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NO-1886 (ibrolipim), a lipoprotein lipase–promoting agent, accelerates the expression of UCP3 messenger RNA and ameliorates obesity in ovariectomized rats

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Abstract

The synthetic compound NO-1886 (ibrolipim, [4-(4-bromo-2-cyano-phenylcarbamoyl)-benzyl]-phosphonic acid diethyl ester, CAS 133208-93-2) is a lipoprotein lipase (LPL)–promoting agent that decreases plasma triglycerides, increases high-density lipoprotein cholesterol levels, and prevents fat accumulation in high fat-fed rats. However, the effect of NO-1886 on body weight, fat accumulation, and energy expenditure in ovariectomized (OVX) rats is not clear. The primary aim of this study was to ascertain whether NO-1886 ameliorated obesity in OVX rats and to examine the effects on fatty acid oxidation–related enzymes. NO-1886 decreased accumulation of visceral fat and suppressed the increase in body weight resulting from the ovariectomy. NO-1886 decreased the respiratory quotient and increased expression of the fatty acid translocase messenger RNA (mRNA) in the liver, soleus muscle, and mesenteric fat. NO-1886 also increased the expression of fatty acid–binding protein mRNA in the liver and soleus muscle and the expression of the uncoupling protein 3 (UCP3) mRNA in the heart, soleus muscle, and mesenteric fat, but not in the brown adipose tissue. Furthermore, NO-1886 did not affect UCP1 and UCP2 in brown adipose tissue. Therefore, amelioration of obesity by NO-1886 in OVX rats is possibly because of an the increased expression of fatty acid oxidation–related enzymes and UCP3, both of which are related to fatty acid transfer and fat use. Our study indicates that the LPL-promoting agent NO-1886 may be potentially beneficial in the treatment of obesity and obesity-linked health problems in postmenopausal women.

1. Introduction

Postmenopausal women have a high risk of atherosclerosis accompanied by obesity. Therefore, amelioration of obesity in postmenopausal women is very important. Obesity is by definition an accumulation of body fat [1] and is linked to many health problems such as non–insulin-dependent diabetes mellitus, insulin resistance, atherosclerosis, kidney disease, coronary heart disease, hyperlipemia, and hypertension in humans [2]. One of the main causes of obesity in humans is a decreased basal metabolism associated with aging, which results in a decrease in the daily total energy expenditure. It has been observed in aged postmenopausal women that hyperlipemia is common and is accompanied by body fat accumulation with an associated decrease in estrogen, a decrease in basal metabolism, or both. The experimental ovariectomized (OVX) animal model is often used to study osteoporosis and/or obesity [3,4]. Ovariectomy results in a decrease of basal metabolism and a concomitant increase in body weight, whereas estradiol replacement reverses these effects. Loss of estrogen produced by the ovary affects the expression of leptin, a satiety factor responsible for the regulation of appetite in rodents. Increased food intake in OVX rats may be due in part to the loss of appetite regulation in these animals, thereby resulting in the obese phenotype [5,6].

The chemical compound NO-1886, a lipoprotein lipase (LPL)–promoting agent, lowers plasma triglycerides (TGs) and increases high-density lipoprotein cholesterol when administered to rats [7]. Tsutsumi et al [7] have demonstrated that increasing LPL activity results in the elevation of high-density lipoprotein cholesterol and that long-term administration of NO-1886 protects against the development of atherosclerosis in rats fed with an atherogenic diet.

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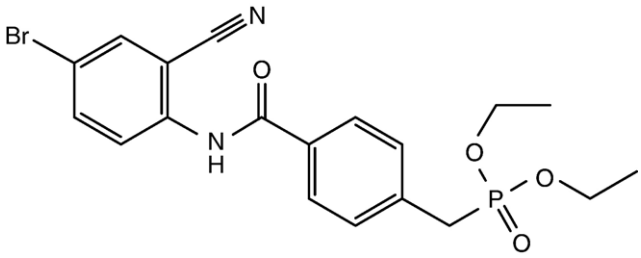

Fig. 1. Chemical structure of NO-1886 (ibrolipim).

Some investigators have suggested that increasing LPL activity in adipose tissue results in increased fat accumulation [8,9], whereas other scientists have suggested that increasing LPL activity in skeletal muscle results in decreased fat accumulation [10,11]. On the other hand, Kusunoki et al [12] have reported that NO-1886 treatment suppresses fat accumulation in a high-fat diet–fed rat model. However, the mechanism for the suppression of fat accumulation is unclear.

In the current study, the primary aim was to examine the effects of NO-1886 on body weight, fat accumulation, and energy expenditure in aged OVX obese rats. A further aim was to examine the expression profiles of suppressed body fat accumulation-related genes in the peripheral tissues.

2. Materials and methods

2.1. Materials

Agent NO-1886 (generic name: ibrolipim, [4-(4-bromo-2-cyano-phenylcarbamoyl)-benzyl]-phosphonic acid diethyl ester) was synthesized in the New Drug Research Laboratory at the Otsuka Pharmaceutical Factory, Tokushima, Japan. The chemical structure is shown in Fig. 1. All other chemicals used were high-grade commercially available products.

2.2. Animals and treatment

Ten-week-old female Sprague-Dawley rats from Charles River Japan (Yokohama, Japan), weighing 200 to 260 g, were maintained under conditions of constant humidity and temperature (22°C ± 2°C) and a 12-hour light-dark cycle (light from 7:00 AM to 7:00 PM). The animals were fed a standard diet (CRF-1, Oriental Yeast, Tokyo, Japan) and water ad libitum. The rats were randomly divided into 3 experimental groups after a 6-week breeding under these conditions. Two groups underwent an experimental ovariectomy treatment and other group underwent a sham operation while anesthetized by an intraperitoneal injection of 40 mg/kg sodium pentobarbital. Animals in the ovariectomy treatment groups were considered obese when body weight was 20% higher as compared with averaged sham-operated rats. Obesity conditions occurred 5 weeks after ovariectomy treatment.

2.3. Experimental protocol

After the ovariectomy treatment or sham operation, the rats were weighed, and food intake volume was measured every week. Then, NO-1886 suspended in 5% arabic gum solution was orally administered at a dose of 100 mg/kg per day to 1 OVX group from 5 to 13 weeks after the operation, and other 1 OVX group was treated with vehicle (arabic gum) from 5 to 13 weeks after the operation. At 13 weeks after the ovariectomy or sham operation (after 8 weeks of treatment with NO-1886 or vehicle), rats were killed under sodium pentobarbital anesthesia, and tissue samples were collected for measuring net weight. The portion of the tissue samples to be used in the messenger RNA (mRNA) determinations were immediately frozen in liquid nitrogen and stored at −80°C until use. The tissue samples collected included liver, heart, soleus muscle, perirenal and retroperitoneal white adipose tissue (WAT), mesenteric WAT, subcutaneous WAT, the brown adipose tissue (BAT) depot from the occipital region of the head, and the BAT depot from the interscapulum. Blood samples were assayed for serum glucose, insulin, and leptin levels after centrifugation and supernatant collection.

2.4. Measurement of respiratory quotient

The rats' whole-body oxygen consumption and carbon dioxide production were measured using a 6-chamber Oxycongamma and data analyzing system (Fukuda Denshi

Table 1
Oligonucleotide sequences of gene-specific primers and probe in rat

mRNA	GenBank accession no.	TaqMan probe and primers
FAT/CD36	AF111268	Probe: 5′-FAM-CTCTTCCACATTTCCTACATGCAAGTCCTG-TAMRA-3′ Forward: 5′-TGTGCTGGACATTGGCAA-3′ Reverse: 5′-AAGCCTTCGATAGGTTCTGAGAC-3′
FABP	M18034	Probe: 5′-FAM-TTTGCCACCAGACAGGTCGCTAGCAT-TAMRA-3′ Forward: 5′-TACATGAAGTCACTCGGTGTGG-3′ Reverse: 5′-CTCAATGATTGTGGTCGGCT-3′
UCP2	NM_019354	Probe: 5′-FAM-TTTCCCTCTAGACACCGCCAAAGTCC-TAMRA-3′ Forward: 5′-GCAGCCTGTATTGCAGATCTCA-3′ Reverse: 5′-TGACTCTCTCCTTGGATCTGCAG-3′
UCP3	NM_013167	Probe: 5′-FAM-TGCCTCCCACAACAGTTGTAAAGTTCCTGG-TAMRA-3′ Forward: 5′-GGTTGGACTTCAGCCATCAGAA-3′ Reverse: 5′-AGATCAGCAAAACAGGCTGCA -3′

Table 2
Effects of NO-1886 treatment (100 mg/kg per day PO) for 8 weeks on plasma glucose, insulin, and leptin in sham-operated rats, OVX rats, and OVX + NO-1886 rats

	Sham	OVX	OVX + NO-1886
Glucose (mg/dL)	172.2 ± 6.2	177.5 ± 9.7	157.3 ± 8.4
Insulin (ng/mL)	6167 ± 945	8015 ± 2192	4277 ± 1018
Leptin (ng/mL)	3.81 ± 0.87	10.10 ± 1.04	4.20 ± 0.84*

Values are means ± SEM (n = 6).
* $P < .01$, significantly different from the respective values in OVX rats.

and Arco System, Tokyo and Chiba, Japan, respectively). Temperature was maintained at 22°C ± 2°C, and lights were on from 7:00 AM to 7:00 PM. System settings included a flow rate of 1.6 L/min and a measurement period of 60 seconds. The respiratory quotient (RQ) for the rats was calculated by dividing the carbon dioxide production by the oxygen consumption and was measured 4 hours after NO-1886 administration. Each rat was placed in a separate calorimetry chamber (each with a volume of 15.8 L). There were 6 rats per group (sham-operated, vehicle OVX, and NO-1886–treated OVX groups), and each group had ad libitum access to chow and water. The rats were monitored at 3, 5, and 13 weeks after the ovariectomy or sham operation.

2.5. RNA preparation

Total RNAs were extracted from the 30-mg tissue samples using the Qiagen RNeasy mini-kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. The extracted total RNAs were dissolved in 100 μL of RNase-free water and stored at −80°C until use.

2.6. Real-time quantitative reverse transcription–polymerase chain reaction

Real-time quantitative reverse transcription–polymerase chain reaction (RT-PCR) was performed to determine the relative expression levels of the rat fatty acid oxidation enzymes in the rat tissues. The TaqMan probe consisted of an oligonucleotide with a 5′-reporter dye and a downstream 3′-quencher dye. The fluorescent reporter dye, FAM (6-carboxy-fluorescein) or VIC (Appelera Corp), was covalently linked to the 5′ end of the oligonucleotide. This reporter dye was quenched by TAMRA (6-carboxy-tetramethyl-rhodamine), which is typically located at the 3′ end. Fluorescence quenching depended on the spatial proximity of the reporter and quencher dyes. All oligonucleotide primers and TaqMan probes (shown in Table 1) were designed using Primer Express software version 1.0 (Applied Biosystems, Tokyo, Japan), except for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for which we used the TaqMan rodent GAPDH Control Reagents kit (Applied Biosystems).

Reverse transcriptase–polymerase chain reaction was carried out in a 50-μL volume reaction using a TaqMan One-step RT-PCR Master Mix Reagents kit (Applied Biosystems) in 96-well plates for the rat fatty acid oxidation enzymes and the endogenous control, GAPDH mRNA. TaqMan assay reaction buffer contained 1× Master Mix reagents, 1× MultiScribe and RNase Inhibitor Mix (TaqMan One-step RT-PCR Master Mix Reagents), 300 nmol/L forward primer, 900 nmol/L reverse primer, 200 nmol/L TaqMan probe, and 50 ng total RNA. The rodent GAPDH mRNA reaction buffer contained all the above, but was modified as follows: 100 nmol/L forward primer, 100 nmol/L reverse primer, and 200 nmol/L TaqMan probe.

Reverse transcriptase reaction conditions were 48°C for 30 minutes and 95°C for 10 minutes for 1 cycle. Polymerase chain reaction conditions were 95°C for 15 seconds and 60°C for 1 minute for 40 cycles on an ABI PRISM 7700 Sequence Detector (Applied Biosystems). Standard curve linearity for the mRNA of the enzymes was determined for the 0.16- to 100-ng total RNA range by using a standard sample prepared from the tissues of the

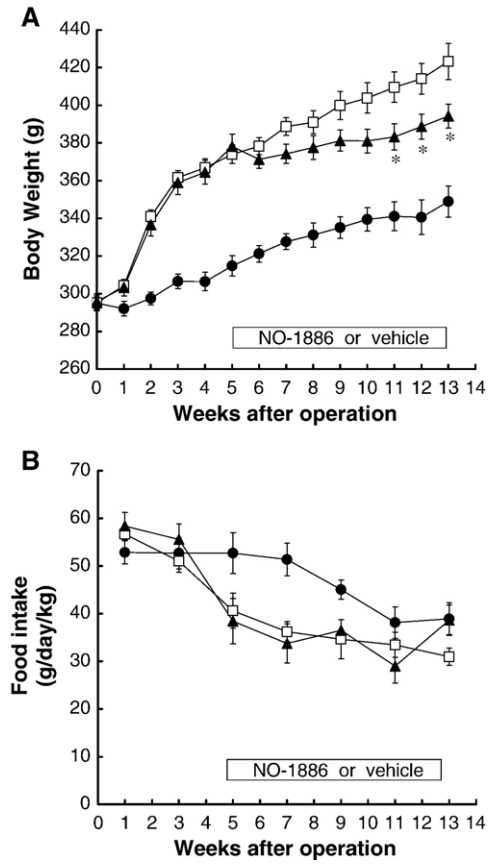

Fig. 2. Effects of NO-1886 on body weight and on food intake. Effects of NO-1886 on body weight (A) and on food intake (B) in sham-operated rats (black circles), OVX rats (open squares), and OVX + NO-1886 rats (black triangles). Data are expressed as means ± SEM (n = 6). Daily administration of vehicle or NO-1886 at a dose of 100 mg/kg of body weight was started 5 weeks after the operation and was continued for 8 weeks. *$P < .05$, significant difference from the values seen in the OVX rats.

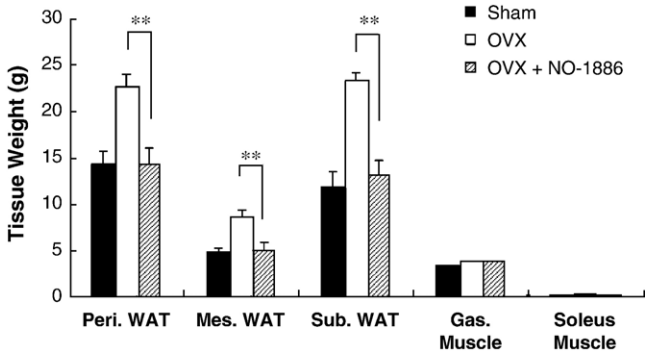

Fig. 3. Effects of NO-1886 on the weights of WAT and skeletal muscle. Effects of NO-1886 on the weight of adipose tissues (perirenal and retroperitoneal adipose tissues, mesenteric adipose tissues, and subcutaneous adipose tissues) and skeletal muscles (gastrocnemius and soleus muscles). All tissues were obtained from the rats shown in Fig. 2. Data are expressed as means ± SEM (n = 6). $^{**}P < .01$, significant difference from the values seen in the OVX rats. Peri. WAT indicates perirenal and retroperitoneal adipose tissues; Mes. WAT, mesenteric adipose tissues; Sub. WAT, subcutaneous adipose tissues; Gas. Muscle, gastrocnemius muscle.

control rats. The results for the expression of mRNA were presented relative to the expression of the control gene, rodent GAPDH mRNA.

2.7. Statistical analysis

StatView (version 5) statistical analysis software supported by SAS Institute (Cary, NC) was used for data analysis. Values were expressed as means ± SEM. The statistical significance of the values was analyzed between OVX rats and OVX + NO-1886 rats by the Student *t* test. *P* values of less than .05 were considered significant.

3. Results

3.1. Effects of NO-1886 on plasma glucose, insulin, and leptin

Table 2 shows plasma parameters for the sham-operated rats, OVX rats, and OVX + NO-1886 rats. Plasma glucose and insulin levels did not significantly differ between the OVX rats and OVX + NO-1886 rats. The leptin levels of the OVX + NO-1886 rats were significantly lower than those seen in the OVX rats.

3.2. Effects of NO-1886 on body weight and food intake

Ovariectomized rats showed a marked body weight increase as compared with sham-operated rats. The reduction in body weight in OVX rats treated with NO-1886 was significant after 6 weeks of administration (Fig. 2A). NO-1886 did not alter food intake in the OVX rats (Fig. 2B).

3.3. Effects of NO-1886 on WAT and skeletal muscles weights

The OVX rats treated with NO-1886 showed a significant reduction in the weight of adipose tissue (perirenal and retroperitoneal WAT, mesenteric WAT, and subcutaneous WAT), reaching levels close to those in the sham-operated rats (Fig. 3). The skeletal muscle weights (gastrocnemius muscle and soleus muscle) did not differ between the OVX and OVX + NO-1886 rats.

3.4. Effects of NO-1886 on RQ

Fig. 4 shows the RQ in sham-operated, OVX, and OVX + NO-1886 rats. RQ was measured at the pretreatment point (3 weeks after the ovariectomy operation), 1-week treatment point (5 weeks after the ovariectomy operation), and 8-week treatment point (13 weeks after the OVX operation). The RQ of the OVX + NO-1886 rats was significantly lower than that seen in the OVX rats (0.888 ± 0.014 vs 0.837 ± 0.008, $P < .05$, n = 8) at the 8-week administration point (Fig. 4).

3.5. Effects of NO-1886 treatment on the mRNA levels of the fatty acid transporter enzymes

The liver mRNA levels of the fatty acid translocase (FAT/CD36) decreased to one fourth the level in sham rats. NO-1886 administration in OVX rats caused a significant increase in the FAT/CD36 mRNA levels in the liver, soleus muscle, and mesenteric fat as compared with the OVX rats (Fig. 5). NO-1886 caused no significant changes in the FAT/CD36 mRNA levels in the heart. The mRNA levels of fatty acid–binding protein (FABP) in the liver and soleus muscle in the OVX + NO-1886 rats were significantly increased compared with the OVX rats. NO-1886 caused no significant changes in FABP in the heart and mesenteric fat.

3.6. Effects of NO-1886 treatment on mRNA levels of uncoupling proteins and FoF1-ATPase

Fig. 6 shows the mRNA levels of uncoupling proteins 2 and 3 (UCP2 and UCP3, respectively) in the liver, heart,

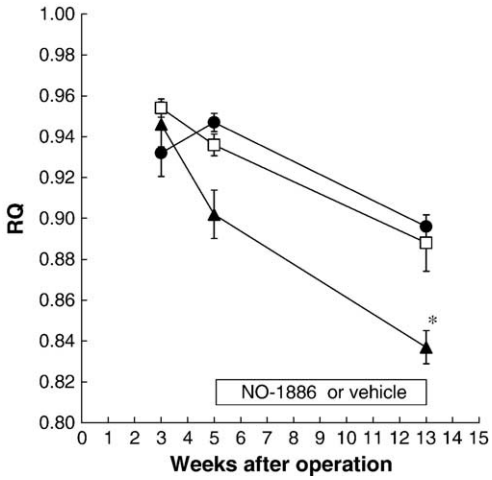

Fig. 4. Effects of NO-1886 on RQ. Effects of NO-1886 on RQ in sham-operated rats (black circles), OVX rats (open squares), and OVX + NO-1886 rats (black triangles). The RQ of the rats was measured 4 hours after NO-1886 or vehicle administration in the animals. Data are expressed as means ± SEM (n = 6). $^{*}P < .05$, significant difference from the values seen in the OVX rats.

soleus muscle, and mesenteric fat. NO-1886 did not modify either the UCP1 mRNA (data not shown) or the UCP2 mRNA levels in the liver, heart, soleus muscle, or mesenteric fat. NO-1886 also did not significantly affect UCP3 mRNA in the liver. NO-1886 caused no significant changes in the mRNA levels of UCP1, UCP2, and UCP3 in BAT (data not shown). For UCP3 mRNA levels, NO-1886 caused a 1.7-fold induction in the heart ($P < .01$), a 1.8-fold induction in the soleus muscle ($P < .05$), and a 2.5-fold induction in the mesenteric fat ($P < .001$), as compared with that seen in the OVX rats. NO-1886 caused no significant changes in the mRNA levels of FoF1-ATPase in the liver, heart, soleus muscle, and mesenteric fat compared with the OVX rats (data not shown).

4. Discussion

NO-1886, an LPL-promoting agent, is known to increase LPL mRNA and LPL activity in adipose tissue and skeletal muscle [7,13–15]. Kusunoki et al [12] have reported that NO-1886 prevents fat accumulation in both visceral and subcutaneous adipose tissue in high fat-fed induced obese rats without affecting food intake. Furthermore, Hara et al [16] have reported that NO-1886 decreases the plasma TG level but does not affect tissue TG accumulation in fructose-fed rats. These authors have also reported that long-term administration of NO-1886 causes a reduction in the RQ in both diabetic and obese rats [12,16]. Recently, Yin et al [17] reported that supplementing 1% NO-1886 to the high-fat/

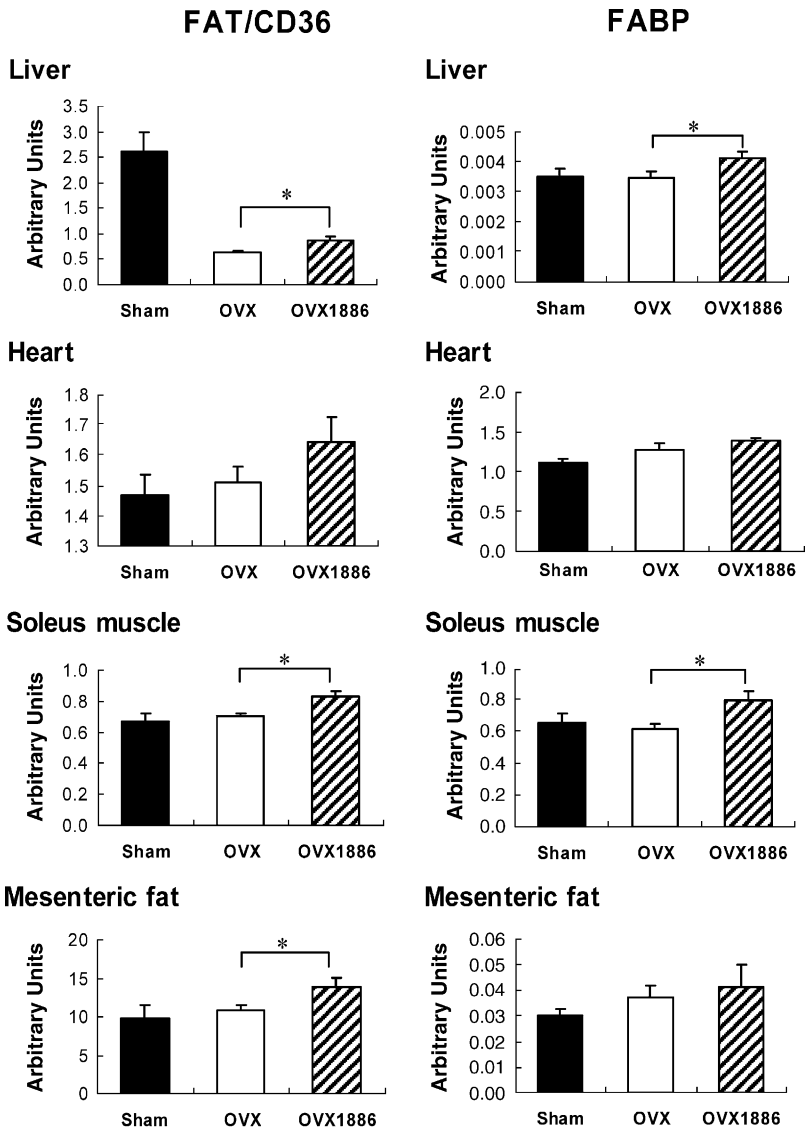

Fig. 5. Effects of NO-1886 treatment on the fatty acid transporter enzyme mRNA levels. The expression of FAT/CD36 and FABP mRNA in the liver, heart, soleus muscle, and mesenteric fat from sham-operated, OVX, and OVX + NO-1886 rats. All tissues were obtained from the rats shown in Fig. 2. The mRNA expression levels were analyzed by the real-time RT-PCR method. Details of the experiments are described in Materials and methods. Data are expressed as means ± SEM (n = 6). *$P < .05$, significant difference from the values seen in the OVX rats.

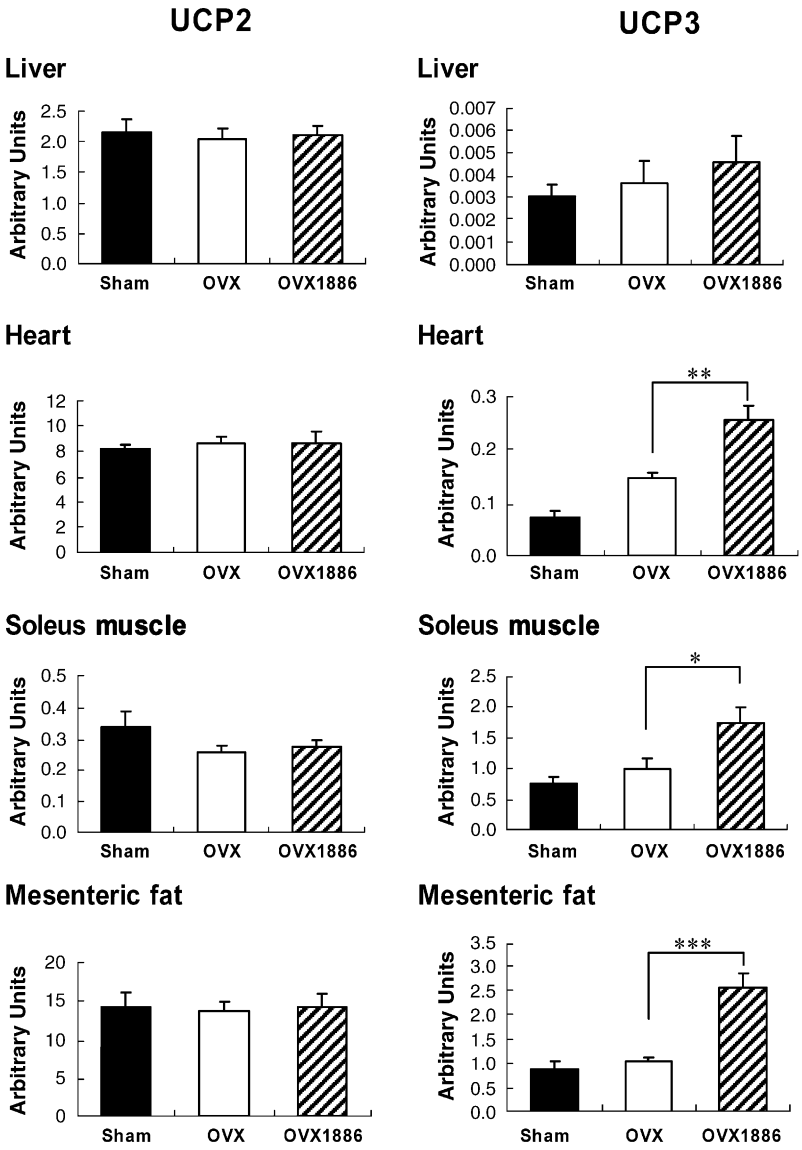

Fig. 6. Effects of NO-1886 treatment on the UCP mRNA levels. The expression of UCP2 and UCP3 mRNA in the liver, heart, soleus muscle, and mesenteric fat from sham-operated, OVX, and OVX + NO-1886 rats. All tissues were obtained from the rats shown in Fig. 2. The mRNA expression levels were analyzed by the real-time RT-PCR method. The details of the experiments are described in Materials and methods. Data are expressed as means ± SEM (n = 6). *$P < .05$, **$P < .01$, ***$P < .001$, significant difference from the values seen in the OVX rats.

high-sucrose diet increased whole-body glucose clearance and inhibited adipocyte enlargement in high-fat/high-sucrose diet–induced diabetic minipigs. These data suggest that NO-1886 improves the glucose metabolism by decreasing fat deposit. The aims of the current study were to ascertain whether NO-1886 has an antiobesity action in OVX rats, which is the postmenopausal rat model, and to clarify the mechanism of this action if present.

In this study, NO-1886 decreased both visceral and subcutaneous fat accumulation and suppressed the increase in body weight that normally follows ovariectomy without affecting food intake. Furthermore, plasma leptin levels were elevated compared with sham rats, and the effects caused by leptin were attenuated in the NO-1886–administered OVX rats. Because NO-1886 did not significantly affect plasma concentrations of glucose and insulin, it was likely that NO-1886 did not significantly affect glucose metabolism in the OVX rats. However, it can be presumed that the decreased plasma leptin level caused by the NO-1886 administration is due to the reduction in the weight of the adipose tissue from which leptin is secreted [18]. On the other hand, NO-1886 did not significantly increase nonesterified fatty acid in the rats (data not shown). Furthermore, the RQ values for the NO-1886–treated rats were less than those for the OVX group. These results may indicate that NO-1886 accelerates fatty acid oxidation, leading to a reduction in the RQ and a subsequent suppression of fat accumulation in the OVX rats.

The plasma membrane lipid transporters FAT/CD36 and FABP are known to be the principal molecules that determine the rate and capacity of fatty acid uptake and transport [19,20]. NO-1886 has been reported to accelerate the mRNA expression of fatty acid transporter, oxidation enzymes, and UCP2 in the normal rat liver [21]. In this study, we determined the mRNA levels of fatty acid transporter in OVX rats and in NO-1886–administered OVX rats. As compared with that seen in the OVX rats, NO-1886 administration significantly increased the mRNA levels of the plasma membrane fatty acid transporter enzymes (either FAT/CD36 or FABP) in the liver, soleus muscle, and mesenteric fat.

UCPs are inner mitochondrial membrane transporters that uncouple substrate oxidation from ATP synthesis, converting fuel to heat. Recently, however, in addition to its role as a UCP, UCP3 has also been identified as being involved in transporting fatty acid anions [22], preventing reactive oxygen species [23], and regulating fatty acids and glucose [24]. Although UCP3 is not a primary regulator of thermogenesis such as UCP1, UCP3 might influence energy expenditure because of a secondary effect that is due to the uncoupling of its physiological function [25].

Because up-regulation of UCP3 mRNA levels has been observed during fasting [26], high-fat feeding [27], exercise [28], and lipid infusion [29], it has been proposed that UCP3 is responsible for the regulation of lipids as fuel substrates. Although many studies of UCP3 expression have been reported, it has not been established as to whether UCP3 plays an important role in thermogenesis. In obese Zucker *fa/fa* rats [30] and Wistar fatty rats [31], the UCP3 expression level in skeletal muscle has been found to be decreased relative to the control lean rats. In Pima Indians, it has been reported that there is both a negative correlation between skeletal muscle UCP3 expression and body mass index and a positive correlation between the UCP3 mRNA level and the resting metabolic rate [32]. In humans, stable weight reduction was associated with a decrease in UCP3 mRNA in skeletal muscle [33]. Furthermore, overproduction of UCP3 in skeletal muscle reduced the body weight despite an increased intake of food [34]. Therefore, UCP3 is an interesting possible target for the prevention and treatment of obesity. In a recent study that used mutant mice that overexpressed or lacked LPL, UCP3 mRNA levels were increased in the mice with the highest levels of LPL in the skeletal muscle and down-regulated in the mice that lacked LPL in the skeletal muscle [35]. These findings indicate that increased LPL activities occurring in the skeletal muscle contribute to the induction of UCP3 expression by promoting an increased uptake of fatty acids.

In the present study, NO-1886 significantly increased UCP3 mRNA compared with that seen in OVX rats in the heart, soleus muscle, and mesenteric fat, but did not affect UCP1 or UCP2 mRNA. However, in BAT, UCP1, UCP2, or UCP3 mRNAs were not affected by NO-1886 administration. Therefore, it is theorized that increased fatty acids, which are degraded by the LPL-promoting agent NO-1886, are immediately incorporated into the cell via the fatty acid transporter enzymes, resulting in the acceleration of fatty acid oxidation and activation of UCP3, and the conversion of fuel to heat in the peripheral tissues in the OVX rats. These actions may explain the antiobesity effect of NO-1886.

NO-1886 does not have an affinity for human estrogen receptors in binding assays and does not have an effect on estrogen action (data not shown). Our study indicates that NO-1886 might potentially be beneficial in the treatment of obesity and obesity-linked health problems for postmenopausal women and may protect against the development of atherosclerosis, as previously reported by Tsutsumi et al [7].

In summary, NO-1886 ameliorated obesity in OVX rats. This is possibly due to an increased expression of fatty acid oxidation–related enzymes and UCP3, which are related to fatty acid transfer and fat use.

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