Lipoprotein Lipase Activator NO-1886 (ibrolipim) Accelerates the mRNA Expression of Fatty Acid Oxidation–Related Enzymes in Rat Liver

Masako Doi, Yasunori Kondo, and Kazuhiko Tsutsumi

The lipoprotein lipase (LPL) activator NO-1886 (ibrolipim) has been shown to have potential benefits for the treatment of obesity in rats. However, the anti-obesity mechanism of NO-1886 has not been clearly understood. To address this, we studied the effects of NO-1886 on the mRNA expression of fatty acid oxidation–related enzymes in rats. The respiratory quotient (RQ) in rats administered a single oral dose of NO-1886 was significantly lower than control rats under both fed and fasted conditions. NO-1886 orally administered to rats for 7 days caused 1.54-fold increase in carnitine palmitoyl transferase II (CPTII) mRNA in the carnitine palmitoyl transferase system. Furthermore, NO-1886 caused a 1.47-fold increase in long-chain acyl-CoA dehydrogenase (LCAD) mRNA, a 1.49-fold increase in acetyl-CoA acyltransferase 2 (ACAA2) mRNA, and a 1.24-fold increase in enoyl-CoA hydratase (ECH) mRNA in rats, all which are liver β-oxidation enzymes. NO-1886 also increased uncoupling protein-2 (UCP2) mRNA levels in liver by 1.42-fold when compared to the control group. These results suggest that the LPL activator NO-1886 may accelerate the expression of fatty acid oxidation–related enzymes, resulting in a reduction of RQ.

THE SYNTHESIZED compound NO-1886 (ibrolipim) is a lipoprotein lipase (LPL) activator.[1-4] Kusunoki et al have reported that NO-1886 prevented fat accumulation in high-fat–fed rats by increasing fat oxidation.[5] However, the mechanism for this prevention of fat accumulation remained unclear. NO-1886 is known to cause a reduction of respiratory quotient (RQ) in carbohydrate-induced hyperlipidemic rats and high-fat-fed–induced obese rats.[5,6] RQ is the steady-state ratio of carbon dioxide production to oxygen consumption by whole body metabolism. Therefore, in general, a decrease in RQ means an increase in fat oxidation.[6]

We hypothesized that NO-1886 may activate the fatty acid oxidation–related enzymes in rats resulting in a decrease in RQ. In this study we examined whether NO-1886 accelerated the expression of mRNA of fatty acid oxidation–related enzymes and uncoupling protein-2 (UCP2) in rat liver.

MATERIALS AND METHODS

Materials

Agent NO-1886 (ibrolipim), 4-diethoxyphosphorylmethyl-N- (4-bromo-2-cyanophenyl) benzamide, was synthesized in the New Drug Research Laboratory at Otsuka Pharmaceutical Factory, Tokushima, Japan. The chemical structure is shown in Fig 1. All other chemicals used were high-grade commercially available products.

Animals

Twenty-four male Sprague-Dawley rats from Charles River Japan, Inc (Yokohama, Japan), weighing 80 to 100 g, were maintained under constant humidity and temperature (22 ± 2°C) and a 12-hour light-dark cycle (light from 7 AM to 7 PM). They were fed a standard diet, CRF-1 (Oriental Yeast, Tokyo, Japan), and water ad libitum.

Measurement of Respiratory Quotient

After 3 weeks, 12 rats at 7 weeks of age, weighing 320 to 360 g, were stratified by body weight and divided into 4 groups of 3 rats each. NO-1886 suspended in 5.0% arabic gum solution was orally administered at a dose of 100 mg/kg to 2 groups (NO-1886–treated fasted or fed group). A 5.0% arabic gum solution was administrated to the other 2 groups (control fasted or fed group). The RQ in the rats was measured 4 hours after NO-1886 administration.

Oxygen consumption and carbon dioxide production were measured using a 6-chamber Oxycongamma (Fukuda Denshi, Tokyo, Japan). Temperature was maintained at 22 ± 2°C, and lights were on from 7 am to 7 pm. System settings included a flow rate of 1.6 L/min, and a measurement period of 60 seconds. The RQ of rats was measured 4 hours after NO-1886 administration from 10 am to 2 pm. Rats were placed in separate calorimetry chambers, each with a volume of 15.8 L. Six rats (control fasted group and NO-1886–treated fasted group) had access only to water, and the other 6 rats (control fed group and NO-1886–treated fed group) had ad libitum access to chow and water.

RNA Preparation

The remaining 12 rats were stratified by body weight and divided into 2 groups of 6 rats each. NO-1886 suspended in 5.0% arabic gum solution was orally administered at a dose of 100 mg/kg/d to one group for 7 days (NO-1886–treated group). A 5.0% arabic gum solution was administered to the other group for 7 days (control group). At 2 PM, 4 hours after the last administration, the control and NO-1886–treated rats were anesthetized with sodium pentobarbital. The liver tissues were then excised and immediately frozen in liquid nitrogen and stored at −80°C until use.

Total RNA was extracted from a 30-mg tissue sample of each liver by 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.

Real-Time Quantitative Reverse-Transcriptase Polymerase Chain Reaction

Real-time quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) was performed to determine the relative expression levels of the rat fatty acid oxidation enzymes in liver.

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, was covalently linked to the

From the Division of Pharmacology, Drug Safety and Metabolism, and Research and Development, Otsuka Pharmaceutical Factory, Inc, Tokushima, Japan.

Submitted December 5, 2002; accepted July 16, 2003.

Address reprint requests to Masako Doi, Division of Pharmacology, Drug Safety and Metabolism, Otsuka Pharmaceutical Factory, Inc, Muya-cho, Naruto, Tokushima 772-8601, Japan.

0026-0495/03/5212-0012$30.00/0
doi:10.1016/j.metabol.2003.07.007

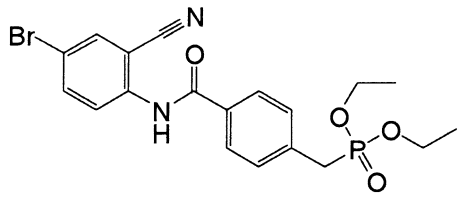

Fig 1. Chemical structure of NO-1886 (ibrolipim).

5′-end of the oligonucleotide. This reporter dye was quenched by TAMRA (6-carboxy-tetramethyl-rhodamine) typically located at the 3′-end. Fluorescence quenching depended on the spatial proximity of the reporter and quencher dyes.

Oligonucleotide primers and TaqMan probes (shown in Table 1) were designed using Primer Express software version 1.0 (Applied Biosystems, Tokyo, Japan), except for TaqMan 18S ribosomal RNA Control Reagents kit (Applied Biosystems).

The RT-PCR reaction was performed in a 30-μL volume reaction using 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 18S ribosomal RNA. 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. However, 18S ribosomal RNA reaction buffer contained all the above, except for the following differences: 50 nmol/L forward primer, 50 nmol/L reverse primer, and 50 nmol/L TaqMan probe.

RT reaction conditions were 48°C for 30 minutes, and 95°C for 10 minutes for 1 cycle; PCR conditions were 95°C for 15 seconds, and 60°C for 1 minute for 40 cycles on an ABI PRISM 7700 Sequence Detector System (Applied Biosystems). Linearity of the standard curve for the rat β-oxidation enzymes mRNA was obtained for 0.16 to 100 ng total RNA range using a standard sample prepared from liver of control rats.

The results for the expression of mRNA were presented relative to the expression of the control gene, 18S ribosomal RNA.

Statistical Analysis

Comparisons of 2 groups were made by paired Student's *t* test. Statistical significance is defined as $P < .05$. Values are mean ± SD.

RESULTS

Effects of NO-1886 on RQ

The RQ of NO-1886–treated rats was significantly lower than that of control rats both under fed (5.59% decrease) and fasted (4.03% decrease) conditions (Table 2).

Effects of NO-1886 on mRNA Levels of Fatty Acid Oxidation Enzymes in Rat Liver

In liver, NO-1886 administration caused no significant changes in the mRNA levels of fatty acid translocase (CD36/

Table 1. Oligonucleotide Sequences of Gene-Specific Primers and Probe in Rat Liver

mRNA		Genbank Accession No.	TaqMan Probe and Primers
CD36/FAT	Fatty acid translocase	AF111268	Probe:5′-FAM- CTCTTCCACATTTCCTACATGCAAGTCCTG-TAMRA-3′ Forward: 5′- TGTGCTGGACATTGGCAA-3′ Reverse: 5′- AAGCCTTCGATAGGTTCTGAGAC-3′
LC-ACS	Long-chain acyl-CoA synthetase	D90109	Probe: 5′-FAM-AGAGTGAACAGAACGAAACCCAAGCCTCC-TAMRA-3′ Forward: 5′-GCCTAAAAGCTCTGGAGGATCTT-3′ Reverse: 5′-GGTTGCCTGTAGTTCCACTTGTG-3′
CPTI	Carnitine palmitoyl transferase I	NM_031559	Probe: 5′-FAM-AGGCAGAACTTGCCCATGTCCTTGTAATG-TAMRA-3′ Forward: 5′-CCATAGTGCAGGAGCGTACAGT-3′ Reverse: 5′-CGAGTCCCGATGCCTTCAT-3′
CPTII	Carnitine palmitoyl transferase II	J05470	Probe: 5′-FAM-TGGTGTCGAACTTCTCCTTGGCAGC-TAMRA-3′ Forward: 5′-GCTCTCAAGGCTGGCATCAC-3′ Reverse: 5′-TGGAACTGAATGGAGTCAATGCT-3′
LCAD	Long-chain acyl-CoA dehydrogenase	J05029	Probe: 5′-FAM-CGTCACCAATCGTGAAGCTCGATCG-TAMRA-3′ Forward: 5′-GGCTGGTTAAGTGATCTCGTGAT-3′ Reverse: 5′-TCTCCACCAAAAAGAGGCTAATG-3′
ECH	Enoyl-CoA hydratase	X15958	Probe: 5′-FAM-TGACCGAATTTCAGCACAGGATGCC-TAMRA-3′ Forward: 5′-AGCAATGGAGATGGTCCTCACT-3′ Reverse: 5′-CACATTGGATGGCCTCTTCA-3′
HAD	3-Hydroxyacyl CoA dehydrogenase	AF095449	Probe: 5′-FAM-TGAACCGTCTCTTGGTGCCATACCTCATAG-TAMRA-3′ Forward: 5′-GAAGCATCCTGTTTCCTGCAA-3′ Reverse: 5′-GCGGTGTCGATGTCTTCCTTA-3′
ACAA2	Acetyl-CoA acyltransferase 2 (3-oxoacyl CoA thiolase)	NM_130433	Probe: 5′-FAM-ATCCAGAATCACCGCACACCTGGTTC-TAMRA-3′ Forward: 5′-AACGTGAGTGGAGGTGCCATA-3′ Reverse: 5′-CAAGCTGATCCCACTGCGTAT-3′
UCP2	Uncoupling protein 2	NM_019354	Probe: 5′-FAM-TTTCCCTCTAGACACCGCCAAAGTCC-TAMRA-3′ Forward: 5′-GCAGCCTGTATTGCAGATCTCA-3′ Reverse: 5′-TGACTCTCTCCTTGGATCTGCAG-3′

Table 2. Effects of NO-1886 on Respiratory Quotient in Rats

	Control Group	NO-1886 Group
Fasting	0.795 ± 0.004	0.763 ± 0.014*
Feeding	0.948 ± 0.032	0.895 ± 0.003*

NOTE. RQ was measured 4 hours after NO-1886 administration in rats under fed and fasted conditions. Data are expressed as means ± SD.

Significantly different from the value in the respective control group: *$P < .05$.

FAT), mitochondrial long-chain acyl-coenzyme A (CoA) synthetase (LC-ACS), and carnitine palmitoyl transferase I (CPTI). However, NO-1886 administration did cause a 1.54-fold induction ($P < .01$) in carnitine palmitoyl transferase II (CPTII) mRNA levels compared to the control group. Expression of long chain acyl-CoA dehydrogenase (LCAD) mRNA was also significantly increased 1.47-fold. The level of enoyl-CoA hydratase (ECH) mRNA was significantly increased 1.24-fold, whereas the level of 3-hydroxyacyl-CoA dehydrogenase (HAD) mRNA was not changed. Regarding acetyl-CoA acyltransferase 2 (ACAA2), the enzyme responsible for the final step of fatty acid oxidation in mitochondria, its mRNA was significantly increased 1.49-fold in liver (Fig 2).

Effects of NO-1886 on mRNA of Rat UCP2 in Liver

In liver, NO-1886 administration caused 1.42-fold induction ($P < .01$) in UCP2 mRNA levels in liver compared to the control group.

DISCUSSION

NO-1886, an LPL activator, is known to increase LPL mRNA and LPL activity in adipose tissue and skeletal muscle.[1-4] Kusunoki et al 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.[5] Furthermore, Hara et al have reported that NO-1886 decreases plasma triglycerides level but does not affect tissue triglycerides accumulation in fructose-fed rats.[6] They have also reported that long-term administration of NO-1886 causes a reduction in RQ in diabetic and obese rats.[5,6] Based on this information, we hypothesized that NO-1886 may activate fatty acid oxidation in rats. In this study, we determined that administration of NO-1886 caused a reduction in RQ in normal rats. Furthermore, NO-1886 increased the mRNA expression of liver β-oxidation enzymes LCAD, ECH, and ACAA2. In general, a decrease in RQ means an increase in fat oxidation. Therefore, these results indicate that NO-1886 may cause a reduction in RQ by accelerating liver β-oxidation.

NO-1886 also increased the mRNA expression of CPTII, an enzyme in the carnitine palmitoyl transferase (CPT) system. The CPT system plays a role in the entrance of acyl moieties into mitochondria, with CPTI located on the outer mitochondrial membrane and CPTII located on the inner mitochondrial membrane.[7] These results may mean that NO-1886 accelerates the transfer of free fatty acids (FFA) into mitochondria.

UCP is considered to be a mitochondrial carrier that stimulates heat production by dissipating the proton gradient generated across the inner mitochondrial membrane during respiration and therefore uncouples respiration from adenosine triphophate (ATP) synthesis.[8] The *UCP1* gene is expressed in brown adipose tissue, the *UCP2* gene is expressed in almost all tissues, and the *UCP3* gene is expressed in skeletal muscle.[9] In this study, NO-1886 increased the mRNA expression of UCP2 by about 42% in rat liver. These results may indicate that NO-1886 accelerates the CPT system resulting in an increase in fatty acid transfer into mitochondria, and an acceleration of β-oxidation and FFA process through UCP2 resulting in a reduction of RQ.

Ferraro et al have reported that RQ is inversely correlated

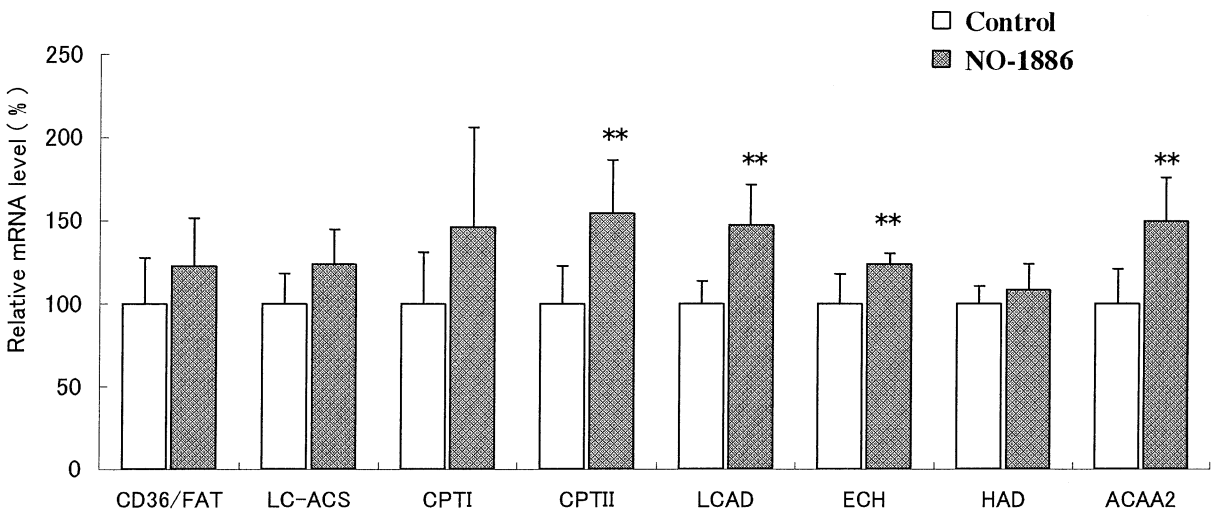

Fig 2. Effects of NO-1886 on rat fatty acid oxidation–related enzyme mRNA in liver. Rats were administered either control solution (5.0% arabic gum) or NO-1886 suspended in 5.0% arabic gum solution (100 mg/kg/d, orally) for 7 days. Total RNA was isolated from liver. Rat fatty acid oxidation–related enzyme mRNA levels were determined by real-time quantitative RT-PCR using 18S ribosomal RNA as an internal standard. Data are expressed as means ± SD (n = 6). Significantly different from the value in the respective control group: **$P < .01$.

with LPL activity in skeletal muscle in Pima Indians.[10] Jensen et al have reported that overexpression of human LPL in skeletal muscle prevents diet-induced obesity in transgenic mice.[11] Furthermore, Tsutsumi et al recently reported that RQ and tissue LPL activity showed circadian rhythms in rats and that RQ was inversely correlated with skeletal muscle LPL activity.[12] These reports indicate that the reduction of RQ is caused by the elevation of skeletal muscle LPL activity. NO-1886 is an LPL activator that increases skeletal muscle LPL activity.[2] In this study, we found that NO-1886 accelerated fatty acid oxidation. Therefore, these results may indicate that elevation of skeletal muscle LPL activity induces the expression of fatty acid oxidation mRNAs resulting in the reduction of RQ. More detailed studies are needed to show the relation between LPL activity in skeletal muscle and fatty acid oxidation in liver.

We did not determine the effects of NO-1886 on glucose metabolism and on peroxisome proliferatior–activated receptor (PPAR) in this study. Recently, Yong-Xu et al have reported that PPAR delta, and not only PPAR alpha and gamma, regulates fatty acid metabolism. PPAR delta stimulates fatty acid oxidation and prevents obesity.[14] In our earlier studies on gene transactivation, NO-1886 did not show agonistic effects against PPAR alpha, delta, gamma, and retinoid X receptor (RXR) alpha (data not shown). These results suggested that activating effect of NO-1886 on fatty acid oxidation did not involve PPARs.

In this study, NO-1886 increased the mRNA expression of UCP2 in liver. Weigle et al reported that elevation of circulating FFA levels in animals given an Intralipid plus heparin infusion caused significant increases in UCP3/actin mRNA ratio in skeletal muscle and concluded that FFAs were potential mediators for the increase in muscle UCP3 expression.[13] NO-1886 is an LPL activator that does not affect FFA in circulation, but may increase FFA concentration in tissue. Recently, we found that NO-1886 reduced fat weight in ovariectomized obese rats, and increased UCP3 expression in skeletal muscle by 1.6-fold compared with control rats (unpublished data). Therefore, NO-1886 increases not only liver UCP2, but also muscle UCP3. NO-1886 may reduce fat weight in obese rats by increasing liver UCP2 and muscle UCP3.

In summary, we determined that NO-1886 accelerates the expression of fatty acid oxidation–related enzymes, resulting in a reduction of RQ.

REFERENCES

1. Tsutsumi K, Inoue Y, Shima A, et al: The novel compound NO-1886 increases lipoprotein lipase activity with resulting elevation of HDL-cholesterol, and long-term administration inhibits atherogenesis in the coronary arteries of rats with experimental atherosclerosis. J Clin Invest 92:411-417, 1993

2. Tsutsumi K, Inoue Y, Shima A, et al: 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, 1995

3. Tsutsumi K, Inoue Y, Hagi A, et al: The novel compound NO-1886 elevates plasma high density lipoprotein cholesterol levels in hamsters and rabbits by increasing lipoprotein lipase without any effect on cholesteryl ester transfer protein activity. Metabolism 46:257-260, 1997

4. Chiba T, Miura S, Sawamura F, et al: Antiatherogenic effects of a novel lipoprotein lipase enhancing agent in cholesterol-fed New Zealand white rabbits. Arterioscler Thromb Vasc Biol 17:2601-2608, 1997

5. Kusunoki M, Hara T, Tsutsumi K, et al: The lipoprotein lipase activator, NO-1886, suppresses fat accumulation and insulin resistance in rats fed a high-fat diet. Diabetologia 43:875-880, 2000

6. Hara T, Cameron-Smith D, Cooney GJ, et al: The actions of novel lipoprotein lipase activator, NO-1886, in hypertriglyceridemic fructose-fed rats. Metabolism 47:149-153, 1998

7. Eaton S, Bartlet K, Pourfarzam M: Mammalian mitochondrial β-oxidation. Biochem J 320:345-357, 1996

8. Argiles JM, Busquets S, Lopez-Soriano FJ: The role of uncoupling proteins in pathophysiological states. Biochem Biophys Res Commun 293:1145-1152, 2002

9. Pecqueur C, Couplan E, Bouillaud F, et al: Genetic and physiological analysis of the role of uncoupling proteins in human energy homeostasis. J Mol Med 79:48-56, 2001

10. Ferraro RT, Eckel RH, Larson DE, et al: Relationship between skeletal muscle lipoprotein lipase activity and 24-hour macronutrient oxidation. J Clin Invest 92:441-445, 1993

11. Jensen DR, Schlaepfer IR, Morin CL, et al: Prevention of diet-induced obesity in transgenic mice overexpressing skeletal muscle lipoprotein lipase. Am J Physiol 273:R683-R689, 1997

12. Tsutsumi K, Inoue Y, Kondo Y: The relationship between lipoprotein lipase activity and respiratory quotient of rats in circadian rhythms. Biol Pharm Bull 25:1360-1363, 2002

13. Weigle DS, Selfridge LE, Schwartz MW, et al: Elevated free fatty acids induce uncoupling protein 3 expression in muscle. Diabetes 47:298-302, 1998

14. Yong-Xu Wang, Chih-Hao Lee, Sambath Tiep, et al: Peroxisome-proliferatior-activated receptor delta activated fat metabolism to prevent obesity. Cell 113:159-170, 2003