NO-1886 decreases ectopic lipid deposition and protects pancreatic β cells in diet-induced diabetic swine

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Abstract

The synthetic compound NO-1886 (ibrolipim) is a lipoprotein lipase activator that has been proven to be highly effective in lowering plasma triglycerides. Recently, we found that NO-1886 also reduced plasma free fatty acids and glucose in high-fat/high-sucrose diet-induced diabetic rabbits. In the current study, we investigated the effects of NO-1886 treatment on ectopic lipid deposition and the islet pathology in miniature swine fed a high-fat/high-sucrose diet. Our results showed that feeding this diet to miniature swine caused insulin resistance, increased lipid deposition in non-adipose tissue, such as in the heart,

skeletal muscle, liver and pancreas, and also caused pancreatic β cell damage. However, supplementing 1% NO-1886 (200 mg/kg per day) into the high-fat/high-sucrose diet decreased ectopic lipid deposition, improved insulin resistance, and alleviated the β cell damage. These results suggest that improvement of lipid disorder, non-adipose tissue steatosis and insulin resistance may be very important for the protection of β cell damage. Therefore, NO-1886 is potentially beneficial for the treatment of insulin-resistance syndrome.

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Introduction

Reductions in both insulin sensitivity and β cell function are present early in the course of the development of type 2 diabetes (Kahn 1994, 2003, Poitout & Robertson 1996). In recent years, there has been a proposed glucolipotoxicity hypothesis where both elevated glucose 'glucotoxicity' and lipid 'lipotoxicity' have been implicated in insulin resistance and the failure of β cells in type 2 diabetes (Poitout & Robertson 2002, Prentki *et al.* 2002). Circulating free fatty acids (FFAs) and triglyceride (TG) concentrations are commonly elevated in type 2 diabetes (Poitout & Robertson 2002, Pratley & Weyer 2002, Prentki *et al.* 2002). Lowering TG and FFA levels has been shown to decrease hyperglycemia in type 2 diabetes because of both a suppression of glucose production and an accompanying increase in glucose utilization (Deems *et al.* 1998).

It has previously been reported that NO-1886 (generic name: ibrolipim (WHO 2003)) increases lipoprotein lipase (LPL) mRNA in tissues and LPL activity in post-heparin

plasma, and decreases plasma TG levels with concomitant elevation of high-density lipoprotein cholesterol (HDL-C) levels in streptozotocin-induced diabetic rats (Tsutsumi *et al.* 1995). Recently, we found that NO-1886 also reduced plasma FFA and glucose in the high-fat/high-sucrose diet (HFSD)-induced diabetic rabbit (Yin *et al.* 2003). The lipotoxicity concept assumes that high plasma levels of FFA and TG cause excess accumulation of fatty acid (FA) and TG (steatosis) in non-adipocytes, which results in cellular injury. If this assumption were true, prevention of hyperglycemia by NO-1886 would be associated with reduced steatosis in various non-adipose tissues.

The lack of suitable large animal models has hindered investigation in the field of diabetes, particularly in the study of non-insulin-dependent diabetes mellitus and the accompanying complications. Swine have many characteristics similar to humans that make them a suitable species to model human diseases. Swine are omnivores, are easy to handle, raise few ethical considerations, offer similar size to adult humans, have several organ systems

very similar to humans in terms of anatomy, physiology and metabolism, and test compounds can be administered through all routes of delivery (Larsen *et al.* 2002, Boullion *et al.* 2003, Otis *et al.* 2003). Therefore, the current study was designed to determine whether NO-1886 treatment prevented non-adipose tissue steatosis and the functional and morphological impairment of pancreatic β cells in swine with diabetes induced by feeding an HFSD.

Material and Methods

Material

Agent NO-1886 ([4-(4-bromo-2-cyano-phenylcarbamoyl)-benzyl]-phosphonic acid diethyl ester, CAS 133208-93-2; Lot. No. C99H74SM) was synthesized in the New Drug Research Laboratory of Otsuka Pharmaceutical Factory Inc., Tokushima, Japan. Sucrose was obtained from the Liuzhou Sugar Company (Guangxi, China) and lard was obtained from the Hengyang Meat Product Company (Hunan, China).

Animals and diets

Fifteen male Chinese Guizhou swine, 3–4 months of age, were obtained from the barrier unit at the Laboratory Animal Center of Chongqing Medical University (Chongqing, China). Animals were randomized into three groups with similar body weight ($n=5$ in the normal control diet (CD) group; $n=5$ in the HFSD group; $n=5$ in the HFSD supplemented with NO-1886 (HFSD+NO-1886) group). The HFSD used in this study was normal swine diet supplemented with 10% lard and 37% sucrose. Animals were housed in single pens under controlled conditions (temperature between 18 °C and 22 °C, relative air humidity 30–70%, with four air changes per hour) and fed twice daily on a restricted schedule with either CD, HFSD or HFSD+NO-1886. The total study period was 8 months. All swine were fed HFSD or CD diet for the first 3 months. From the beginning of month 4, 1% NO-1886 (200 mg/kg per day) was supplemented into the HFSD diet for the swine assigned to the HFSD+NO-1886 group.

Body weights were recorded monthly. Blood samples for TG, FFA and glucose 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, following an overnight fast. The animals were killed at the end of month 8. The liver and pancreas were dissected from adjacent tissues and frozen in liquid nitrogen. Institutional guidelines for animal care and use were followed. The local animal ethics committee of Nanhua University approved all animal experiments.

Oral glucose tolerance test (OGTT)

An OGTT was performed after 8 months of feeding. After an 18 h overnight fast, animals were offered a glucose

Figure 1 Effects of NO-1886 treatment on (A) body weight, (B) fasting blood glucose, and (C) fasting serum insulin. Untreated HFSD-fed swine are compared with NO-1886-treated swine. Means $\pm$ S.D., $n=5$ per group.

tolerance test mixed meal of 25 g swine fodder and 2 g/kg glucose. The meal was eaten from a bowl under supervision. Blood samples were obtained without anesthesia from the orbital sinus at 0, 30, 60, and 90 min relative to the glucose load.

Insulin sensitivity assay

Insulin sensitivity was evaluated after 6 months of feeding. The swine were starved overnight and injected i.p. with

Table 1 Abdominal fat weight of swine (g/kg body weight). Swine were fed HFSD or normal diet (CD) during an 8-month study period. From the beginning of month 4, 1% NO-1886 was added to the HFSD diet (HFSD-NO-1886 group). Data ($n=5$ /group) are expressed as means $\pm$ s.d.

Group	Retroperitoneal	Epiploon	Mesenteric	Total
CD	2.41 $\pm$ 2.10	1.91 $\pm$ 0.90	5.43 $\pm$ 1.98	9.76 $\pm$ 4.40
HFSD + 1886	12.89 $\pm$ 3.97	2.88 $\pm$ 1.37	10.82 $\pm$ 1.66	26.63 $\pm$ 5.84
HFSD	26.61 $\pm$ 7.64**	6.80 $\pm$ 1.90**	14.35 $\pm$ 2.88	47.76 $\pm$ 12.22**

** $P<0.01$, significantly different from the values in respective NO-1886 treated swine.

pork insulin (Shanghai Biochemistry factory, Shanghai, China) at a dose of 0.75 IU/kg body weight. Insulin was diluted appropriately with sterile saline before injection. Plasma glucose was quantified prior to and at 15, 30 and 90 min after insulin injection. The insulin sensitivity index (Ki) was evaluated as the slope of the fall in glucose over 30 min: $Ki = ([\text{glucose}]_{t=0} - [\text{glucose}]_{t=30}) / 30$.

Plasma measurement

Glucose was determined by commercially available enzymatic method test kits (Shanghai Rongsheng Biotech Inc., Shanghai, China). Plasma FFAs were measured by a colorimetric method (kit supplied by Nanjing Jianchen Biotech Inc., Nanjing, China). Plasma TGs were measured with a TG kit also from Shanghai Rongsheng Biotech Inc. Plasma insulin was determined by RIA using Insulin Radioimmunity kits (China Institute of Atomic Research, Beijing, China).

Detection of fat in non-adipose tissues

For Oil Red O staining, frozen myocardium, skeletal muscle, pancreas and liver samples were mounted, sectioned (4 μ m), fixed to a histological slide, and placed in absolute propylene glycol (2 min). Slides were moved into Oil Red O solution for 1 h, differentiated in 85% propylene glycol (1 min), rinsed twice in distilled water, counterstained with hematoxylin (10 s) and mounted in glycerin.

Immunostaining and morphometry of islet β cells

To examine the effects of HFSD feeding and NO-1886 treatment on insulin-producing β cells, samples of pancreas were fixed in formalin, paraffin embedded, sectioned, and stained with an antibody to porcine insulin. After immunostaining, sections were photographed, and the percent area of pancreas in the section occupied by insulin-producing cells was measured in the three groups by planimetry with Image J software (<http://rsb.info.nih.gov/ij/>).

Electron microscopy

For electron microscopy, pancreatic fragments were fixed in 4% cacodylate-buffered (0.2 M) glutaraldehyde, pH

7.4, for 24 h at 4 °C, rinsed in Millonig's phosphate buffer and post-fixed in buffered 1% osmic acid (2 h at 20 °C). The specimens then were processed for epoxy embedding (Polybed R 812; Fluka). Thin sections were stained with uranyl acetate and lead citrate and photographed by a Philips (Eindhoven, The Netherlands) LS 420 electron microscope.

Determination of pancreatic insulin

To determine pancreatic insulin content, pancreata were excised and homogenized individually in 20 volumes of cold acidic ethanol (75% ethanol, 1.5% concentrated HCl) followed by 48 h of agitation at 4 °C. Insulin was then quantified in the supernatants of the samples by RIA as described above. Protein in tissue extracts was determined by the Bradford method.

Statistical analysis

Results are expressed as means $\pm$ s.d. Statistical calculations were performed using a MANOVA. Statistical significance was obtained when P values were less than 0.05.

Results

Effect of NO-1886 on body and fat weight

Figure 1A shows the mean body weights of the three groups throughout the study. HFSD feeding resulted in a faster increase in body weight. However, supplementing HFSD with 1% NO-1886 inhibited body weight gain throughout the study and this was significantly different at month 6 and month 8. The amounts of retroperitoneal, epiploon, and mesenteric fats (g/kg body weight) were significantly increased in swine from the HFSD group compared with swine from CD and HFSD+NO-1886 groups. NO-1886 decreased abdominal fat accumulation by 57% (Table 1).

Effect of NO-1886 on plasma glucose, insulin and lipid levels

HFSD feeding elevated fasting plasma glucose levels significantly (Fig. 1B). After 3 months of HFSD feeding,

Figure 2 Effects of NO-1886 on (A) blood FFA and (B) serum TG. Untreated HFSD-fed swine are compared with NO-1886-treated swine. Means $\pm$ S.D., $n=5$ per group.

the fasting plasma glucose levels in HFSD and HFSD+NO-1886 groups (at this time point the treatment with NO-1886 had not been started) increased to 121 ± 24 and 131 ± 31 mg/dl respectively. These values were significantly higher than in the CD group (51 ± 10 mg/dl). When treatment with NO-1886 was started, the elevation of blood glucose in the HFSD+NO-1886 group had been efficiently and continuously suppressed when measured at the later time points during the treatment period ($P<0.05$, HFSD+NO-1886 vs HFSD) (Fig. 1B). Plasma fasting insulin levels in swine from the HFSD group also increased significantly ($P<0.05$, HFSD vs CD). NO-1886 treatment alleviated the change in fasting plasma insulin (Fig. 1C).

Three months of HFSD feeding significantly increased the plasma FFA and TG levels compared with swine fed

the CD. Supplementing NO-1886 into the HFSD (HFSD+NO-1886 group) induced a decrease in FFA concentration compared with swine in the HFSD group. After 4 months, the FFA levels in swine receiving NO-1886 approached values similar to those in the CD group (Fig. 2A). NO-1886 also decreased plasma TG levels (Fig. 2B).

Effect of NO-1886 on glucose tolerance

In order to analyze the effects of chronic treatment with NO-1886 on glucose tolerance, we performed an OGTT test in overnight-fasted swine from the three groups at the end of month 8. The change in plasma glucose and insulin concentrations after the glucose load at the end of month 8 is shown in Fig. 3. Swine from the

Figure 3 Effects of NO-1886 treatment on (A) blood glucose and (B) serum insulin in an OGTT. Untreated HFSD-fed swine are compared with NO-1886-treated swine. Means $\pm$ S.D., $n=5$ per group.

HFSD group exhibited marked glucose intolerance compared with those from both the CD and HFSD+NO-1886 groups (Fig. 3A). The deficient glucose removal seen in the HFSD group may have been caused by impairment of acute insulin secretion (absence of the first phase of insulin secretion) in response to the glucose load (Fig. 3B).

Effect of NO-1886 on insulin resistance

Glucose tolerance is a function of glucose-stimulated insulin secretion, hepatic glucose output and tissue insulin sensitivity. The contribution of insulin sensitivity to the difference in glucose tolerance in swine from the three

groups was explored by evaluating the clearance of plasma glucose as a function of time after insulin injection. This measure of whole body insulin sensitivity can be conveniently expressed as a Ki. Swine were injected i.p. with insulin and Ki values were determined (Fig. 4). Since Ki reflects the rate of glucose removal, higher values indicate greater tissue insulin sensitivity. Insulin sensitivity was nearly 2-fold greater in the CD and HFSD+NO-1886 groups compared with the HFSD group ($P<0.001$). Plasma glucose levels were significantly higher for swine in the HFSD group at each time point, indicating that swine from the HFSD group were in a severe insulin-resistant state, and that NO-1886 increased insulin sensitivity in the HFSD+NO-1886 group.

Figure 4 Changes in blood glucose concentrations during an insulin sensitivity test in HFSD-, HFSD+NO-1886- and CD-fed swine examined at the end of month 6. The whole-body insulin sensitivity was expressed as a K_i . Higher K_i values indicate greater tissue insulin sensitivity. Insulin sensitivity was nearly 2-fold greater in the CD and HFSD+NO-1886 groups compared with the HFSD group ($P < 0.001$). Plasma glucose levels were significantly higher for swine from the HFSD group at each time point, indicating that swine from the HFSD group were in a severe insulin-resistant state, and that NO-1886 increased insulin sensitivity in the HFSD+NO-1886 group. $n=5$ per group.

Effect of NO-1886 on non-adipose tissue steatosis

Oil red O staining exhibited many lipid droplets in myocardium, skeletal muscle, pancreas and liver sections of HFSD-fed animals, whereas few or no lipid droplets were seen in the myocardium, skeletal muscle, pancreas and liver sections from the CD and HFSD+NO-1886 groups (Fig. 5).

Effect of NO-1886 on pancreatic structure and insulin content

The pancreatic islets of normal swine from the CD group were oval or round with a smooth circumference (Fig. 6A), whereas the islets from the HFSD group became fibrotic with irregular contours, sparse and shrunken islet cells, and an abundance of large vacuolations in the islets themselves (Fig. 6B). The islets from the HFSD+NO-1886 group appeared quite normal (Fig. 6C). Mean volume density of β cells in the HFSD group was 19% lower than that in HFSD+NO-1886 group and 24% lower than that in swine from the CD group.

At the ultrastructural level, a striking abnormality in the islets from the HFSD group was the abundance of lipid droplets. In addition, there was marked degranulation of the β cells (Fig. 7C). In contrast, in the islets from the HFSD+NO-1886 group, no lipid droplets were observed, and insulin granules were still abundant (Fig. 7B).

The above morphological findings were confirmed by determination of pancreatic insulin content, which

showed reduced pancreatic insulin content in the HFSD group and preserved pancreatic insulin in the HFSD+NO-1886 group (Fig. 6D).

Discussion

It has previously been reported that NO-1886 increases LPL mRNA in adipose tissue, and myocardial and skeletal muscle. NO-1886 also increases LPL protein and LPL activity in post-heparin plasma, and produces a reduction in plasma TG levels with concomitant elevation of HDL-C levels in animals with lipid disorder (Tsutsumi *et al.* 1995, Hagi *et al.* 1997, Kusunoki *et al.* 2000, Yin *et al.* 2002, 2003). Kusunoki *et al.* (2000) reported that NO-1886 inhibited fat accumulation and reduced insulin resistance in high-fat-induced obesity diabetes type 2 animal model rats. Recently, Yin *et al.* (2002, 2003) discovered that NO-1886 lowered plasma glucose in HFSD-induced rabbit diabetes. According to the 'glucolipotoxicity' hypothesis, we speculated that the efficacy of NO-1886 in lipid disorder would account for its glucose-lowering action.

In this study, NO-1886 suppressed body weight gain and fat accumulation in HFSD-fed swine. These results were consistent with those obtained in high-fat-induced obesity rats by NO-1886 treatment (Kusunoki *et al.* 2000). NO-1886 may induce the acceleration of FFA oxidation

Figure 5 Oil red O staining in heart, skeletal muscle, pancreas, and liver tissues. Magnification: $\times 400$.

and the stimulation of uncoupling protein 3 (Doi *et al.* 2003). Therefore, this may explain why NO-1886 decreased plasma FFA levels in this study.

In this study, we observed that the HFSD-fed swine were hyperlipidemic, hyperglycemic, hyperinsulinemic and insulin resistant. Furthermore, this swine model had liver steatosis, lipid accumulation in heart, muscle and islets and degranulation of β cells. As Schaffer (2003) reviewed, excess lipid accumulation in non-adipose tissues

may arise in the setting of high plasma FFAs or TGs. Alternatively, lipid overload results from mismatch between FFA import and utilization. Evidence from human studies and animal models suggests that lipid accumulation in the heart, skeletal muscle, pancreas, liver and kidney play an important role in the pathogenesis of heart failure, obesity and diabetes. Excess FFAs may impair normal cell signaling, causing cellular dysfunction. In some circumstances, excess FFAs induce apoptotic

Figure 6 Immunostaining for insulin (A) in islets of a CD-fed swine, (B) in islets of an HFSD-fed swine, (C) in islet of an HFSD+NO-1886-fed swine. Compared with the CD- and NO-1886-treated condition, the untreated swine shows a reduced number of dispersed insulin-staining cells. Magnification: $\times 400$. (D) Pancreatic insulin content; $*P=0.002$, compared with the other two groups, means $\pm$ s.d., $n=5$ per group.

cell death. Therefore, lipid overstorage in non-adipose tissues, especially in β cells, will cause a process termed lipotoxicity that impairs both insulin action and secretion. The lard we used in the HFSD contained mainly saturated FAs. Lipotoxicity from accumulation of long-chain FAs is specific for saturated FAs (Listenberger *et al.* 2001, Maedler *et al.* 2001). We think that hyperlipidemia, hyperglycemia, hyperinsulinemia and insulin resistance may cause a disorder of the pancreas.

The major findings of this study are that NO-1886 inhibited elevation of plasma TG and FFA, alleviated non-adipose tissue steatosis, and maintained the function and the structure of β cells. The severe lipid accumulation in islets and the degranulation of β cells strikingly visible in HFSD-fed swine, are minimized by NO-1886 treatment. Functional rescue was observed simultaneously with the morphological rescue of β cells, as reflected by the

glucose-stimulated insulin response that was absent in HFSD-fed swine. This study seems to support the lipotoxicity theory.

In this study, HFSD-fed swine had higher blood glucose levels both in the fasting state and under glucose load relative to CD- and NO-1886-treated swine, suggesting the presence of stronger hyperglycemic stress in HFSD-fed swine. Interestingly, fasting plasma insulin levels in these swine were almost twice the levels of CD-fed swine. HFSD feeding thus forced islet β cells to secrete high insulin levels to compensate for the high glucose. Such hyperglycemic stress was reflected structurally by vacuolation and degranulation of islet β cells and reduced immunoreactions to insulin, showing structural deficits of β cells. However, all of these abnormalities were minimized by treatment with NO-1886.

Figure 7 Islet ultrastructure of (A) a thin section of a normal β cell from a CD-fed swine islet, (B) thin section of a β cell from an HFSD+NO-1886-fed swine islet – reduction in the number of dense core secretory granules was alleviated and no lipid droplets were observed, (C) thin section of a β cell from an HFSD-fed swine islet, showing reduction in the number of dense core secretory granules and areas of diffuse lipid particles (white arrows). Magnification: $\times 8000$.

In summary, HFSD-fed swine were hyperlipidemic, hyperglycemic, hyperinsulinemic and insulin resistant. Furthermore, these swine had liver steatosis, lipid accumulation in heart, muscle, and islets and degranulation of β cells. NO-1886 administration resulted in improvement of hyperlipidemia, hyperglycemia, hyperinsulinemia, insulin resistance, liver steatosis, lipid accumulation in non-adipose tissue and degranulation of β cells in HFSD-fed swine. These results may suggest that improvement of lipid disorder and insulin resistance is beneficial for protection of pancreatic β cells. Therefore, the LPL activator NO-1886 is potentially beneficial for protection of pancreatic β cells.

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