with and without NO-1886 groups were linearly elevated with time. A significant increase in HDL cholesterol was observed as early as at the end of the 1st month in comparison with the control group (HFSCD group: 1.07 ± 0.16 mmol/L; HFSCD1886 group: 1.23 ± 0.24 mmol/L vs. 0.80 ± 0.16 mmol/L in the control group). Furthermore, the HFSCD1886 group had a statistically significantly higher level of HDL cholesterol from month 2 compared to the HFSCD group and remained so for the duration. NO-1886 administered to normal diet-fed animals also caused an increase of HDL level in CD1886 group (1.40 ± 0.33 mmol/L in CD1886 group vs. 0.99 ± 0.12 mmol/L in CD group, at the end of month 3, $P < 0.05$, Table 3). The changing patterns of plasma apoA-I were similar to HDL-C. Along with the experiment carrying on, apoA-I levels increased gradually and significantly in NO-1886 supplemented groups (Table 3).

Plasma TC/HDL-C ratio

The TC/HDL-C ratio predicts coronary heart disease risk regardless of the absolute TC and HDL-C. We detected that plasma TC/HDL-C ratio descended in NO-1886 supplemental groups. As show in Table 3, the data of plasma TC/HDL-C ratio suggested that the increase in plasma total cholesterol in NO-1886 supplemental groups was primarily a reflection of an increase in HDL cholesterol.

Plasma TG

Plasma triglyceride concentrations were significantly increased in miniature pigs fed the HFSCD from the 2nd month of the experimentation, and this persisted for the remainder of the study period and reached a plateau at month 5. Administration of NO-1886 markedly decreased plasma triglyceride ($1.09\pm 0.25\text{mmol/L}$ in HFSCD1886 group vs. $2.10\pm 0.25\text{mmol/L}$ in HFSCD group, $P < 0.01$, Table 3). Similar to this, plasma triglyceride was $0.44\pm 0.06\text{mmol/L}$ in CD1886 group vs. $0.72\pm 0.13\text{mmol/L}$ in CD group at the 5th month ($P < 0.05$, Table 3).

Plasma FFA

There was no difference in plasma free fatty acid among the four groups before the start of the experiment. However, FFA levels in the HFSCD group rapidly elevated at the end of month 3, which was 2.4-fold higher than that in the control group, and rose gradually during the months left. Supplement of NO-1886 significantly lowered the level of FFA approximately to that of the control group ($0.393\pm 0.076\text{mmol/L}$ in HFSCD1886 group vs. $0.982\pm 0.247\text{mmol/L}$ for the HFSCD group, $P < 0.01$), but there was no statistical difference between CD1886 group and CD group (Table 3).

Plasma glucose

Plasma glucose concentration in HFSCD fed animals was linearly elevated with time and reached a plateau of $10.1\pm 2.3\text{mmol/L}$ at month 5, which was 1.0-fold higher than that in the CD group. Administration of NO-1886 markedly lowered the level of plasma glucose ($5.5\pm 1.2\text{mmol/L}$ in HFSCD1886 group vs. $10.1\pm 2.3\text{mmol/L}$ in

HFSCD group, $P < 0.01$, Table 3).

Effect of NO-1886 on fatty streak formation

As it shown in figure 1, the aortas were prone to develop fatty streak lesions in HFSCD-fed pigs. Relative aortic fatty streak lesion area was $43.6 \pm 12.8\%$ for HFSCD group and $16.4 \pm 8.2\%$ for HFSCD1886 group ($P < 0.01$). Thus, supplementing NO-1886 into high-fat/high-sucrose/high-cholesterol diet resulted in a remarkable amelioration in aortic fatty streak lesions. No fatty streak was observed in aortas of the pigs from CD1886 and CD groups.

Chemical analysis of common carotid arteries

To further evaluate the extent of atherosclerosis, common carotid arteries from four dietary groups were analyzed for TC and cholesterol ester deposition (figure 2). Total cholesterol contents of the arteries were 76.7% higher in the HFSCD group than in pigs from HFSCD1886 group ($P < 0.01$), cholesterol ester levels also increased by 97.8% ($P < 0.01$) in the HFSCD group. Free cholesterol content in the common carotid artery was modestly increased by 52.7% in the HFSCD group ($P < 0.05$).

Up-regulation of ABCA1 in the tissues of NO-1886 treated minipigs

To cast light on the effect of NO-1886 on ABCA1, we investigated expression levels of mRNA and protein for ABCA1 in tissue samples from the liver, retroperitoneal adipose tissue and aorta of the pigs. ABCA1 mRNAs levels were the

highest in the tissues of HFSCD1886 group compared with pigs in HFSCD, CD1886 and CD groups. ABCA1 levels in tissues of CD group animals represented the basal expression of ABCA1 in pigs. High-fat/high-sucrose/high-cholesterol feeding increased ABCA1 mRNA levels in the tissue samples from HFSCD group. Moreover, administration of NO-1886 stimulated further higher protein expression of ABCA1 in HFSCD1886 group; the levels of ABCA1 protein in the liver, retroperitoneal adipose tissue and aorta of HFSCD1886 group were 236.9%, 18.6% and 108.6% higher than that in HFSCD group respectively (Figure 3). All protein levels were assessed by densitometry with β -actin as a control.

Up-regulation of LXR α protein levels in the tissues of NO-1886 treated minipigs

ABCA1 expression is dependent on the nuclear receptors LXR α (12), so we next examined whether LXR α was affected by NO-1886. Figure 4 shows that LXR α protein levels were markedly increased in the liver, retroperitoneal adipose and aorta of the pigs from HFSCD1886 group compared with the pigs from HFSCD, CD1886 and CD groups. High-fat/high-sucrose/high-cholesterol feeding induced increased LXR α protein levels in tissues of HFSCD group animals. Moreover, administration of NO-1886 stimulated more expression of LXR α protein in HFSCD1886 group than that in HFSCD group. The levels of LXR α protein in the liver of HFSCD1886 group were 63.5% higher than that in HFSCD group (Figure4). Moreover, the protein levels of LXR α were the highest in the retroperitoneal adipose tissue and aorta of HFSCD1886 group in comparison with pigs from HFSCD, CD1886 and CD groups.

The levels of LXR α protein in the retroperitoneal adipose tissue of HFSCD1886 group were 44.9% higher than that in HFSCD group (Figure 4). Furthermore, in the aorta, LXR α protein was 39.0% higher than that in HFSCD group (Figure 4). In addition, normal diet plus NO-1886 slightly increased LXR α protein level in CD1886 group compared with CD group (Figure 4). All protein levels were assessed by densitometry with β -actin as a control.

Change of SR-BI protein levels in liver

SR-BI was the first molecularly well-defined cell-surface HDL receptor to be described. The liver expresses the highest levels of total tissue SR-BI protein, a finding consistent with the major role of the liver in selective HDL-cholesterol uptake. The level of SR-BI protein was increased by NO-1886 in the liver of the pigs from HFSCD1886 group, which was 57.4% higher than that of the pigs from HFSCD group. But the levels of SR-BI protein in normal diet groups were lower despite of supplement of NO-1886 or not. All protein levels were assessed by densitometry with β -actin as a control.

Plasma LPL and ABCA1 of aorta were independently associated with lesion area

We next performed multivariate linear regression analysis to evaluate the relationship among levels of ABCA1, SR-BI, LPL and lesion areas of the aorta in HFSCD fed minipigs (Table 4). The levels of LPL in plasma (standard correlation coefficients [B] = -0.445, P = 0.028) and the levels of ABCA1 in Artery (B = -0.390,

P = 0.036) were independently associated with lesion areas.

Discussion

The high cholesterol, high TG, and low HDL-C are important risk factors for coronary artery disease (CAD). TC/HDL ratio can be calculated and used to determine the risk of developing atherosclerosis and consequent CAD. In the present study, we fed minipigs a high-fat/high- sucrose/high- cholesterol diet (HFSCD) to establish dyslipidemia and atherosclerosis, and investigated the effects of NO-1886 treatment. Our results showed that feeding HFSCD caused obvious atherosclerotic lesions and increased plasma TC, TG, and FFA levels. Administration of NO-1886 decreased plasma triglycerides, FFA, glucose and TC/HDL-C ratio, and increased plasma HDL-C and apoA-I levels.

HFSCD induced an atherogenic lipoprotein profile, such as hypertriglyceridemia, low HDL, and serious hypercholesterolemia. This characteristic plasma lipid profile may be attributed to increased production of VLDL-triglyceride and apolipoprotein B (apoB) in the liver (15, 16). Availability of substrates, in particular FFA, is thought to be an important factor in increased hepatic VLDL production (17). The complexation, down-regulating the expression of ABCA1 and LXR α , would worsen the hypercholesterolemia and serious atherosclerosis observed in HFSCD group of this study.

It was previously reported that NO-1886 increased LPL mRNA levels, increased LPL protein mass and LPL activity in post-heparin plasma, and reduced plasma triglyceride levels with concomitant elevation of HDL-C levels in animals with lipid

disorder. Recently, we found that NO-1886 also had a plasma glucose-reducing action in high-fat/high-sucrose diet-induced diabetic rabbits and improves the glucose metabolism in high-fat/high-sucrose diet-induced diabetic minipigs by decreasing fat deposit, and suppressing plasma $\text{TNF}\alpha$ (1, 18, 19). In the present study, we have found that NO-1886 also elevated ABCA1, the gatekeeper for eliminating excess tissue cholesterol (20), in minipigs. Administration of NO-1886 also increases LXR α levels. Multivariate linear regression analysis showed that the level of ABCA1 in artery ($B = -0.390$, $P = 0.036$) independently associated with lesion area on the abdominal portion of the aorta. It is therefore speculated that NO-1886 may play an important role in the 'first step' of RCT in vivo and suppressing atherosclerosis related to upregulating ABCA 1.

In the current study, we confirmed that hyperlipemia was ameliorated by NO-1886 administration as we found in our previous studies. Furthermore, this time, we observed that NO-1886 increased plasma ApoA-I levels. ApoA-I is a prototypical cholesterol acceptor. It is thought that free cholesterol effluxed from macrophage foam cells is transferred to the surface of HDL, where it can be esterified by lecithin cholesterol acyltransferase and incorporated into the HDL core. Castro G, et al. suggested that HDL containing human apoA-I in human apoA-I transgenic mice is an effective participant in the postulated early steps in RCT (21). Burger D, et al. demonstrated that HDL-associated apoA-I is a specific inhibitor of cytokine production in monocyte-macrophages upon contact with stimulated T cells. HDL-associated apoA-I might play the role of a constitutive anti-inflammatory factor.

The decrease in plasma levels of HDL-associated apoA-I in acute inflammation may be a sign of the possible development of chronic inflammation (22). Clay MA, et al. suggested HDL containing apoA-I mediated inhibition of vascular cell adhesion molecule (VCAM)-I and E-selectin expression in the development of atherosclerosis (23). Deckert V, et al. demonstrated that atherogenic lipoproteins can impair the endothelium-dependent arterial relaxation, and circumstantial evidence suggests a beneficial role of plasma HDL and apoA-I in counteracting the endothelium dysfunction (24). Therefore, the effect of NO-1886 on plasma apoA-I level is considered to be significantly important to protect against the development of atherosclerosis in the studied animals.

RCT is the process in which peripheral cells release cholesterol to an extracellular acceptor such as HDL, which then mediates cholesterol delivery to the liver for excretion. RCT represents a physiological mechanism by which peripheral tissues are protected against excessive accumulation of cholesterol. The first step in RCT is the interaction of the cells with lipoprotein particles, a process that results in both the cellular uptake and release of cholesterol. ABCA1 mediates the cellular efflux of phospholipids and cholesterol to lipid-poor apoA-I and plays a significant role in HDL metabolism and process of RCT (20). ABCA1's role in the causation of Tangier disease, characterized by absent HDL and premature atherosclerosis, has implicated this transporter and its regulators, LXRs as new candidates potentially influencing the progression of atherosclerosis (12). Liver X receptors (LXR α and LXR β) are members of the un-fully understood receptor superfamily and intermediates in the

cholesterol synthetic pathway. The pivotal role of LXRs in the metabolic conversion of cholesterol to bile acids is well established. It has been confirmed that LXRs regulates a number of target genes involved in both cholesterol and fatty acid metabolism in liver, macrophages and intestine such as ABCA1, ABCG5 and ABCG8 (25-27). The observation that LXR α is responsive to fatty acids and is expressed in metabolic tissues suggests that it also plays a general role in lipid metabolism. The results in the present study show that a long term feeding of HFSCD increases ABCA1 and LXR α slightly compared with the control diet group. This may be a sort of compensation to resist the toxic effect of excess cholesterol. NO-1886 increased ABCA1 and LXR α markedly in the HFSCD1886 group compared with the HFSCD group. Up-regulation of ABCA1 and LXR α by NO-1886 would induce more cholesterol efflux from the vessel smooth muscle cell and macrophage, inhibiting the formation of foam cell and reduce aortic fatty streak lesion area. Moreover, adipose cells that specialize in energy storage, and contain large intracellular triglyceride-rich lipid droplets, are enriched with free cholesterol, and express sterol-regulated transcription factors such as liver X receptor (LXR). High cholesterol content in adipose tissue may affect the reverse cholesterol transport mediated by HDL. Impairments in this system may be one possible factor favoring the development of atherosclerosis in obesity. The alterations in the first step of RCT are tightly associated with the abdominal distribution of fat mass (28). Up-regulation of ABCA1 and LXR α by NO-1886 would induce more cholesterol efflux from retroperitoneal adipose and postpone development of atherosclerosis.

The scavenger receptor class B type I (SR-BI) was the first molecularly well-defined cell-surface HDL receptor to be described. SR-BI mediates selective HDL cholesterol uptake by formation of a productive lipoprotein/receptor complex, which requires specific structural domains and conformation states of apolipoprotein A-I present in HDL particles. The importance of SR-BI in overall HDL cholesterol metabolism and its antiatherogenic activity *in vivo* has been definitively established by SR-BI gene manipulation in mice (29). The overexpression of SR-BI studies clearly demonstrate that hepatic overexpression of SR-BI can be antiatherogenic. This may be due to changes in structures and quantities of circulating lipoproteins or increases in HDL-cholesterol flux to the liver, i.e., increased reverse cholesterol transport (30). In our study, high-fat/high-sucrose/high-cholesterol feeding induced an increase of plasma levels of HDL and upregulation of hepatic SR-BI expression in the animals. SR-BI expression was further enhanced by NO-1886 administration, implicating HDL cholesteryl ester clearance, biliary cholesterol content and transport of cholesterol from the liver into the bile could be increased.

In summary, HFSCD-fed minipigs developed hyperlipidemic condition, and induced distinct fatty streak lesion in the aortas. The administration of the LPL activator NO-1886 significantly increased the levels of plasma HDL-C and apoA-I and expression of ABCA1 that contributes to alleviating cholesterol toxicity and ameliorating lipid disorder, results in protection against the development of atherosclerosis.

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References

1. Tsutsumi K, Inoue Y, Shima A, Iwasaki K, Kawamura M, and Murase T. 1993. The novel compound NO-1886 increases lipoprotein lipase activity with resulting elevation of high density lipoprotein cholesterol, and long-term administration inhibits atherogenesis in the coronary arteries of rats with experimental atherosclerosis. *J Clin Invest.* 92: 411-417.
2. Tsutsumi K, Inoue Y, Shima A, and Murase T. 1995. Correction of hypertriglyceridemia with low high-density lipoprotein cholesterol by the novel compound NO-1886, a lipoprotein lipase-promoting agent, in STZ-induced diabetic rats. *Diabetes.* 44: 414-417.
3. Garfagnini A, Devoto G, Rosselli P, Boggiano P, and Venturini M. 1995. Relationship between HDL-cholesterol and apolipoprotein A1 and the severity of coronary artery disease. *Eur. Heart J.* 16: 465-470.
4. Zampogna A, Luria MH, Manubens SJ, and Luria MA. 1980. Relationship between lipids and occlusive coronary artery disease. *Arch Intern Med.* 140:1067-1069.
5. Kannel WB. 2000. The Framingham Study: ITS 50-year legacy and future promise. *J Atheroscler Thromb.* 6:60-66.
6. Fielding CJ, Fielding PE. 1995. Molecular physiology of reverse cholesterol transport. *J Lipid Res.* 36:211-228.
7. Rothblat GH, de la Llera-Moya M, Atger V, Kellner-Weibel G, Williams DL, and Phillips MC. 1999. Cell cholesterol efflux: integration of old and new observations provides new insights. *J. Lipid. Res.* 40(5): 781-796.
8. Tall AR. 1998. An overview of reverse cholesterol transport. *Eur Heart J.* 19 Suppl A: A31-35.
9. Finking G, Hanke H. 1997. Nikolaj Nikolajewitsch Anitschkow(1885-1964) established the cholesterol-fed rabbit as a model for atherosclerosis research. *Atherosclerosis* 135: 1-7.
10. Murase T, Uchimura H. 1980. A selective decline of post-heparin plasma hepatic

- triglyceride lipase in hypothyroid rats. *Metabolism*. 29: 797-801.
11. Staprans I, Rapp JH, Pan XM, Hardman DA, and Feingold KR. 1996. Oxidized lipids in the diet accelerate the development of fatty streaks in cholesterol-fed rabbits. *Arterioscler Thromb Vasc Biol*. 16: 533-538.
 12. Folch J, Lees M, and Sloane Stanley GH. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem*. 226:497-509.
 13. Rapp JH, Connor WE, Lin DS, Inahara T, and Porter JM. 1983. Lipids in human atherosclerotic plaques and xanthomas: clues to the mechanism of plaque progression. *J Lipid Res*. 24:1329-1335.
 14. Singaraja RR, Bocher V, James ER, Clee SM, Zhang LH, Leavitt BR, Tan B, Brooks-Wilson A, Kwok A, Bissada N, Yang YZ, Liu G, Tafuri SR, Fievet C, Wellington CL, Staels B, and Hayden MR. 2001. Human ABCA1 BAC transgenic mice show increased high density lipoprotein cholesterol and ApoAI-dependent efflux stimulated by an internal promoter containing liver X receptor response elements in intron 1. *J Biol Chem*. 276:33969-33979.
 15. Nikkila EA, Kekki M. 1973. Plasma triglyceride transport kinetics in diabetes mellitus. *Metabolism*. 22:1-22.
 16. Cummings MH, Watts GF, Umpleby AM, Hennessy TR, Naoumova R, Slavin BM, Thompson GR, and Sonksen PH. 1995. Increased hepatic secretion of very-low-density lipoprotein apolipoprotein B-100 in NIDDM. *Diabetologia*. 38: 959-967.
 17. Lewis GF. 1997. Fatty acid regulation of very low density lipoprotein production. *Curr Opin Lipidol*. 8:146-153.
 18. Yin W, Liao D, Kusunoki M, Xi S, Tsutsumi K, Wang Z, Lian X, Koike T, Fan J,

- Yang Y, and Tang C. 2004. NO-1886 decreases ectopic lipid deposition and protects pancreatic beta cells in diet-induced diabetic swine. *J Endocrinol.* 180: 399-408.
19. Yin W, Liao D, Wang Z, Xi S, Tsutsumi K, Koike T, Fan J, Yi G, Zhang Q, Yuan Z, and Tang K. 2004. NO-1886 inhibits size of adipocytes, suppresses plasma levels of tumor necrosis factor-alpha and free fatty acids, improves glucose metabolism in high-fat/high-sucrose-fed miniature pigs. *Pharmacol Res.* 49:199-206.
20. Oram JF, Lawn RM. 2001. ABCA1: The gatekeeper for eliminating excess tissue cholesterol. *J Lipid Res.* 42: 1173-1179.
21. Castro G, Nihoul LP, Dengremont C, de Geitere C, Delfly B, Tailleux A, Fievet C, Duverger N, Deneffe P, Fruchart JC, and Rubin EM. 1997. Cholesterol efflux, lecithin-cholesterol acyltransferase activity, and pre-beta particle formation by serum from human apolipoprotein A-I and apolipoprotein A-I/apolipoprotein A-II transgenic mice consistent with the latter being less effective for reverse cholesterol transport. *Biochemistry.* 36: 2243-2249.
22. Burger D, Dayer JM. 2002. High-density lipoprotein-associated apolipoprotein A-I : the missing link between infection and chronic inflammation? *Autoimmunity Rev.* 1:111-117.
23. Clay MA, Pyle DH, Rye KA, Vadas MA, Gamble JR, and Barter PJ. 2001. Time sequence of the inhibition of endothelial adhesion molecule expression by reconstituted high density lipoproteins. *Atherosclerosis.* 157: 23-29.
24. Deckert V, Lizard G, Duverger N, Athias A, Palleau V, Emmanuel F, Moisant M, Gambert P, Lallemand C, and Lagrost L. 1999. Impairment of endothelium dependent arterial relaxation by high fat feeding in apoE-deficient mice: toward normlization by human apoA- I expression. *Circulation.* 100: 1230-1235.
25. Kruit JK, Plosch T, Havinga R, Boverhof R, Groot PH, Groen AK, and Kuipers F. 2005. Increased fecal neutral sterol loss upon liver X receptor activation is independent of biliary sterol secretion in mice. *Gastroenterology.* 128(1): 147-156.
26. Hoekstra M, Kruijt JK, Van Eck M, and Van Berkel TJ. 2003. Specific gene

- expression of ATP-binding cassette transporters and nufully understood hormone receptors in rat liver parenchymal, endothelial, and Kupffer cells. *J Biol Chem.* 278: 25448-25453.
27. Repa JJ, Berge KE, Pomajzl C, Richardson JA, Hobbs H, and Mangelsdorf DJ. 2002. Regulation of ATP-binding cassette sterol transporters ABCG5 and ABCG8 by the liver X receptors alpha and beta. *J Biol Chem.* 277: 18793-18800.
28. Aufran D, Guerci B, Paul JL, Moulin P, Verges B, Durlach V, and Girard-Globa A. 2001. Basal and postprandial serum-promoted cholesterol efflux in normolipidemic subjects: Importance of fat mass distribution. *Metabolism.* 50: 1330-1335.
29. Trigatti BL, Krieger M, and Rigotti A. 2003. Influence of the HDL receptor SR-BI on lipoprotein metabolism and atherosclerosis. *Arterioscler Thromb Vasc Biol.* 23: 1732-1738.
30. Rigotti A, Miettinen HE. and Krieger M. 2003. The Role of the High-Density Lipoprotein Receptor SR-BI in the Lipid Metabolism of Endocrine and Other Tissues. *Endocrine Reviews.* 24: 357-87.

Figure legends

Figure 1. Scatterplot showing the lesion area on the abdominal portion of the aorta of Chinese Bama miniature pigs fed control diet (◆), control diet plus NO-1886 (●), HFSCD diet (▲) and HFSCD diet plus NO-1886 (▼), n=7 for CD and CD1886 groups, n=8 for HFSCD and HFSCD1886 groups.

Figure 2. Bar graphs showing cholesterol and cholesterol ester deposition in the common carotid arteries of the pigs fed control diet, control diet +NO-1886, HFSCD diet, HFSCD diet supplemented NO-1886 for 5 months. Data are expressed as mean \pm S.D. in μmol cholesterol per g arterial tissue (wet weight) ** indicates $P < 0.01$, * indicates $P < 0.05$. n=7 for CD and CD+NO-1886 group, n=8 for HFSCD and HFSCD+ NO-1886 group.

Figure 3. ABCA1 mRNA levels in Liver, retroperitoneal adipose tissue and aorta as determined by quantitative real-time PCR. Ratios of the ABCA1 to β -actin transcripts are expressed as a percentage of the β -actin control. * indicates $P < 0.05$, ** indicates $P < 0.01$. Data are expressed as mean \pm S.D. from three independent experiments, each performed in triplicate.

Figure 4. Western blotting analysis of ATP-binding cassette transporter (ABCA1), Liver X Receptor α (LXR α), and scavenger receptor- type B class 1 (SR-BI) protein

levels in the liver, retroperitoneal adipose and aorta. The details of experiments are described in Materials and methods. All protein levels were assessed by densitometry with β -actin as a control. (A) ABCA1, LXR α , and SR-BI proteins were analyzed from various tissues. (B) Statistical graphs of ABCA1, LXR α , and SR-BI protein levels. * indicates $P < 0.05$, ** indicates $P < 0.01$. Data are expressed as mean \pm S.D. from three independent experiments, each performed in triplicate.

Figure 1

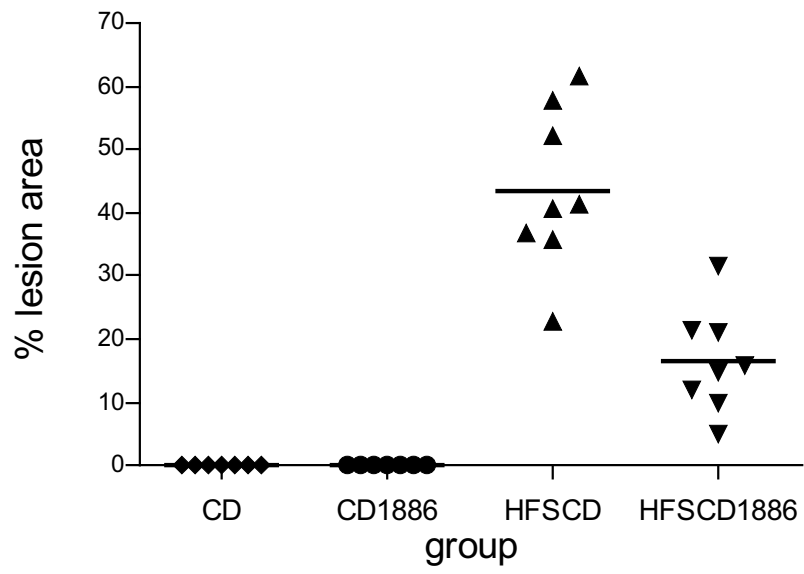


Figure 2

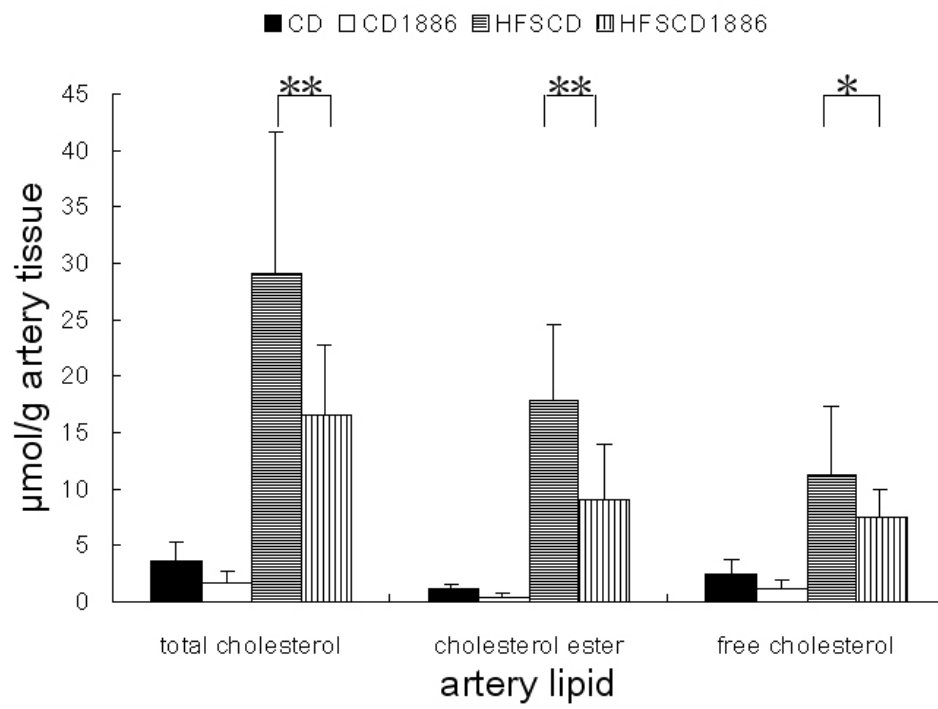


Figure 3

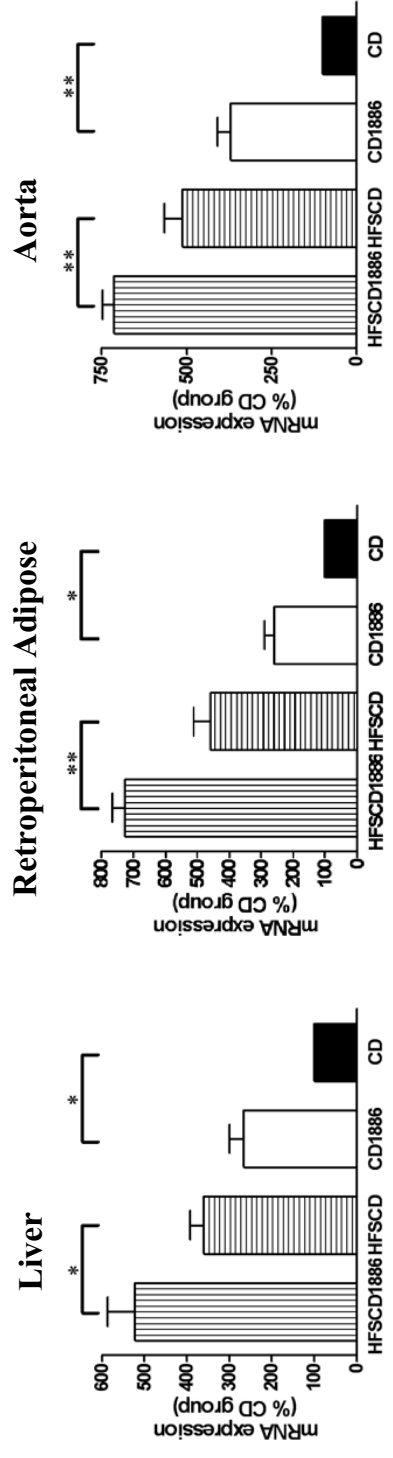


Figure 4A

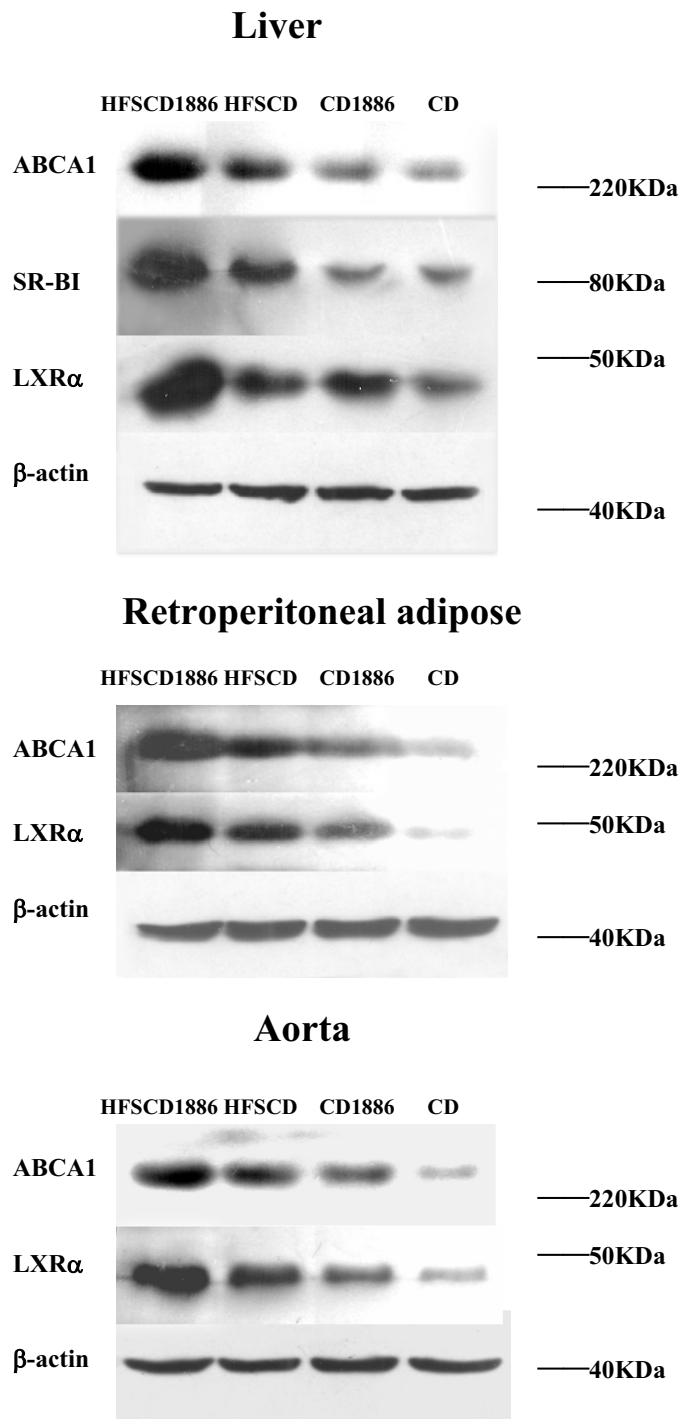
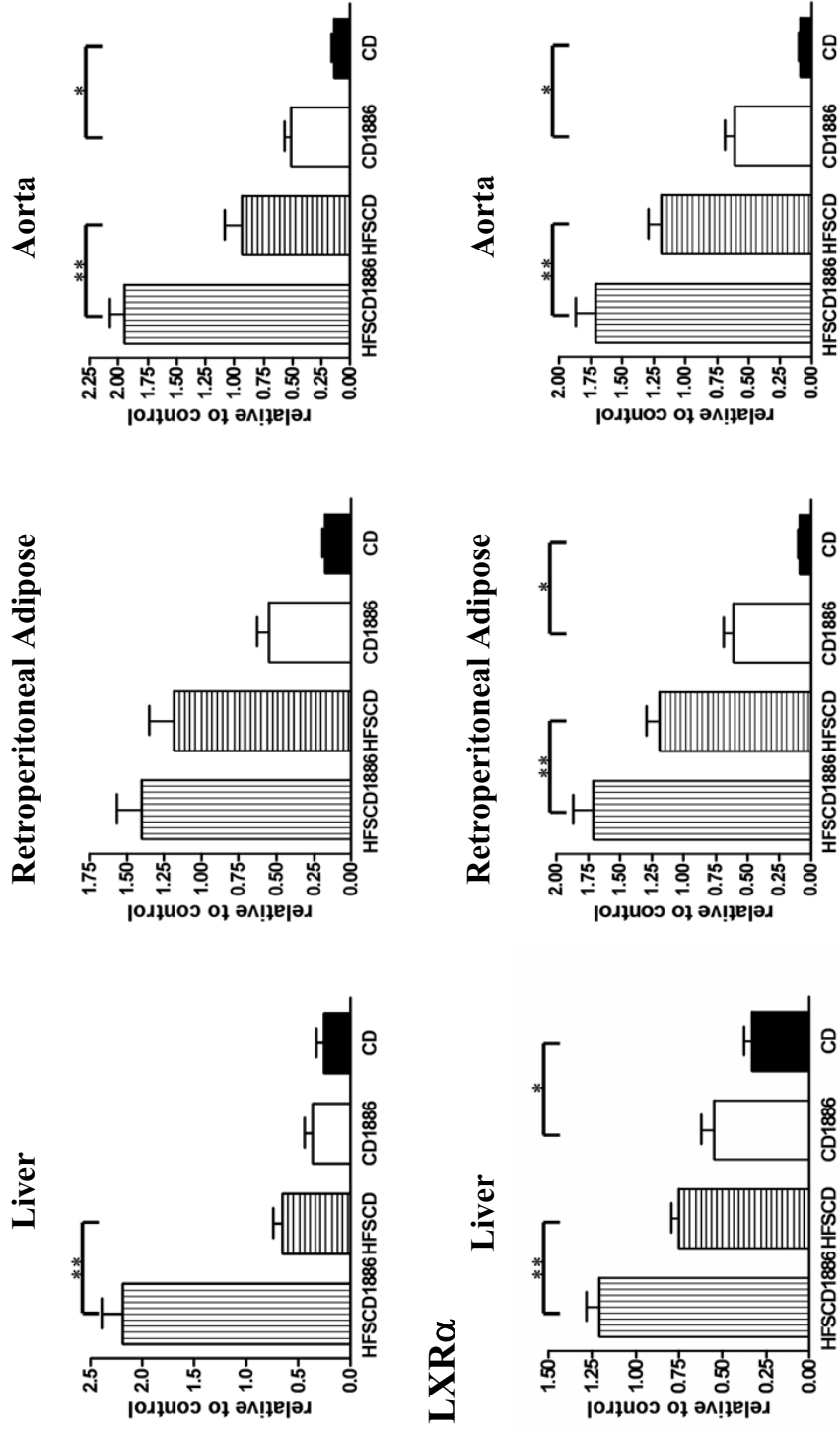


Figure 4B
ABCA1



SR-BI

Liver

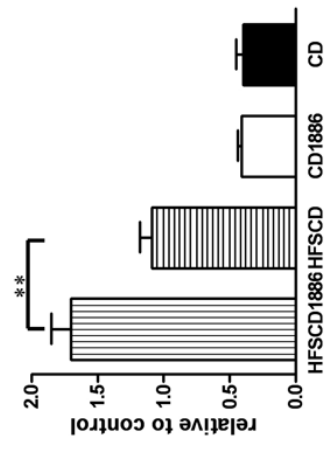


Table 1: The components of the diets

Components	Control diet (%)	High-fat /high-sucrose/ high-cholesterol diet (%)
Rice	64.11	31.98
Wheat bran	10.51	5.57
Soybean meal	11.98	6.35
Cottonseed meal	4	2.12
Colza meal	4	2.12
Fish powder	2	1
Bone powder	1.1	0.5
Calcium bicarbonate	0.8	0.8
Salt	0.5	0.5
Trace elements	0.5	0.5
Vitamins	0.5	0.5
Pork lard		10
Sucrose		37
Cholesterol		2

Table 2: Postheparin plasma LPL activity (nmol/ml/min)

	HFSCD1886	HFSCD	CD1886	CD
Postprandial	47.6±9.6 ^{*#}	31.9±8.1 *	16.4±4.2 *	7.3±1.7
Fasting	9.3±2.7 *	8.6±1.8 *	5.6±1.1	4.1±0.8

**P*<0.05 vs. CD group; #*P*<0.05 vs. HFSCD group, n=7 for CD and CD1886 groups, n=8 for HFSCD and HFSCD1886 groups.

Table 3: Fasting plasma lipid and glucose concentrations

Parameters	0	1 month	2 months	3 months	4 months	5 months
Total cholesterol (mmol/L)						
CD	2.1±0.4	2.0±0.3	1.9±0.3	1.7±0.3	1.8±0.5	2.1±0.5
CD1886	2.1±0.5	2.0±0.4	2.2±0.4	2.0±0.5	2.0±0.5	2.6±0.6
HFSCD	2.2±0.5	6.3±1.4**	9.1±1.2**	11.2±2.3**	14.9±1.7**	20.5±3.3**
HFSCD1886	2.3±0.5	7.9±1.9#	11.2±2.2##	12.8±3.0	16.2±3.4	21.4±4.0
High density lipoprotein (mmol/L)						
CD	0.77±0.12	0.80±0.16	0.87±0.15	0.99±0.12	0.87±0.15	0.94±0.23
CD1886	0.82±0.12	0.85±0.18	1.12±0.37	1.40±0.33*	1.34±0.30*	1.36±0.28*
HFSCD	0.78±0.12	1.07±0.16*	1.41±0.31**	1.81±0.44**	1.96±0.45**	1.94±0.45**
HFSCD1886	0.79±0.15	1.23±0.24	2.41±0.38##	2.87±0.47##	3.01±0.46##	2.86±0.46##
Apoprotein A I (g/L)						
CD	0.098±0.020	0.102±0.021	0.094±0.023	0.094±0.044	0.089±0.036	0.105±0.027
CD1886	0.103±0.017	0.107±0.018	0.114±0.024	0.126±0.041	0.198±0.047*	0.188±0.044
HFSCD	0.100±0.020	0.360±0.105**	0.415±0.116**	0.557±0.132**	0.504±0.085**	0.519±0.084**
HFSCD1886	0.094±0.015	0.471±0.092##	0.528±0.106#	0.719±0.164##	0.705±0.127##	0.756±0.125##
Total cholesterol /High density lipoprotein ratio						
CD	2.8±0.8	2.6±0.6	2.3±0.5	1.8±0.3	2.3±0.9	2.3±0.7
CD1886	2.6±0.6	2.4±0.6	2.1±0.5	1.5±0.3	1.5±0.3	2.0±0.7
HFSCD	2.6±0.7	6.0±1.7**	6.7±1.6**	6.4±1.5**	7.3±1.4**	10.4±2.9**
HFSCD1886	3.0±1.1	6.5±1.3	4.8±1.4##	4.5±0.8##	5.3±0.5##	7.3±1.2##
Triglyceride (mmol/L)						
CD	0.59±0.05	0.60±0.05	0.63±0.08	0.68±0.14	0.69±0.12	0.72±0.13
CD1886	0.57±0.05	0.41±0.08**	0.42±0.09**	0.44±0.08**	0.44±0.09	0.44±0.06*
HFSCD	0.59±0.04	0.97±0.11**	1.50±0.20**	1.87±0.18**	1.90±0.36**	2.10±0.25**
HFSCD1886	0.58±0.05	0.60±0.03##	0.75±0.13##	0.89±0.17##	1.01±0.20##	1.09±0.25##
Free fatty acids (mmol/L)						
CD	0.111±0.024	0.222±0.040	0.240±0.035	0.281±0.037	0.288±0.056	0.293±0.045
CD1886	0.112±0.014	0.196±0.090	0.231±0.046	0.285±0.059	0.289±0.059	0.294±0.053
HFSCD	0.110±0.021	0.253±0.055	0.315±0.048**	0.759±0.166**	0.998±0.176**	0.982±0.247**
HFSCD1886	0.136±0.041	0.193±0.042	0.253±0.031##	0.366±0.092##	0.450±0.124##	0.393±0.076##

Glucose (mmol/L)	4.7±0.5	4.5±0.8	4.8±0.7	5.2±0.9	4.7±0.8	4.9±0.5
CD	4.7±0.5	4.5±0.8	4.8±0.7	5.2±0.9	4.7±0.8	4.9±0.5
CD1886	4.8±0.6	4.7±0.8	4.5±0.6	4.5±1.0	5.0±1.0	4.7±0.7
HFSCD	4.8±0.8	5.9±0.9*	7.3±1.8**	9.1±2.0**	9.7±2.3**	10.1±2.3**
HFSCD1886	4.9±0.9	4.7±1.7#	5.0±1.2##	6.1±1.1##	5.3±1.2##	5.5±1.2##

Values are means ± S.D.; * $P < 0.05$ vs. CD; ** $P < 0.01$ vs. CD; # $P < 0.05$ vs HFSCD group, ## $P < 0.01$ vs HFSCD group, n=7 for CD and CD1886 groups, n=8 for HFSCD and HFSCD1886 groups

Table 4: Multivariate linear regression analysis of relationships between the levels of ABCA1, SR-BI, LPL and the lesion area of the aorta

Independent variables	Standardized Coefficients (B)	P
(Constant)	0	<0.001
LPL	-0.445	0.028
ABCA1 (Liver)	-0.076	0.572
ABCA1 (Adipose)	-0.094	0.425
ABCA1 (Artery)	-0.390	0.036
SR-BI (Liver)	-0.051	0.725

Dependent variable: lesion area. $R^2 = 0.969$, $F = 61.607$, $P < 0.001$.