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Lipoprotein lipase activator ameliorates the severity of dietary steatohepatitis

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Abstract

Dietary model of steatohepatitis was established by feeding mice a methionine choline deficient (MCD) diet. Mice on MCD or control diet for 3 weeks were treated with or without NO-1886, a newly synthetic lipoprotein lipase (LPL) activator. In a separate experiment, NO-1886 was given after pre-treatment with 3 weeks of MCD diet. NO-1886 significantly reduced MCD-induced inflammation by repressing levels of hepatic lipid peroxides and pro-inflammatory tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and cyclooxygenase-2 (COX-2). In addition, NO-1886 dampened hepatic steatosis via accelerating fatty acid oxidation caused by enhanced expression of PPARα, cytochrome P450-10 (Cyp4a10), and Acyl-CoA oxidase (ACO). It failed to regulate genes of fatty acid uptake and synthesis pathways. In conclusion, NO-1886 ameliorated and induced regression of experimental steatohepatitis via increasing endogenous LPL activation resulting in suppression on pro-inflammatory factors and reduction of hepatic fatty acids. These findings indicate that NO-1886 is a potential therapeutic agent for steatohepatitis.

Keywords: Lipoprotein lipase; NO-1886; Nutritional steatohepatitis; Cytokines; Peroxisome proliferator-activated receptor

Nonalcoholic steatohepatitis (NASH) is an emerging epidemic disease that may lead to fibrosis, cirrhosis, and even hepatocellular carcinoma [1,2]. The relationship of fatty liver to the metabolic syndrome is now well established. One common feature of NASH and metabolic syndrome is an increase in serum triglycerides, free fatty acids (FFAs), and decreased levels of high-density lipoprotein (HDL) cholesterol. The enhanced uptake of fatty acids in excess of metabolic requirements results in accumulation of triglycerides and is one factor leading to steatosis in NASH [1]. In turn, fatty acids can trigger oxidative stress and stimulate inflammatory recruitment to cause steatohepatitis [1–6]. There is currently no specific therapy for the treatment of NASH. The only potential therapeutic options are insulin sensitizers such as metformin and thiazolidinediones, but the long-term clinical efficacy of these agents has not been confirmed.

NO-1886 is a novel compound that has been reported to increase lipoprotein lipase (LPL) mRNA and activity, resulting in a reduction in plasma triglyceride levels and elevation of HDL in animals [7]. NO-1886 also reduces plasma FFA and glucose concentration in rabbits fed a high-fat/high sucrose diet [8]. Furthermore, NO-1886 inhibits fat accumulation and reduces insulin resistance in type 2 diabetes [9]. However, whether NO-1886 could be a potential therapeutic agent for NASH is still unknown. The present study was designed to investigate whether induction of LPL activity by NO-1886 would ameliorate

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the progression of steatohepatitis. The mechanisms of action are also studied.

Materials and methods

Animal treatments. Male C57BL/6J mice (8 weeks old) were housed in a temperature-controlled room under a 12-h light/12-h dark cycle and had free access to food and water. Experimental diets included: MCD diet (cat. No. 960439; ICN, Aurora, OH); control diet (MCD diet supplemented with DL-methionine (3 g/kg) and choline chloride (2 g/kg; cat. NO. 960441; ICN, Aurora, OH); and MCD diet supplemented with the LPL selective inducer, NO-1886 (500, 1000, and 2000 ppm, respectively) [10], for up to 3 weeks. In a separate experiment, mice were treated with NO-1886 in the diet at 2000 ppm after fed MCD for 3 weeks, and the experiment was continued for additional 2 weeks. At the end of the experiment, animals were sacrificed without fast. All the protocols and procedures were approved by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong.

Determination of serum and hepatic chemistry levels. Serum alanine aminotransferase (ALT), triglyceride, HDL, and cholesterol levels were determined using spectrophotometric assay kits (Sigma, St. Louis, MO). Liver triglyceride content was estimated according to the manufacturer's instruction using the triglyceride E-test kit (Wako Pure Chemical Industries, Osaka, Japan). Liver lipoperoxide levels were estimated using the thiobarbituric acid-reactive substances (TBARS) assay [11].

Determination of hepatic mRNA expression. Total RNA was isolated from liver using TRIzol (Invitrogen, Carlsbad, CA). First strand cDNA synthesis was performed by reverse transcription of 5 μg of total RNA. Hepatic mRNA expression of LPL, pro-inflammatory factors including tumor necrosis factor-alpha (TNF-α), interleukin (IL-6), intercellular adhesion molecule-1 (ICAM-1), and cyclooxygenase-2 (COX-2); and fatty acid metabolism-related regulators including acyl-CoA oxidase (ACO), fatty acid synthase (FAS), long-chain acyl-CoA dehydrogenase (LCAD), liver X receptors-alpha (LXRα) and -beta (LXRβ), stearoyl coenzyme A desaturase-1 (SCD1), sterol regulatory element binding protein-1c (SREBP-1c), peroxisome proliferator-activated receptor-alpha (PPARα) and -gamma (PPARγ), and cytochrome P4504a10 (Cyp4a10) and Cyp4a14 was quantified by real-time reverse transcriptase (RT)-PCR using SYBRGreen Master Mix (Applied Biosystems, Foster, CA).

LPL activity assay. Liver tissues (~40 mg) were homogenized in ice-cold buffer and the intracellular LPL activities determined according to the manufacturer's instructions (Jiancheng Bioengineeringm, Nanjing, China).

Statistical analysis. Data are presented in this study as means ± SD. Comparisons between groups were performed with the Student's *t*-test or ANOVA. The Mann–Whitney rank sum test was performed for histological analysis. A two-side *P* value less than 0.05 was considered statistically significant.

Results

Effect of NO-1886 on MCD diet-induced steatohepatitis

Similar to previous reports, mice fed the MCD diet had significant weight loss [3,11,12], and administration of NO-1886 did not rectify the body weight loss. Weight loss was similar in the MCD group and MCD supplemented with NO-1886 group. In contrast, mice in the control group gained a minimal amount of weight during the study period.

Mice fed the control diet exhibited normal liver histology (Fig. 1A), serum ALT (Table 1), and total hepatic lipid content (Table 1). Feeding the MCD diet for 3 weeks induced moderate hepatic steatosis with diffuse scattered lobular inflammatory cells throughout the hepatic parenchyma as well as in inflammatory foci (Fig. 1B and Table 1). This was associated with a marked elevation of serum ALT levels (Table 1) and reduced serum triglyceride, HDL, and cholesterol concentrations (Table 1). In contrast, in mice fed MCD diet supplemented with NO-1886 at 1000 and 2000 ppm, serum ALT levels were significantly lower than mice receiving MCD diet only (Table 1). The severity of liver necroinflammation was significantly reduced in those receiving high doses NO-1886 (Fig. 1 and Table 1). Blood chemistry analysis showed that NO-1886 treatment of MCD diet–fed mice reduced serum triglyceride (Table 1) and restored serum HDL (Table 1) and total cholesterol levels (Table 1). Histological grading of liver sections confirmed that NO-1886 significantly ameliorated steatosis (Fig. 1 and Table 1), as supported by the significant reduction in intrahepatic triglyceride levels (Table 1).

Effect of NO-1886 on oxidative stress

Hepatic TBARS was analyzed as a marker of oxidative stress. Mice fed MCD had significantly greater TBARS compared with mice on control diet (Table 1). Increased hepatic lipid peroxidation levels induced by the MCD diet were significantly modified by dietary supplementation with NO-1886 at concentrations of 1000 and 2000 ppm, respectively (Table 1).

Effect of NO-1886 on LPL mRNA expression and LPL activity

Compared to control animals, co-administration of NO-1886 upregulated LPL mRNA expression. As shown in Table 1, the amount of LPL mRNA was increased by NO-1886 in a dose-dependent manner. With NO-1886 in doses of 500, 1000, and 2000 ppm, the levels of LPL mRNA was raised by 3-, 4-, and 6.2-fold, respectively. As compared with the MCD diet mice, a significant induction of LPL mRNA was found in mice fed with NO-1886 1000 and 2000 ppm, respectively (Table 1). Consistent with this finding, LPL activity was also enhanced in these animals (Table 1).

Effects of NO-1886 on the expression of inflammatory factors

To elucidate the mechanisms of the effect of NO-1886 on steatohepatitis, we investigated mRNA expression levels of pro-inflammatory factors including TNF-α, IL-6, COX-2, and ICAM-1. Hepatic TNF-α, IL-6, COX-2, and ICAM-1 genes were upregulated in the MCD group relative to the control group (Table 2). Administration of NO-1886 at the dose of 2000 ppm caused a significant decrease of TNF-α (2.4-fold), IL-6 (1.6-fold), and COX-2 mRNA (3.5-fold) compared to MCD diet alone (Table 2). The level of ICAM-1 was not changed.

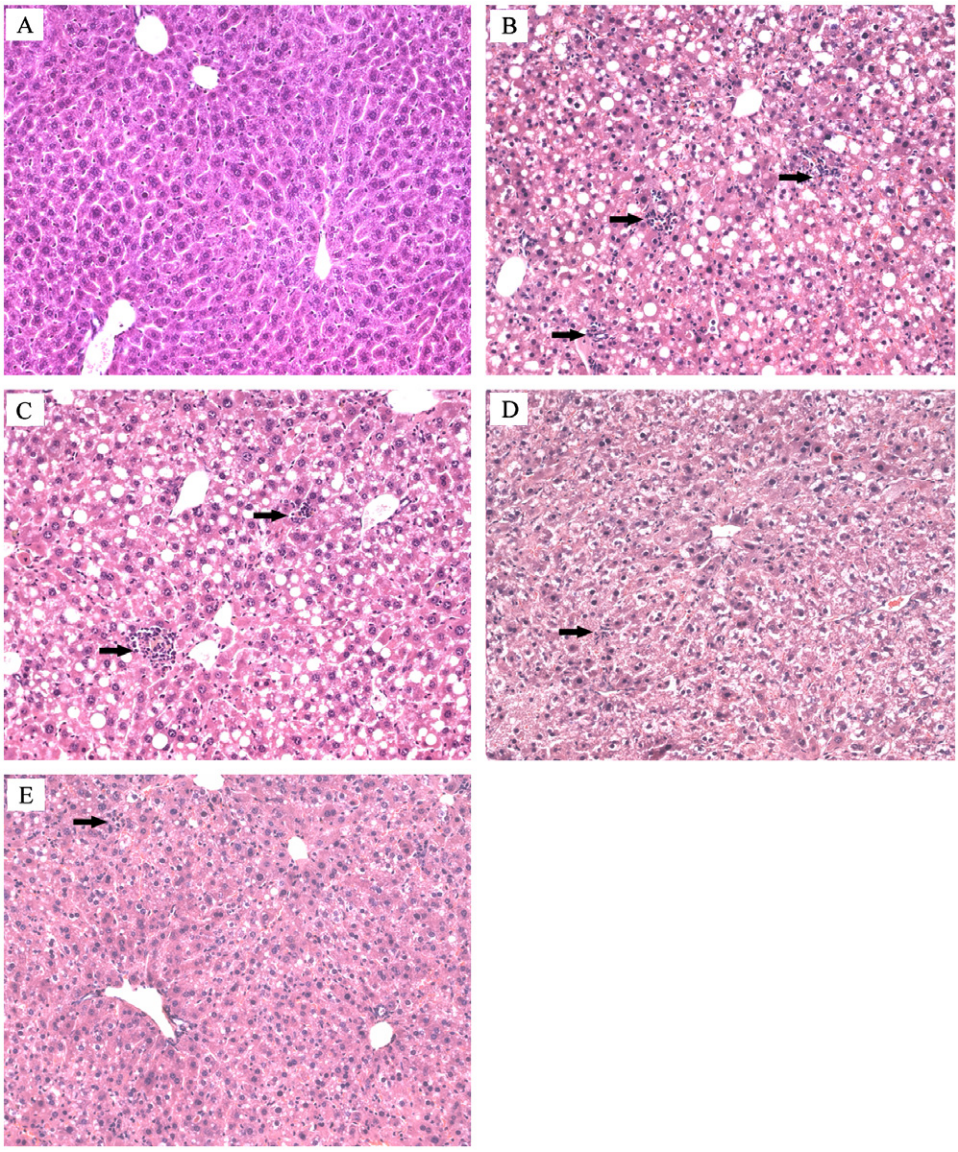

Fig. 1. Effect of treatment with NO-1886 on MCD diet-induced steatohepatitis. Hematoxylin and eosin-stained liver sections from mice fed: (A) the control diet, (B) MCD diet, (C) MCD diet supplemented with NO-1886 (500 ppm), (D) with NO-1886 (1000 ppm), and (E) with NO-1886 (2000 ppm). Experimental duration is 3 weeks. Slides are representative of 5–6 separate experiments (original magnification 100×).

Effects of NO-1886 on the expression of genes involved in fatty acid regulation

Steatosis occurs because of imbalance between hepatic uptake of fatty acids and/or increased hepatic fatty acid synthesis (lipogenesis) on one hand, and impaired fat disposal, such as via fatty acid oxidation (lipolysis), on the other [2,13–16]. To seek an explanation for the serum and hepatic triglyceride-lowering effects of NO-1886, we assessed the hepatic expression levels of the lipogenic genes PPARγ, SREBP1c, FAS, LXRα, LXRβ, and SCD1, and lipolytic genes involved in the β-oxidation of fatty acid, such as ACO, LCAD, PPARα, and PPARα downstream target molecules Cyp4a10 and Cyp4a14. Compared with mice fed the MCD diet alone, administration of NO-1886 with the MCD diet had no effect on mRNA levels for any of the lipogenic genes (Table 3), However, NO-1886 significantly induced mRNA levels for selected lipolytic genes ACO (2.2-fold), PPARα (2.8-fold), and Cyp4a10 (2.3-fold) (Table 3). LCAD and Cyp4a14 were not changed by NO-1886 treatment (Table 3).

NO-1886 dampened established steatohepatitis

To assess whether NO-1886 could ameliorate the histological severity of established steatohepatitis, mice administered the MCD diet for 3 weeks were treated with NO-1886. We used the dose of 2000 ppm because this conferred clear preventive effects on the development of steatohepatitis in this study. NO-1886 treatment was initiated in mice after feeding MCD diet for 3 weeks, and the experiment was terminated 2 weeks later. A remarkable improvement of liver histology (Fig. 2A–C) concomitant with reduced serum ALT (Fig. 2D), diminished hepatic inflammatory

Table 1
Effect of MCD diet and treatment with NO-1886

	Control	MCD	MCD + NO-1886 500 ppm	MCD + NO-1886 1000 ppm	MCD + NO-1886 2000 ppm
Serum ALT (U/L)	58 ± 5.7	628 ± 248.6***	534 ± 257.3	181 ± 84.5##	290 ± 84.9#
Serum TG (mmol/L)	1.03 ± 0.15	0.69 ± 0.07***	0.72 ± 0.17	0.54 ± 0.14	0.43 ± 0.10#
Serum HDL (mmol/L)	2.24 ± 0.36	1.24 ± 0.26**	1.55 ± 0.41	1.97 ± 0.27#	2.03 ± 0.33##
Serum Cholesterol (mmol/L)	3.58 ± 0.21	1.41 ± 0.23***	1.5 ± 0.12	1.79 ± 0.06#	1.82 ± 0.13##
Steatosis	0.00 ± 0.00	.75 ± 0.76***	2.20 ± 0.67	1.00 ± 0.61##	0.9 ± 0.65##
Necroinflammation	0.3 ± 0.27	2.00 ± 0.32***	1.60 ± 0.42	1.10 ± 0.22#	1.3 ± 0.45#
Hepatic TG (μg/g liver)	33 ± 11	116 ± 16***	51 ± 9	40 ± 18###	31 ± 9###
Hepatic TBARs (μmol/g liver)	0.29 ± 0.03	3.05 ± 0.37***	2.45 ± 0.35	2.21 ± 0.38#	2.36 ± 0.51#
LPL mRNA	1 ± 0.28	2.10 ± 0.60	2.98 ± 0.73	3.98 ± 1.05#	6.19 ± 0.85##
LPL activity (U/mg protein)	0.44 ± 0.04	0.59 ± 0.04*	0.82 ± 0.08##	0.89 ± 0.09###	0.91 ± 0.12###

Values of hepatic steatosis and necroinflammation are means ± SD ($n = 4$–5/group).
* $P < 0.05$ control *vs.* MCD diet.
** $P < 0.01$ control *vs.* MCD diet.
*** $P < 0.001$ control *vs.* MCD diet.
# $P < 0.05$ MCD *vs.* MCD + NO-1886-treated mice.
$P < 0.01$ MCD *vs.* MCD + NO-1886-treated mice.
$P < 0.0001$ MCD *vs.* MCD + NO-1886-treated mice.

Table 2
Effect of MCD diet with or without NO-1886 on hepatic inflammatory mediators TNF-α, IL-6, COX-2, and ICAM-1 mRNA expression levels

	Control	MCD	MCD + NO-1886 2000 ppm
TNF-α	1.00 ± 0.49	4.32 ± 0.90***	2.42 ± 1.21#
IL-6	1.00 ± 0.4	3.89 ± 1.38**	1.62 ± 0.67##
COX-2	1.00 ± 0.56	6.11 ± 1.48***	3.46 ± 1.94#
ICAM-1	1 ± 0.26	1.98 ± 0.68*	1.70 ± 0.50

Data are means ± SD ($n = 5$–6/group).
* $P < 0.05$ control *vs.* MCD diet.
** $P < 0.001$ control *vs.* MCD diet.
*** $P < 0.0001$ control *vs.* MCD diet.
# $P < 0.05$ MCD *vs.* MCD + NO-1886.
$P < 0.01$ MCD *vs.* MCD + NO-1886.

Table 3
Effect of MCD diet with or without NO-1886 on the expression of genes involved in fatty acid regulation

	Control	MCD	MCD + NO-1886 2000 ppm
SREBP-1c	1.00 ± 0.55	0.16 ± 0.06**	0.19 ± 0.11
FAS	1.00 ± 0.4	0.41 ± 0.23**	0.27 ± 0.12
PPARγ	1.00 ± 0.59	0.65 ± 0.16	0.71 ± 0.72
LXRα	1.00 ± 0.50	0.49 ± 0.17*	0.37 ± 0.07
LXRβ	1.00 ± 0.45	0.60 ± 0.28	0.47 ± 0.09
SCD1	1.00 ± 0.48	0.05 ± 0.03****	0.08 ± 0.03
LCAD	1.00 ± 0.35	0.53 ± 0.18*	0.56 ± 0.20
ACO	1.00 ± 0.33	0.17 ± 0.06****	0.37 ± 0.07#
PPARα	1.00 ± 0.43	0.23 ± 0.06***	0.64 ± 0.08#
Cyp4A10	1.00 ± 0.57	3.08 ± 0.93**	7.19 ± 1.44##
Cyp4a14	1.00 ± 0.31	1.66 ± 0.32*	1.48 ± 0.30

Data are means ± SD ($n = 5$–6/group).
* $P < 0.05$ control *vs.* MCD diet.
** $P < 0.01$ control *vs.* MCD diet.
*** $P < 0.001$ control *vs.* MCD diet.
**** $P < 0.0001$ control *vs.* MCD diet.
# $P < 0.01$ MCD *vs.* MCD + NO-1886.
$P < 0.0001$ MCD *vs.* MCD + NO-1886.

score (Fig. 2E), fat score (Fig. 2F) and lowered intrahepatic triglyceride (Fig. 2G) and TBARs (Fig. 2H) was documented in NO-1886 treated mice as compared with MCD only mice.

Discussion

Mice fed MCD diet consistently developed steatohepatitis within weeks. The histology exhibited steatosis, cellular inflammatory infiltrate, and hepatocellular necrosis, mimicking the pathology of NASH in humans. Administering the LPL selective inducer NO-1886 (1000 or 2000 ppm) in MCD fed mice increased liver LPL mRNA and LPL activity. This resulted in attenuation of the steatohepatitis, as evident by decreased ALT levels and diminished histologic evidence of inflammation. In addition, mice administered NO-1886 had less hepatic steatosis and hepatic triglyceride content was lower than in MCD diet-alone fed mice. NO-1886 was associated with a significant reduction of intrahepatic oxidative stress, and its antioxidant effect appears to play a role in the attenuation of steatohepatitis.

We demonstrated here that the novel compound NO-1886, which acts to increase hepatic LPL activity, significantly reduced plasma triglyceride levels with a concomitant increase in HDL cholesterol. Studies have revealed that NO-1886, through an enhancement of LPL activity, plays an important role in lowering plasma triglyceride and in generating HDL cholesterol in rodents [7,10,17]. In humans, hypertriglyceridemia with low HDL cholesterol is an important component of metabolic syndrome. There is increasing evidence of an association between hypertriglyceridemia and increased risk of NASH, and an inverse relationship between plasma HDL levels and development of NASH has been observed [18–21].

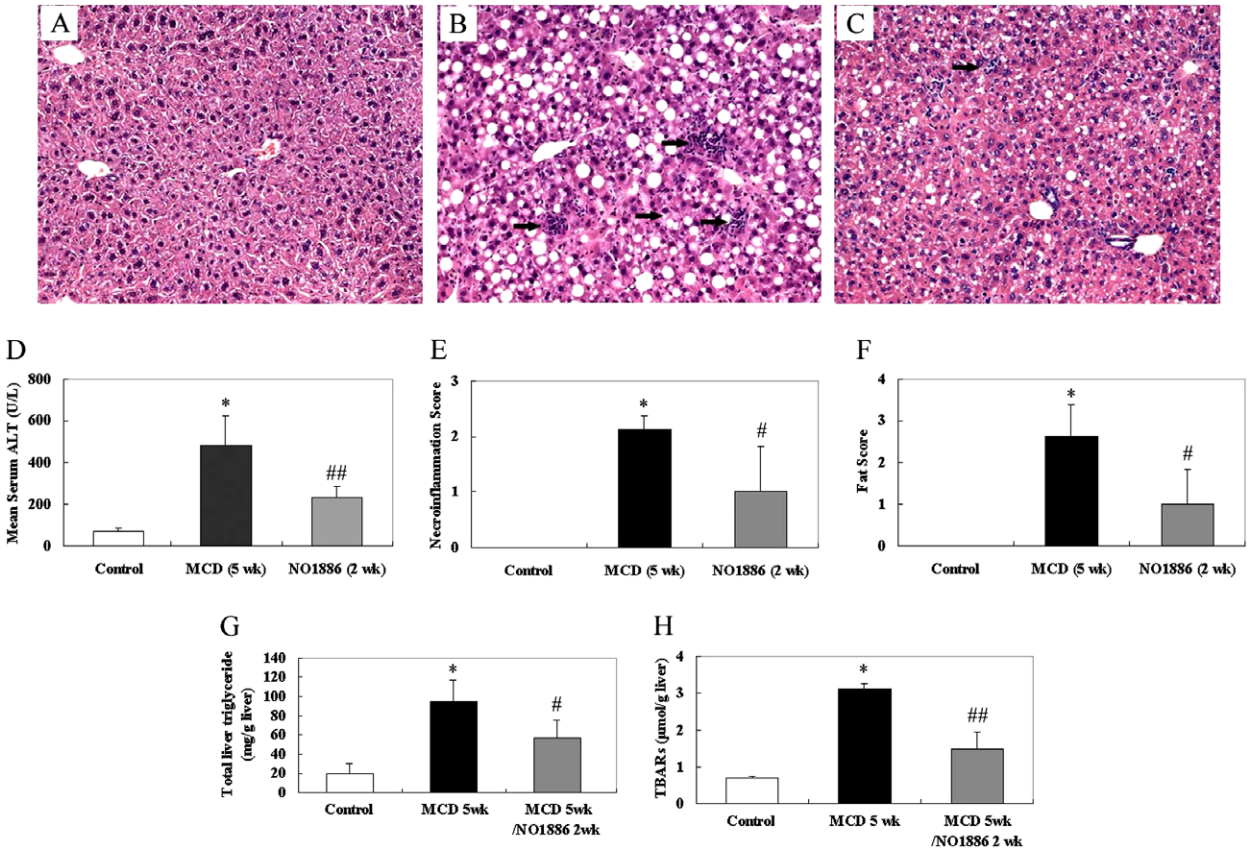

Fig. 2. Effect of NO-1886 on established steatohepatitits induced by the MCD diet. Hematoxylin and eosin-stained liver sections from mice fed (A) the control diet, (B) MCD diet for 5 weeks, and (C) MCD diet supplemented with NO-1886 (2000 ppm) for the final 2 weeks. Slides are representative of 4–5 separate experiments. (D) The group data for serum ALT, (E) steatosis, (F) inflammatory scores, (G) hepatic triglyceride, and (H) lipoperoxide contents were assessed in mice fed the control, MCD diet for 5 weeks or MCD diet supplemented with NO-1886 (2000 ppm) in the last 2 weeks (total 5 weeks). Data are means ± SD with four to five animals in each group. $^{*}P < 0.0001$, MCD compared with control diet–fed mice. $^{\#}P < 0.05$, $^{\#\#}P < 0.001$, NO-1886-treated MCD fed compared with the mice fed the MCD diet.

These data suggest that the protective effect of NO-1886 in steatohepatitis might result, at least partly, from remodeling the serum lipoprotein profile though an enhancement of LPL activity. Such an effect might be advantageous for patients with NASH.

TNF-α, IL-6, ICAM-1, and COX-2 are key inflammatory factors involved in the development of steatohepatitis [2,6,22,23]. All of these genes have pro-inflammatory properties and have been implicated in other hepatic inflammatory processes induced by alcohol, viruses, toxins, or ischemia–reperfusion injury [24–26]. These pro-inflammatory regulators are mediated, at least in part, through oxidative stress [2]. In this study, NO-1886 administration significantly suppressed oxidative stress and blunted TNF-α, IL-6, and COX-2 gene expression. Consistent with our findings, Yin et al. [27] reported that NO-1886 lowered plasma TNF-α in pigs with high-fat/high-sucrose diet-induced diabetes. Further, Niho et al. demonstrated that NO-1886 reduced COX-2 mRNA levels in the small intestine of Min mice [28]. The anti-inflammatory effects of NO-1886 may be partly related to inhibition of hepatic lipoperoxide and reductions in the mRNA expression levels of these regulators. Despite inhibition of these inflammatory regulators, the protection against diet-induced steatohepatitis conferred by NO-1886 remained incomplete as transcription of individual mediators of inflammation was not equally dampened by NO-1886. For instance, NO-1886 failed to alter hepatic expression of ICAM-1, a downstream target of nuclear factor-kappaB (NF-κB), which appears, play a pivotal role in the initial inflammatory recruitment in experimental steatohepatitis [22].

Hepatic steatosis represents an excessive accumulation of triglycerides in the hepatocytes of the liver. The underlying cause of fat accumulation in steatosis is mostly attributable to enhanced uptake and synthesis of fatty acids, and to inhibition of fatty acid oxidation. Administration of NO-1886 did not alter the expression of transcription factors (SREBP1c, PPARγ) and genes of fatty acid uptake (LXRa, LXRβ), synthesis (FAS, SCD1) pathways that favor import of lipid into the liver [13,15,29–32], Thus NO-1886 may not effect the fat input pathways.

In the liver, fatty acid oxidation occurs via mitochondrial and peroxisomal β-oxidation and CYP4A-catalyzed ω-oxidation [33]. ACO and LCAD are the key enzymes of these three fatty acid oxidation systems in liver and are regulated by the lipolytic transcription factor PPARα

[15,33]. In this study, expression of PPARα, ACO, and LCAD was reduced following administration of the MCD diet to mice. Similar observations have been reported both in MCD fed mice [3] and ethanol fed mice [34]. Interestingly, supplementation of the MCD diet with NO-1886 increases fatty acid oxidation via enhanced expression of PPARα, ACO, and Cyp4a10. It has been shown that PPARα stimulation is efficacious against steatohepatitis in MCD fed mice [3,6]. Doi et al. [35] reported that NO-1886 accelerated β-oxidation in rat liver. Thus, the amelioration of hepatic steatosis obtained with NO-1886 could have been a consequence of PPARα stimulation, and accelerated fatty acid oxidation catalyzed by LPL and PPARα regulated genes. Lipoprotein lipase hydrolyzes triglycerides and thereby plays a central role in the catabolism of triglyceride-rich lipoproteins [35].

In conclusion, NO-1886, a LPL selective inducer, attenuated the development of steatohepatitis and induced regression of established steatohepatitis in MCD diet–fed mice. Multiple mechanisms could be involved, including antioxidant effects, anti-inflammatory properties, and induction of fatty acid oxidation in the liver, via enhancing LPL activity. LPL activator NO-1886 is potentially beneficial for the treatment of steatohepatitis. Whether the effects of LPL activation on experimental steatohepatitis occur directly through modulation of fatty acid turnover or result from its effects on enhanced degradation of inflammatory mediators needs further experimentation to clarify.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.02.129.

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