NO-1886 suppresses diet-induced insulin resistance and cholesterol accumulation through STAT5-dependent upregulation of IGF1 and CYP7A1

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Abstract

Insulin resistance and dyslipidemia are both considered to be risk factors for metabolic syndrome. Low levels of IGF1 are associated with insulin resistance. Elevation of low-density lipoprotein cholesterol (LDL-C) concomitant with depression of high-density lipoprotein cholesterol (HDL-C) increase the risk of obesity and type 2 diabetes mellitus (T2DM). Liver secretes IGF1 and catabolizes cholesterol regulated by the rate-limiting enzyme of bile acid synthesis from cholesterol 7 α -hydroxylase (CYP7A1). NO-1886, a chemically synthesized lipoprotein lipase activator, suppresses diet-induced insulin resistance with the improvement of HDL-C. The goal of the present study is to evaluate whether NO-1886 upregulates IGF1 and CYP7A1 to benefit glucose and cholesterol metabolism. By using human hepatoma cell lines (HepG2 cells) as an *in vitro* model, we found that NO-1886 promoted IGF1 secretion and CYP7A1 expression through the activation of signal transducer and activator of

transcription 5 (STAT5). Pretreatment of cells with AG 490, the inhibitor of STAT pathway, completely abolished NO-1886-induced IGF1 secretion and CYP7A1 expression. Studies performed in Chinese Bama minipigs pointed out an augmentation of plasma IGF1 elicited by a single dose administration of NO-1886. Long-term supplementation with NO-1886 recovered hyperinsulinemia and low plasma levels of IGF1 suppressed LDL-C and facilitated reverse cholesterol transport by decreasing hepatic cholesterol accumulation through increasing CYP7A1 expression in high-fat/high-sucrose/high-cholesterol diet minipigs. These findings indicate that NO-1886 upregulates IGF1 secretion and CYP7A1 expression to improve insulin resistance and hepatic cholesterol accumulation, which may represent an alternative therapeutic avenue of NO-1886 for T2DM and metabolic syndrome.

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Introduction

The metabolic syndrome (syndrome X) refers to the aggregation of a cluster of risk factors, including insulin resistance/hyperinsulinemia and dyslipidemia (increased low-density lipoprotein cholesterol (LDL-C), and depressed high-density lipoprotein cholesterol (HDL-C)), and hypertension (Moller & Kaufman 2005).

Insulin-like growth factor 1 (IGF1) is a protein hormone similar in molecular structure to insulin. Nonetheless, unlike insulin that is secreted from pancreas, IGF1 is produced primarily from liver in response to GH stimulation. IGF1 has been found to play an important role in embryonic and childhood growth. In adults, IGF1 is crucial to function on glucose homeostasis, lipolysis, proteolysis, and protein oxidation (Froesch *et al.* 1996). Decreased levels of circulating

IGF1 found in diabetic patients have been discovered to be associated with insulin resistance (Moller & Flier 1991, Clark *et al.* 1997, Takano *et al.* 2001). IGF1 directly acts on the balance between GH and insulin to the control of glucose homeostasis (Yakar *et al.* 2001). In addition to the critical role on glucose metabolism, IGF1 interferes with the development of diabetic myopathy (Kajstura *et al.* 2001). High levels of IGF1 prevent diabetic complications induced by enhanced oxidative stress observed in type 2 diabetes mellitus (T2DM) subjects (Brownlee 2005). It is thus believed that IGF1 participates in physiological glucose homeostasis.

Cholesterol functions in the membrane of cells to modulate fluidity. It is also the raw material for manufacturing steroid hormones. However, elevation of tissue cholesterol accumulation accelerates the formation of atherosclerotic plaques, leading to heart attacks and strokes (Brown & Goldstein 1986).

The excessive cholesterol in peripheral tissues obtained from a combination of local synthesis and the uptake from LDL and very LDL (VLDL) is reversely transported to the liver through the process of reverse cholesterol transport (RCT) by HDL particles (Fielding & Fielding 1995). Liver maintains whole body cholesterol homeostasis by regulating the biosynthesis of cholesterol, its uptake from plasma, storage and catabolism to bile acids. Conversion of cholesterol to bile acids in the liver and its subsequent fecal excretion represents a major route for the elimination of cholesterol from the body (Turley & Dietschy 1988). Bile acids can be synthesized via 'classical pathway' and 'alternate pathway' utilizing different sequence of initial steps. Compared with the alternative pathway, the classical pathway is quite 'flexible'. Expressional regulation of the rate-limiting enzyme of cholesterol 7 α -hydroxylase (cytochrome P-450, family 7, subfamily A, and polypeptide 1 (CYP7A1, EC1.14.13.17)) represents a major node for hepatic cholesterol catabolism (Chawla *et al.* 2000). Transcription of *CYP7A1* is stimulated by dietary cholesterol in rodents (Horton *et al.* 1995). In mammals, however, dietary cholesterol fails to stimulate hepatic CYP7A1 expression, resulting in the susceptible accumulation of the peripheral cholesterol and atherosclerosis (Chiang *et al.* 2001, Goodwin *et al.* 2003). Recently, transgenic strategies have proved the favorable effects on dyslipidemia by increasing the expression of CYP7A1 (Ratliff *et al.* 2006), whereas, there is still lack of an effective pharmacological drug to activate CYP7A1 in mammals.

The chemically synthesized compound NO-1886 has been known to activate lipoprotein lipase (LPL) by increasing LPL mRNA and activity (Tsutsumi *et al.* 1993). Recently, NO-1886 has been found to elevate plasma HDL-C levels by upregulating ATP-binding cassette transporter A1 to facilitate cholesterol efflux from peripheral tissues to nascent HDL particles, suppressing fat accumulation and insulin resistance induced by high-carbohydrate, high-fat diet regimen in animal models (Tsutsumi *et al.* 1997, Zhang *et al.* 2006).

Although it has been verified that NO-1886 upregulates HDL-C and RCT, what happens to the excessive hepatic cholesterol uptaken from HDL and/or LDL is still awaiting further elucidation. In this study, we investigated the effects of NO-1886 on IGF1 and CYP7A1 expression. Our data indicate that NO-1886 enhances IGF1 secretion and CYP7A1 expression, which ultimately functions to prevent diet-induced insulin resistance and dyslipidemia. These effects are therefore regarded to be involved in the therapeutic mechanisms of NO-1886 on insulin resistance and dyslipidemia.

Materials and Methods

Reagents

DMEM/Hams F-12 (DMEM/F-12) was purchased from Gibco (Invitrogen Corp). The antibody of anti-CYP7A1 (sc-25536) was from Santa Cruz Biotechnology (Santa Cruz,

CA, USA), anti- β -actin (ab6276) was from Abcam (Cambridge, UK), anti-signal transducer and activator of transcription 5 (STAT5) and anti-phospho-STAT5 (Tyr-694/Tyr-699) were from Cell Signaling Technology, Inc. (Danvers, MA, USA). The Janus kinase 2 (JAK2)/STAT pathway inhibitor AG 490 (PHZ1204) was from BioSource International, Inc. (Camarillo, CA, USA). The enhanced chemiluminescence kit (ECL) was from Amersham Biosciences. Sucrose was from Liuzhou Sugar Co. (Guangxi, China) and lard was from Hengyang Meat Product Co. (Hunan, China). Cholesterol was from Sigma-Aldrich. High sensitive RIA kits for insulin, GH and IGF1 were from Jiuding Bio-Engineering Corporation (Tianjing, China). BCA Protein Assay Kit was from Pierce Biotechnology (Rockford, IL, USA). The compound NO-1886 ([4-(4-bromo-2-cyano-phenylcarbamoyl)-benzyl]-phosphonic acid diethyl ester, Chemical Abstracts Service 133208-93-2; lot No. C99L925M) was chemically synthesized in the New Drug Synthesis Section of Otsuka Pharmaceutical factory Inc. (Tokushima, Japan). All of the other chemical reagents were of either reagent or molecular biological grade from Bio Basic Inc. (Markham, ON, Canada).

Cell culture

Human hepatoblastoma cells HepG2 were obtained from American Type Culture Collection (Rockville, MD, USA) and cultured in DMEM/Hams F-12 with 10% (v/v) fetal bovine serum (FBS, Biofluids, Rockville, MD, USA) supplemented with 100 units/ml penicillin, and 100 μ g/ml streptomycin (Biofluids). Cells were maintained at 37 °C in 5% CO₂. Cells were cultured overnight with serum-free medium (DMEM/F12 medium with 0.1% FBS) before each experiment. In transient transfection assays, constructs (50 μ g) of caSTAT5A1*6 expression plasmid (described elsewhere (Onishi *et al.* 1998)) were electroporated into HepG2 cells. These cells were then cultured for 24 h before experiments.

Animal

Fifteen male Chinese Bama minipigs (*Sus scrofa domestica*) were purchased from Experimental Animal Supply Center of the Third Military Medical University (Chongqing, China), aged 30 $\pm$ 5 days, and weighting 6.3 $\pm$ 1.3 kg. The minipigs were housed in five cages covered with sterile glass chips per room in three rooms at constant temperature of 23 $\pm$ 2 °C and 55% humidity, the light/darkness cycle was 12 h. Before experiments, animals were given laboratory standard chow to acclimate for 2 weeks. The animals were observed daily for clinical signs and custody. For the treatment of single dose of NO-1886, after overnight fasting, six male minipigs were randomly divided into two groups according to their body weight (BW); 1 g/kg BW NO-1886 (suspended in 5% Arabic gum) was orally administered within 10 min. Blood was obtained without sedation by pricking an ear vein with a lancet and collecting drops in a hematocrit tube at each hour

for 8 h. The plasma samples kept frozen at -80°C were analyzed within 1 week. For the experiment of long-term treatment of NO-1886, 15 male minipigs were randomly divided into three groups according to their BW. Animals of the first group were given a daily standard chow (control diet, CD); the second group were fed a daily high-fat/high-sucrose/high-cholesterol diet (HFSCD, normal pig diet supplemented with 10% lard, 37% sucrose, and 2% cholesterol); the third group were fed a daily HFSCD supplemented with NO-1886 diet (100 mg/kg BW/day, HFSCD1886), the components were specifically described previously (Zhang *et al.* 2006). Animals were treated for 5 months; blood samples for plasma lipids, glucose, insulin and GH 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 overnight fasting. At the end of treatment period of 5 months, animals were killed by phlebotomy under anesthesia with i.v. injection of sodium pentobarbital (60 mg/kg). Liver tissues were dissected and stocked in liquid nitrogen until western blot analyses and cholesterol quantification within 7 days. All experimental work was conducted in accordance with the humane guidelines for ethical and sensitive care under the Act of Animal Protective Department of Human Province and conformed to the guideline of the UFAW handbook on the care and management of laboratory animals.

RNA preparation and real-time quantitative RT-PCR

Total RNA isolated from cells was reverse transcribed using TaKaRa PrimeScript RT reagent kits (TaKaRa, Kyoto, Japan). Quantitative real-time PCR was performed with the LightCycler system (Roche Diagnostics) using TaKaRa SYBR Premix Ex Taq (TaKaRa) and the gene-specific primers. After the reaction, each PCR product was verified for its single amplification by melting curve analysis. The expression levels of *CYP7A1* (forward: 5'-CCG ATG GAT GGA AAT ACC AC-3'; reverse: 5'-GGC AGC GGT CTT TGA GTT AG-3') were normalized to the expression of 18S rRNA (forward: 5'-AAA CGG CTA CCA CAT CCA AG-3'; reverse: 5'-GGC CTC GAA AGA GTC CTG TA-3') in each sample and were given arbitrary units. Analyses were performed in triplicate.

Western blot

After treatment as described in the figure legends, cells were rinsed three times with ice-cold PBS (NaCl, 137 mmol/l; KCl, 2.7 mmol/l; Na_2HPO_4 , 10 mmol/l; KH_2PO_4 , 2 mmol/l, pH 7.4) and lysed in RIPA buffer (50 mmol/l Tris, 150 mmol/l NaCl, 1 mmol/l EDTA, 0.5% sodium deoxycholate (w/v), 1% Nonidet P-40 (v/v), 0.1% SDS (w/v), 1 $\mu\text{mol/l}$ aprotinin, 10 $\mu\text{mol/l}$ leupeptin, 0.1 $\mu\text{mol/l}$ phenylmethylsulfonyl fluoride, 20 mmol/l sodium fluoride, 20 mmol/l β -glycerolphosphate and 1 mmol/l sodium orthovanadate, pH 7.4). Cell lysates were obtained by

centrifugation at 15 000 *g* and 4°C for 20 min. Equal amounts of proteins were subjected to SDS-PAGE (Laemmli 1970). Proteins were transferred to an Immobilon-P membrane (Millipore, Bedford, MA, USA). Immunoblotting was performed with an ECL PLUS system according to the manufacturer's instructions. ECL images of immunoblots were scanned with the White/u.v. transilluminator (Upland, CA, USA) and quantified with Labworks image analysis software from UVP Inc. (Upland, CA, USA).

Measurement of IGF1, GH, insulin, glucose, LDL-C, HDL-C, and total cholesterol

For the experiment using HepG2 cells, concentrations of IGF1 and GH were measured in triplicate as described previously (Osada *et al.* 1996). Briefly, HepG2 cells that had 80% confluent state in 6-well plates were used. The cells were washed with PBS and incubated in 2 ml of DMEM/F-12 medium containing 1% FBS for 2, 4, 8, 16, and 24 h with NO-1886 treatment. Medium samples for IGF1 determination were obtained and stored at -80°C until measurement by a commercial RIA kit. Data were normalized by protein assay. For the animal experiment, plasma samples stored at -80°C were used to detect the IGF1 and GH concentration by RIA kit; plasma glucose, LDL-C, HDL-C and total cholesterol were directly measured by commercial kits within 3 h after performance of sampling.

Determination of total hepatic cholesterol content

Quantification of tissue cholesterol was carried out using the method of cholesterol oxidase-peroxidase after ethyl acetate extraction. Briefly, samples of ~ 1 g saponified for 1 h were extracted in triplicate with two volumes of ethyl acetate. After accurately taking 1/10 volume to evaporate ethyl acetate under vacuum, the residue was dissolved in 2 ml glacial acetic acid. The well-suspended solution was applied to determine cholesterol content by colorimetric assay with commercial kit.

Statistical analysis

Each experiment was performed in triplicate and representative data are shown. All of the other results were expressed as means $\pm$ S.E.M. Values of *P* were calculated using ANOVA followed with a Scheffé's *post hoc* test, and values of *P* < 0.05 were considered significant.

Results

NO-1886 increased CYP7A1 expression through activation of STAT5 in HepG2 cells

The CYP7A1 expression is restricted to the liver, and the HepG2 cells have been used as a model for studying the regulation of human CYP7A1 (Pandak *et al.* 1996).

We investigated the dose response and time course of activation of CYP7A1 expression with NO-1886 treatment ranging from 0.01 to 3 µg/ml for 4 h in HepG2 cells. As shown in Fig. 1A, treatment of cells with NO-1886 improved CYP7A1 expression up to 0.3 µg/ml and then reached a

plateau at 3 µg/ml. Figure 1B shows the time course of 0.3 µg/ml NO-1886 treatment on CYP7A1 expression in HepG2 cells. The increase in CYP7A1 expression started to be detected from 2nd hour reaching a maximum at 6th hour and gradually decreasing thereafter.

Figure 1 NO-1886 increased CYP7A1 expression through activation of STAT5 in HepG2 cells. After overnight serum starvation, HepG2 cells were left untreated or treated with: (A) NO-1886 under the dose ranging from 0.01 to 3 µg/ml for 4 h; (B and C) NO-1886 at 0.3 µg/ml for 2–24 h; (D) pretreatment with AG490 for 30 min, then cells were treated with 0.3 µg/ml NO-1886 for another 2 h. Cell lysates were subjected to SDS-PAGE and western blot by anti-CYP7A1 antibodies, anti-STAT5 (pTyr694/699) antibodies, anti-STAT5 antibodies and anti- β -actin antibodies. (E) Cells treated as in (D) were applied to determine CYP7A1 gene transcription by a real-time quantitative PCR described in Materials and Methods. (F) Cells electroporated with FLAG-tagged constitutive active STAT5 (pME-caSTAT5-FLAG) or control plasmid (control) were seeded into 6-well plates as described in Materials and Methods followed by the treatment and detection of CYP7A1, FLAG and β -actin as shown in figure by western blot. A representative image from three independent experiments is shown. Results from all the three experiments were also quantified, and the fold increase is shown. Data are means $\pm$ S.E.M. * P <0.05 and † P <0.01 versus control group; NS, no statistical significance.

The expression of hepatic CYP7A1 is regulated by various factors (Lu *et al.* 2000). Among them, we were especially interested in STAT5, a member of the STAT family of transcriptional factors. In response to cytokines, STAT5 is phosphorylated at the site of tyr694/699 by the receptor-associated kinases, then dimerizes and translocates to the nucleus, where they act as transcription activator (Vidal *et al.* 2007). We hypothesized that NO-1886 might activate CYP7A1 expression through STAT5 activation. As shown in Fig. 1C, similar to CYP7A1 expression, STAT5 tyrosine phosphorylation was significantly upregulated with NO-1886 treatment from 2 to 6 h, and then recovered to the base line gradually. In order to prove whether the STAT5 was involved into the regulation of CYP7A1 by NO-1886, the specific JAK2/STAT inhibitor tyrphostin AG 490 was applied to block the activation of STAT5 (Meydan *et al.* 1996). Pretreatment of AG 490 completely abrogated NO-1886-induced transcription and expression of CYP7A1 and STAT5 phosphorylation (Fig. 1D and E). In constitutive active STAT5 transfected cells, however, AG 490 failed to inhibit CYP7A1 expression, suggesting the involvement of STAT5 on AG 490 inhibition of CYP7A1 (Fig. 1F).

NO-1886 increased IGF1 secretion in STAT5-dependent manner in HepG2 cells

It was noted that one of the classical targets downstream of STAT5 is IGF1 in GH signaling (Davey *et al.* 2001, Chia *et al.* 2006). STAT5 activation motivated us to investigate whether or not IGF1 secretion are also activated (Woelfle *et al.* 2003, Woelfle & Rotwein 2004).

For proving the hypothesis, we detected the alteration of IGF1 secretion after treating HepG2 cells with NO-1886. As shown in Fig. 2A, treatment of NO-1886 significantly elevated the concentration of secreted IGF1 which reached a peak at the 8th hour, at a time when STAT5 phosphorylation starting to elapse (Fig. 1C), implicating a further downstream event of STAT5 activation. Pretreatment of AG 490 (10 µg/ml), however, completely abolished IGF1 secretion (Fig. 2A). NO-1886 did not interfere with intracellular concentration of IGF1 (Fig. 2B). However, preventing STAT activation by AG 490 significantly upregulated its intracellular accumulation of IGF1 (Fig. 2B). This may probably be due to the lack of feedback inhibition of IGF1 by secreted IGF1, a condition which has been reported previously in myoblast (Frost *et al.* 2002). These data suggested that STAT5 activation by NO-1886 is responsible for the facilitation of IGF1 secretion, and there might exist other undiscovered mechanisms for IGF1 production.

NO-1886 elevated plasma IGF1 in minipigs

As NO-1886 activated CYP7A1 expression and IGF1 secretion in HepG2 cells, it might provide beneficial effects on dyslipidemia and insulin resistance respectively.

Figure 2 NO-1886 increased IGF1 secretion in STAT5-dependent manner in HepG2 cells. After overnight serum starvation, under the pretreatment of vehicle of DMSO or AG 490 for 30 min (designated as the start time of '0' shown in figure), HepG2 cells were left untreated or treated with 0.3 µg/ml NO-1886 for 2–24 h. At the end of each time, 1 ml medium were stored at -80°C until RIA, after subtracting the contents of control group of vehicle, the data were regarded as the alteration of IGF1 secretion (A). Cells were washed with ice-cold PBS and total protein was extracted into RIPA buffer, samples were stored at -80°C until RIA and protein assay, the control group subtracted data were designated as the changes in intracellular IGF1 (B) Results were controlled by total protein assay ($n=3$ samples per group were analyzed). Data are means $\pm$ S.E.M. ^a $P<0.05$; ^b $P<0.01$ versus starting time point value of 0 (data were analyzed by Student *t*-test). ^c $P<0.05$; ^d $P<0.01$ versus the group of NO-1886+AG 490 on the independent time points.

Thus, in order to create a physiological relevance to the role of NO-1886, the *in vivo* analyses were executed in minipigs, the optimum humanoid diabetes model animals (Xi *et al.* 2004, Bellinger *et al.* 2006).

We first checked the effects of single dose distribution of NO-1886 (1 g/kg) on plasma IGF1 and GH protein levels in minipigs. As Fig. 3A shows, plasma IGF1 levels were upregulated from the 2nd hour and then climbed to a peak at 4 h. Comparing with the data in HepG2 cells (Fig. 2), we found an earlier responsiveness of IGF1 to NO-1886 stimulation. This could be due to the different sensitivity between liver and hepatoma cell line and/or the contribution from other IGF1 secreting cell types, such as skeletal muscle cells (Frost *et al.* 2002). Further investigation found that the upregulation of IGF1 proceeded without similar modification in GH levels (Fig. 3B). Although GH is the main regulator of

Figure 3 NO-1886 elevated plasma IGF1 in minipigs. After overnight fast, six male minipigs divided into two groups were orally administered with 1 g/kg BW NO-1886 (suspended in 5% Arabic gum). Plasma obtained at the end of each hour for 8 h were analyzed to determine plasma IGF1 (A) and GH (B) concentration by RIA. Data are means $\pm$ S.E.M. * $P < 0.05$ versus vehicle of Arabic gum control group.

IGF1 secretion, treatment of NO-1886 induces an important activation of STAT5 and this phenomenon may solely be the mechanism by which IGF1 secretion is promoted rather than through the promotion of GH secretion. However, we could still detect the 'predicted' feedback inhibition by the elevation of IGF1 between 2 and 3 h in NO-1886 treatment group (Fig. 3B).

Long-term distribution of NO-1886 prevented HFSCD-induced hyperglycemia and insulin resistance by raising IGF1 levels

Treatment of NO-1886 enhanced IGF1 secretion in both the HepG2 cells and minipigs and promoted CYP7A1 expression in HepG2 cells. Therefore, whether NO-1886 prevents diabetogenic diet-induced insulin resistance and dyslipidemia was investigated. As shown in Fig. 4A, from the 3rd month, HFSCD decreased fasting plasma IGF1 concentration compared with CD group. However, long-term supplementation of NO-1886 promoted the elevation of fasting plasma IGF1 without interfering with fasting GH levels (Fig. 4B). At the same time, treatment of NO-1886 prevented the HFSCD-induced impairment of insulin sensitivity and

recovered fasting insulin and glucose (Fig. 4C and D). In combination with previous *in vivo* studies on the role of IGF1 on insulin sensitivity, the present work suggests that improvement of IGF1 by NO-1886 prevents the progression of HFSCD-induced hyperglycemia and insulin desensitization.

Figure 4 Long-term distribution of NO-1886 prevented HFSCD-induced hyperglycemia and insulin resistance by raising IGF1 levels. Fifteen male minipigs were randomly divided into three groups (CD, HFSCD, and HFSCD1886) and treated as described in the Materials and Methods section. Blood samples for plasma IGF1 (A), GH (B), glucose (C), and insulin (D) quantification were obtained at the end of each month after overnight fasting. Data are means $\pm$ S.E.M. * $P < 0.05$ versus CD group and * $P < 0.05$ versus HFSCD group.

NO-1886 increased plasma HDL-C and decreased HFSCD-induced elevation of LDL-C, hepatic accumulation of cholesterol through upregulation of CYP7A1 in minipigs

Although enhancement of HDL-C and RCT from peripheral tissues has been reported previously as mechanisms for preventing diabetic angiopathy by NO-1886, the excessive load of cholesterol is toxic to hepatocytes and cells need to catabolize cholesterol for clearance (Zhang *et al.* 2006). Transgenic studies found that overexpression of CYP7A1 increases hepatic LDL receptor expression and decreased plasma LDL-C (Spady *et al.* 1995, Pandak *et al.* 2001). Therefore, for providing the evidence of NO-1886 on cholesterol metabolism, animals treated for 5 months were used to measure LDL-C, HDL-C and total cholesterol in plasma, and the CYP7A1 expression and total cholesterol content in liver. As shown in Fig. 5A, treatment of NO-1886 suppressed HFSCD-induced elevation of LDL-C to about 44% on average. Owing to the elevation of HDL-C (Fig. 5B), total plasma cholesterol concentration was not apparently interfered by NO-1886 (Fig. 5C). Animals treated for 5 months were killed, and the liver tissues were used for CYP7A1 detection and cholesterol quantification. As shown in Fig. 5D and E, HFSCD (cholesterol) failed to induce hepatic CYP7A1 expression in minipigs, leading to a severe accumulation of cholesterol. Treatment with NO-1886, however, significantly enhanced the protein levels of CYP7A1 and cholesterol clearance in minipigs (Fig. 5D and E).

Discussion

In humans, cholesterol homeostasis is maintained by a delicate balance between cholesterol input and output pathways. The development of new mechanisms of action for the metabolic dyslipidemia has focused attention on the cholesterol catabolism (Repa & Mangelsdorf 2000). In liver, plasma HDL-C is transported into hepatocytes through scavenger receptor class B type I in the plasma membrane by a way of passive diffusion (Trigatti *et al.* 2000). Thus, to keep the concentration gradient of cholesterol, the hepatocytes need to catabolize cholesterol for its depletion, a process which is controlled by CYP7A1 (Myant & Mitropoulos 1977,

Figure 5 NO-1886 increased plasma HDL-C and decreased HFSCD-induced elevation of LDL-C, hepatic accumulation of cholesterol through upregulation of CYP7A1 in minipigs. Blood samples taken from 15 male minipigs, treated as described in Materials and Methods section, were used to determine their LDL-C (A), HDL-C (B), and total cholesterol (C) concentration by using commercial kits. At the end of treatment for 5 months, animals were killed, and liver tissues were dissected and stocked in liquid nitrogen until the detection of CYP7A1 expression (D) and hepatic cholesterol content (E). Data are means $\pm$ S.E.M. * $P < 0.05$, $^{\dagger}P < 0.01$ versus vehicle of CD group; $^{\#}P < 0.05$ versus HFSCD group; NS, no statistical significance.

Javitt 1994, Chiang 1998, Lu *et al.* 2000). In the present study, compared with CD group, HFSCD maximally induced an elevation of HDL-C by ~ 2.3 folds in plasma and ~ 6 folds of cholesterol in liver; whereas supplementation of NO-1886 elevates plasma HDL-C by 3.7 folds and only ~ 2.5 folds of hepatic cholesterol (Fig. 5). Inferentially, according to Fick's law of passive diffusion that the flux rate from high concentration to low concentration is proportional to the concentration gradient, treatment of NO-1886 consequently upregulates cholesterol influx rate by:

$$\begin{aligned} N &= J_{\text{HFSCD1886}}/J_{\text{HFSCD}} \\ &= (D \times C_{\text{plasma (HFSCD1886)}}/C_{\text{liver (HFSCD1886)}})/(D \times C_{\text{plasma (HFSCD)}}/C_{\text{liver (HFSCD)}}) \\ &= (3.7/2.5)/(2.3/6) = 3.86(\text{folds}). \end{aligned}$$

Activation of CYP7A1 by NO-1886 may efficiently facilitate RCT.

As reported previously, GH enhances CYP7A1 activity and bile acids synthesis to a powerful magnitude by upregulating CYP7A1 expression. Although specific mechanism is still unclear, the role of STAT5 and peroxisome proliferators-activated receptor α (PPARA) might be implicated, activation of STAT5 negatively regulates the suppressive effects of PPAR α on CYP7A1 (Zhou & Waxman 1999, Patel *et al.* 2000). Similar to GH, NO-1886 improved hepatic cholesterol clearance by raising CYP7A1 expression. Recently, NO-1886-induced LPL activation has been shown to rectify diet-induced hepatic steatosis in mice at the same dose used in the present study (Yu *et al.* 2007). This study also provided the evidence that NO-1886 increased PPARA expression. Thus, further experiments need to be conducted to investigate the role of PPARA on NO-1886-induced CYP7A1 activation.

Insulin resistance, characterized by reduced responsiveness to normal concentration of insulin, is a common feature of many of the patients with T2DM and metabolic syndrome (DeFronzo & Ferrannini 1991, Eckel *et al.* 2005). Low levels of circulating IGF1 are a risk factor for the desensitization of insulin action (Massa *et al.* 1993, Yakar *et al.* 2001). Although GH is important for IGF1 secretion, hypersecretion of GH is related with GH signal desensitization and insulin desensitization (Moller & Flier 1991, Clark *et al.* 1997, Takano *et al.* 2001, Miquet *et al.* 2004). Our present investigation discovered that NO-1886 upregulated hepatic IGF1 secretion without interfering with plasma GH, resulting in the improvement of diet-induced insulin resistance.

NO-1886 has been known as an activator of LPL which increased LPL mRNA and LPL activity (maximum at 3 $\mu\text{g/ml}$) (Tsutsumi *et al.* 1993). However, for the activation of CYP7A1 expression, the effective concentration was observed to be at 0.3 $\mu\text{g/ml}$. These findings imply that, unrelated with the mechanism of LPL activation, there may exist some other activities of the drug responsible for the current observation. On the other hand, although NO-1886

has been proved to prevent diabetic dyslipidemia and atherosclerosis in animal experiments (Yin *et al.* 2003, 2004a,b, Niho *et al.* 2005, Zhang *et al.* 2006), the (patho)physiological role of LPL itself is still under controversy. For example, highly expressed LPL in endothelial cells is regarded as a risk factor for angiopathy and atherosclerosis (Goldberg 1996), which highlights the potential role of NO-1886 as a STAT5 activator to upregulate CYP7A1 and IGF1. This study is consistent with the previous observations regarding the beneficial outcomes of CYP7A1 and IGF1 upregulation.

In summary, NO-1886 promoted hepatic CYP7A1 and IGF1 expression via activation of STAT5. This study discovered a new therapeutic target of NO-1886 on diet-induced insulin resistance and dyslipidemia.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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