ORIGINAL

NO-1886, a lipoprotein lipase activator, attenuates contraction of rat intestinal ring preparations

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Abstract : Various intestinal symptoms or diseases are closely associated with intestinal motility, which may be altered by metabolic disturbances associated with diabetes and obesity. It is therefore important that drugs used in the treatment of metabolic disorders should not have any adverse effects on the intestine. In the present study, we examined whether [4-(4-bromo-2-cyano-phenylcarbamoyl)-benzyl]-phosphonic acid diethyl ester (NO-1886), a lipoprotein lipase activator with anti-diabetic and/or anti-obese activity, affects stimulant-induced intestinal contractility. Administration of NO-1886 to intestinal ring preparations of ileum, rectum and colon isolated from Wistar rats attenuated or relaxed contraction induced by a high K+ environment or acetylcholine (ACh). This effect of NO-1886 was dependent on extracellular Ca^{2+} and intracellular myosin light chain kinase activity. Our results also showed that ACh-induced colonic contraction was significantly higher in the obese Otsuka Long-Evans Tokushima Fatty (OLETF) than in the non-obese Long-Evans Tokushima Otsuka (LETO) rats. The hypercontractility observed in the colons of OLETF rats occurred concomitantly with an elevation in muscarinic M3 ACh receptor protein levels. Administration of NO-1886 attenuated the obesity-induced hypercontractility of the colonic rings of OLETF rats. Thus, intestinal contractile system would be a novel pharmacological target of the lipoprotein lipase activator NO-1886. J. Med. Invest. 55 : 61-70, February, 2008

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INTRODUCTION

Various intestinal symptoms or diseases such as diarrhea, constipation, abdominal pain and mixed irritable bowel syndrome are closely associated with several pathophysiological conditions including stress conditions and diabetes (1, 2). The psychological or neuronal disturbances associated with these diseases are considered to be involved in the etiology of these intestinal symptoms (3, 4). Similarly, metabolic disorders such as obesity have also been reported to be associated closely with these intestinal symptoms (5-7), with increased body mass index correlating with the incidence of diarrhea and abdominal pain in humans (6, 7). Although the precise mechanisms responsible for these intestinal symptoms remain unclear, it is possible abnormal contraction of intestinal smooth muscle cells may be involved in the development of the conditions (8-10). In this regard, Dai, *et al*. (8) reported that colon

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ring strips from rats subjected to cold restraint stress had a higher contractile response to acetylcholine (ACh) compared with non-stressed control animals. ACh receptor-mediated hypercontractility was also observed in intestinal strips of rats with type I diabetes (11). In addition, it has been suggested that the contractile activity of both small and large intestines may be disturbed in obese subjects (12). It is therefore important that drugs used in the treatment of metabolic disorders such as obesity or diabetes should not have any adverse effects on gastrointestinal symptoms. Despite this requirement there have been only a small number of research reports describing the effect of these drugs on gastrointestinal symptoms.

The chemical compound [4-(4-bromo-2-cyanophenylcarbamoyl)-benzyl]-phosphonic acid diethyl ester (NO-1886) is a lipoprotein lipase activator discovered by the Otsuka Pharmaceutical Factory, Inc. (13). Administration of NO-1886 was shown to increase plasma HDL-cholesterol levels and decrease triacylglycerol levels in both the plasma and liver of streptozotocin-induced diabetic rats fed a high fat diet (14). NO-1886 was also shown to suppress fat accumulation and improve insulin resistance in rats fed a high-fat diet (15) and miniature pigs fed a high-fat/high-sucrose-diet (16). While the molecular and intracellular details of the mechanisms responsible for these improvements remain unclear, NO-1886 represents a new candidate drug for the treatment of dyslipidemia or diabetic conditions associated with obesity.

Recently we demonstrated NO-1886 inhibited extracellular Ca^{2+}- and intracellular myosin light chain kinase (MLCK)-dependent contractions of rat aortic smooth muscle (17). This finding implies that NO-1886 may be useful for treating cardiovascular dysfunction associated with increased blood vessel contractility (17). As extracellular Ca^{2+} and MLCK also participate in controlling gastrointestinal smooth muscle contractility (18, 19), we hypothesize that NO-1886 may cause an attenuation of this contractile response. In this paper we show that NO-1886 has an inhibitory effect on stimulant-induced intestinal contractions.

MATERIALS AND METHODS

Agents

Agent NO-1886 was synthesized in the New Drug Research Laboratory of Otsuka Pharmaceutical Factory Inc., Tokushima, Japan. N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7), and (S)-(+)-2-Methyl-1-[(4-methyl-5-isoquinolinyl)sulfonyl]homopiperazine dihydrochloride (H-1152) were purchased from Calbiochem (San Diego, CA, USA). Staurosporine was purchased from Wako Pure Chemical Industries (Osaka, Japan) and 1-(5-Chloronaphthalene-1-sulfonyl)homopiperazine hydrochloride (ML-9) from Biomol International, L.P. (Exeter, UK). NO-1886, staurosporine and ML-9 were dissolved in dimethyl sulfoxide (DMSO), while W7 and H-1152 were dissolved in distilled water.

Animals

Male Wistar rats aged 6 weeks were purchased from Japan SLC, Shizuoka, Japan. Spontaneous type 2 diabetic OLETF (Otsuka Long-Evans Tokushima Fatty) rats and their counterpart control, the LETO (Long-Evans Tokushima Otsuka) rats, aged 4 weeks, were supplied by the Tokushima Research Institute, Otsuka Pharmaceutical, Tokushima, Japan. The rats were housed singly at a constant room temperature of 23 ± 1°C with a 12-hour light/dark cycle, and were fed a standard non-purified diet (Oriental Yeast, Tokyo, Japan) with food and water available ad libitum. Male Wistar rats aged between 7-9 weeks and OLETF/LETO rats aged 17 weeks were used for the experiments. All animal procedures were in accordance with the institutional guidelines for the care and use of laboratory animals of The University of Tokushima.

Metabolic disturbances in the OLETF rats were confirmed by measuring bodyweight, blood glucose levels during the fasting and fed states, blood lipids (triglyceride and total cholesterol), and by an oral glucose tolerance test (OGTT) (20). For the measurement of blood chemicals, the rats were anesthetized by an intraperitoneal injection of pentobarbital (50 mg/kg). Blood samples were collected from the jugular vein. The plasma concentrations of triglyceride and total cholesterol were measured using commercial kits according to the manufacturer's protocols (Wako, Osaka, Japan). The methods are based on the reaction cascade where a triglyceride or a cholesterol produces hydrogen peroxide that yields a blue colored complex upon oxidative condensation of 4-aminoantipyrine with 3,5-dimethoxy-N-ethyl-N-(2'-hydroxy-3'-sulfopropyl)-anilinenatrium in the presence of peroxidase. Mesenteric, epididymal, and retroperitoneal fat samples were collected, weighed and compared with sam-

ples obtained from non-diabetic LETO rats.

Preparation of intestinal rings and tension measurements

The rats were fasted for 24 hours and then anesthetized by an intraperitoneal injection of pentobarbital (50 mg/kg). The intestines were removed and dissected free of connective tissue and then cut into ring segments, 3- to 4-mm in length. Each ring was then placed in a 3 ml organ bath (Micro Easy Magnus, Kishimoto Medical ; Kyoto, Japan) and mounted on two stainless steel wires, one of which was fastened to the bath, while the other was connected to a force transducer that measured isometric tension. The bath was filled with Krebs-Ringer bicarbonate buffer (KRB) solution at 37 ˚C and bubbled with a mixture of 95% O_2-5% CO_2. The KRB contained (in mmol/l) 118 NaCl, 4.6 KCl, 2.5 $CaCl_2$, 24.8 $NaHCO_3$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, and 5.6 glucose. The rings were equilibrated for 60 min under a resting tension of 1.0 g, with the solution being changed at 30 min intervals. The intestinal rings were then contracted by a high K^+ concentration solution (50 mM KCl in bath solution). After recording the contractile force of each ring following stimulation with 50 mM KCl, the baths were washed out with KRB. Each ring was allowed to equilibrate at a resting tension of 1.0 g for 20 min. The contractile responses measured under the following test conditions were expressed relative to those measured in the 50 mM KCl-induced contraction (1^{st} KCl) (21).

Studies on the effect of NO-1886

Test experiments involving NO-1886 were conducted on intestinal rings in which contraction was induced by either KCl (0, 30, 50, or 80 mM) or ACh (100 μM). The concentrations of NO-1886 used were 1, 3 and 10 μM. NO-1886 was added to the incubation system either 20 min prior to or during the contraction induced by each constrictor. Vehicle (DMSO)-treated rings were used as controls. In some experiments, intestinal contraction was performed in the absence of extracellular Ca^{2+} using Ca^{2+}-free KRB containing 1 mM EGTA.

Inhibitor experiments

Several inhibitors were used to examine the role of intracellular signaling pathways in the action of NO-1886. The concentrations of inhibitors used in this study were as follows : staurosporine [protein kinase C (PKC) inhibitor ; 100 nM], H-1152 [Rho kinase (ROK) inhibitor ; 100 nM], W7 (calmodulin inhibitor ; 100 μM), and ML-9 [myosin light chain kinase (MLCK) inhibitor ; 30 μM]. The intestinal rings were pre-treated with each inhibitor for 20 min and then contraction was induced by KCl (50 mM) or ACh (100 μM). Each inhibitor was present in the incubation system during contraction.

Western blot analysis

The colon was isolated from OLETF or LETO rats (each n=4) and dissected free of connective tissue and then cut into ring segments, 3- to 4-mm in length. Tissue protein extracts were prepared by homogenizing tissues in a radio immune precipitation assay (RIPA) buffer containing 20 mM tris (hydroxymethyl) aminomethane-hydrogen chloride (Tris-HCl) (pH7.4), 150 mM NaCl, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1% octylphenolpoly (ethyleneglycolether) (Nonidet-P40), 2 mM ethylenediamine tetraacetic acid (EDTA), and 1 mM phenylmethyl sulfonyl fluoride (PMSF) for 30 min on ice. The tissues were then passed through a 21-gauge needle followed by centrifugation at 13,500 x g for 30 min at 4˚C. The supernatant was used as a protein sample of the tissue extracts. Protein concentration was measured by the bicinchoninic acid (BCA)™ protein assay kit (Pierce, Rockford, IL). This method is based on the reactions where the proteins reduce Cu^{+2} to the cuprous cation (Cu^{+1}) that reacts with BCA to form purple colored product.

A 50 μg aliquot of protein was denatured by boiling for 2 min in loading buffer [4% SDS, 250 mM Tris-HCl, pH 6.8, 1% β-mercaptoethanol, 1% bromophenol blue, and 20% glycerol]. The proteins were subjected to SDS-polyacrylamide gel electrophoresis and transferred onto Immobilon-P membranes (Millipore, Bedford, MA, USA). The membranes were blocked for 1 hour using 5% skim milk in Tris-buffered saline (TBS) containing 0.05% polysorbate 20 (Tween 20, which was added to the blocking buffer to prevent nonspecific binding). Then membranes were incubated with either an anti-myosin light chain (MLC) rabbit polyclonal antibody (1 : 1000, Cell Signaling Technology, Inc., MA, USA) or anti-muscarinic M3 ACh receptor (ACh-M3R) rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc, CA, USA). Visualization of the attached antibodies was carried out using goat anti-rabbit IgG horseradish peroxidase conjugate secondary antibody (1 : 2000, Biosource International, Camarillo, CA) using an ECL Plus detection kit (Amersham

Pharmacia Biotech, Aylesbury, UK).

Statistical analysis

Data are expressed as the mean ± SD. Data were analyzed by analysis of variance and Bonferroni multiple comparison tests. Student's t-test was used for paired data when appropriate. A P value < 0.05 was considered to be statistically significant.

RESULTS

NO-1886 attenuates KCl-induced contraction in rat intestines

The effect of NO-1886 on intestinal contraction induced by KCl was examined initially using rat ileum ring preparations. Administration of NO-1886 to ring preparations under basal conditions did not alter basal tone (data not shown). As shown in Fig. 1A, pretreatment with NO-1886 (10 μM) attenuated subsequent KCl (30-80 mM)-induced maximum contraction of rat ileum. As shown in Fig. 1B, administration of NO-1886 during KCl (50 mM)-induced contraction caused a dose-dependent relaxation in ileal tensions. The maximum relaxing effect for NO-1886 was observed at a concentration of 10 μM. The effect of NO-1886 at concentrations more than 10 μM was not determined as the drug did not dissolve completely in the incubation buffer at these high concentrations. The relaxing effects of NO-1886 on KCl (50 mM)-induced contraction were also observed for other intestinal preparations including colon and the rectum (Fig. 1C).

The relaxing effect of NO-1886 did not occur in the absence of extracellular Ca^{2+}

Our previous study demonstrated that NO-1886 inhibition of KCl-induced vasocontraction of rat aortic rings was mediated by an extracellular Ca^{2+}-dependent mechanism (17). To examine the involvement of extracellular Ca^{2+} in the relaxing effect of NO-1886 on intestinal contraction, ileal ring preparations were exposed for 20 min to Ca^{2+}-free KRB containing 1 mM EGTA. Removal of extracellular Ca^{2+} largely attenuated the subsequent KCl (50 mM)-induced contraction (% attenuation : 64.6 ± 15.2%). Under these conditions, NO-1886 did not cause relaxation (Fig. 2A). This finding indicated NO-1886-induced inhibition of ileal contraction was dependent on extracellular Ca^{2+}.

Effect of inhibitors of signaling molecules on NO-1886-induced ileal relaxation

A rise in intracellular calcium results in the formation of a Ca^{2+}/calmodulin complex, leading to subsequent activation of MLCK, MLC phosphorylation, and finally smooth muscle contraction (19, 22). Additional signaling pathways involving ROK and PKC increase myofilament force sensitivity to $[Ca^{2+}]i$, thereby maintaining vascular contraction (19, 22, 23). NO-1886-induced inhibition of vasocontraction of rat aortic rings has been shown to involve MLCK, but not PKC, calmodulin nor ROK (17). In the present study, the effects of inhibitors of these signaling molecules were examined in ileal ring preparations in the presence or absence of NO-1886. Pretreatment with either staurosporine (PKC inhibitor), H-1152 (ROK inhibitor), W7 (calmodulin inhibitor) or ML-9 (MLCK inhibitor) attenuated KCl-induced contractions [% inhibition ; staurosporine (39.6 ± 3.2%), H-1152 (32.2 ± 6.7%), W7 (36.5 ± 11.2%), and ML-9 (72.6 ± 3.2%)]. As shown in Fig. 2B, neither staurosporine, H-1152 nor W7 influenced the relaxing effect of NO-1886 during KCl-

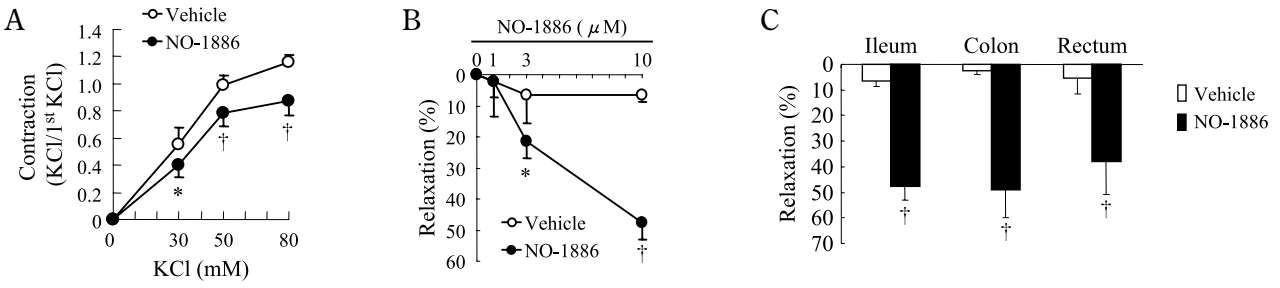

Fig. 1 NO-1886 attenuated KCl-induced contraction in Wistar rat intestines. (A) NO-1886 pretreatment (10 μM for 20 min) attenuated subsequent KCl-induced contraction in rat ileum ring preparations. Vehicle (DMSO) treatment was used as the control. (B) NO-1886 caused a dose-dependent relaxation in the ileal rings which had been contracted by KCl (50 mM). (C) NO-1886 (10 μM) induced the maximum relaxation response in the ileum, colon and rectum ring preparations. Each contractile value in (A) was standardized against the contraction level induced by 50 mM KCl in the absence of pretreatment (1st KCl). Data are expressed as means ± SD for 4-8 intestinal rings in each group. *P < 0.05 and †P < 0.01, compared with vehicle group.

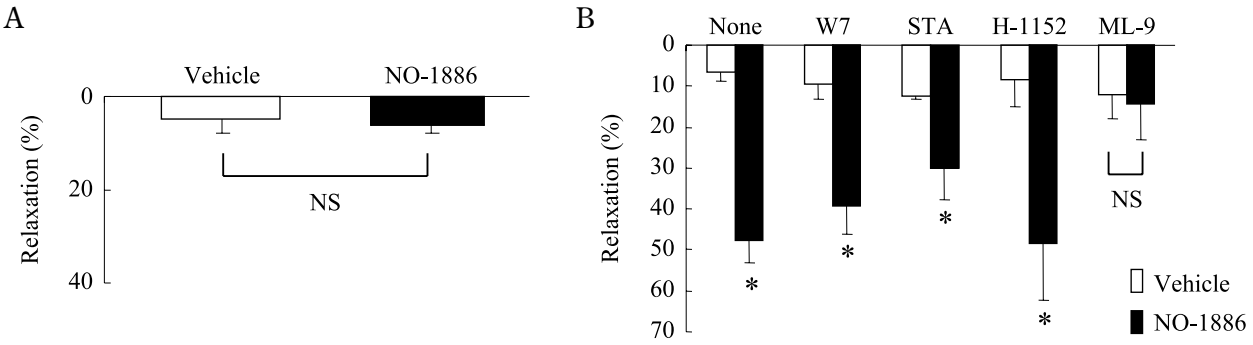

Fig. 2 (A)The effect of removing extracellular Ca^{2+} on NO-1886-induced relaxation in ileal ring preparations. (B) Effects of several inhibitors of signaling molecules on the relaxing effect of NO-1886. The inhibitors used were W 7 (100 μM), Staurosporine (STA, 100 nM), H-1152 (100 nM), and ML-9 (30 μM). Ileal ring preparations from Wistar rats were pre-incubated in Ca^{2+}-free KRB or with each inhibitor for 20 min. Each ring was then contracted by KCl (50 mM). After the contraction had reached a plateau level, NO-1886 (10 μM) was added to the incubation baths. Data are expressed as means ± SD for 4-7 intestinal rings in each group. *P < 0.01, compared with vehicle group.

induced contraction. In contrast, pre-treatment with ML-9, an MLCK inhibitor, completely blocked the relaxing effect of NO-1886 on KCl-induced ileal contraction (Fig. 2B).

NO-1886 attenuates ACh-induced intestinal contraction via an MLCK-dependent pathway

ACh is one of the most important neurotransmitters in the intestine, where ACh released from the enteric nervous system mediates gastrointestinal tract contractions (24). ACh binds to its receptor, (i.e. the muscarinic ACh receptors including ACh-M3R) (25), and increases intracellular Ca^{2+} levels and binding to calmodulin, which in turn induces smooth muscle contraction via activation of MLCK (19, 22). Fig. 3 summarizes the effect of NO-1886 on ACh (100 μM)-induced contraction in colon and rectum ring preparations. In our preliminary experiments, ACh (100 μM) did not induce contraction in the ileal ring preparations (data not shown). As ACh-stimulated rings were unable to sustain their plateau contractile level (data not shown), the maximum contractile levels were used in the data analyses. As shown in Fig. 3, pretreatment with NO-1886 significantly attenuated ACh-stimulated contraction in both colon and rectum preparations. Pretreatment with W7 and ML-9 alone also inhibited the development of subsequent ACh-induced contraction (open bars in Fig. 3). NO-1886 showed an inhibitory effect on ACh-stimulated contraction even in the presence of W7, although this effect disappeared when the rings were pre-incubated with ML-9 (Fig. 3). These data suggest that NO-1886 attenuated ACh-induced intestinal contraction via the MLCK-dependent pathway.

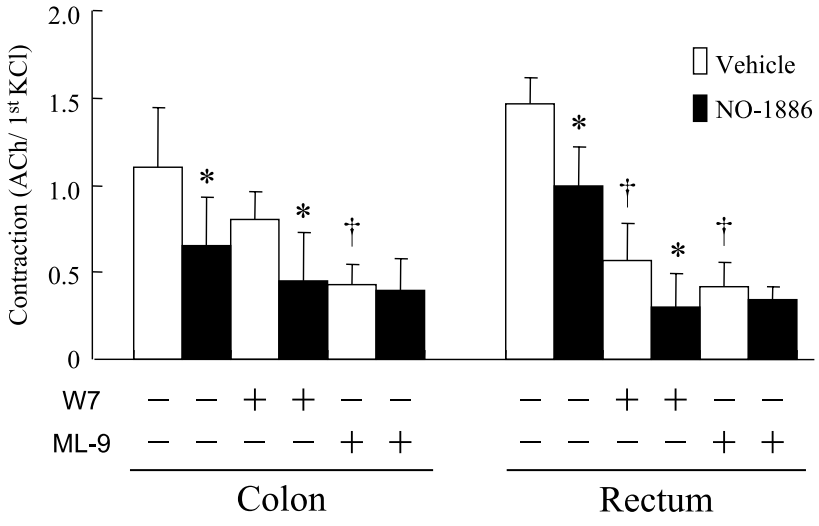

Fig. 3 The effect of NO-1886 on ACh-induced contraction in rat colon and rectum. The colonic and rectal ring preparations from Wistar rats were pre-incubated with NO-1886 (10 μM), W7 (100 μM) and/or ML-9 (30 μM) for 20 min prior to the ACh (100 μM) -induced contraction. Vehicle treatment was used as controls. ACh-induced maximum contraction was recorded for each ring preparation. Each contractile value was standardized against the contraction level induced by 50 mM KCl in the absence of pretreatment (1st KCl). Data are expressed as means ± SD for 4-11 intestinal rings in each group. *P < 0.01, compared with each vehicle group. †P < 0.01, compared with vehicle group without inhibitor treatment.

OLETF, a rat model of obesity associated with glucose intolerance

To examine whether obesity or diabetes affects intestinal contraction, spontaneous obese, type-2 diabetic OLETF rats (26, 27) were used in the subsequent experiments. The clinical and biochemical characteristics of the OLETF and LETO control rats are summarized in Table 1. OLETF rats at 17 weeks of age had higher body weight, visceral fat mass and plasma triglyceride concentration compared to LETO control rats. Blood glucose levels in the fed condition were also higher in the OLETF rats than in the LETO rats. However, fasting levels of blood glucose and plasma total cholesterol were not significantly different from those in LETO rats. Oral glucose tolerance tests revealed that the blood glucose levels of OLETF rats were increased markedly after the oral glucose load (2 g/kg), indicating that this model animal decreased glucose disposal from the circulation (glucose intolerance). The glucose intolerance state observed in our OLETF rats would be attributed to the insulin resistance of the tissues as had been demonstrated by a hyperinsulinemic glucose clamp study (26). The OLETF rats were therefore used in this study as a model for assessing the effect of obesity-related metabolic disorders on intestinal contraction.

Table 1. Physical and biochemical characteristics of the LETO and OLETF rats

	LETO	OLETF
Body weight (g)	402.7±18.8	508.3±37.6*
Fat mass (g)		
Epididymal	5.5±0.9	12.6±0.4†
Retroperitoneal	5.7±0.7	17.9±3.1†
Mesenteric	3.4±0.4	8.0±1.6†
Blood glucose (mg/dl)		
Fed	101±3.6	134.5±23.2*
Fasting	77.7±8.6	80.3±6.0
[After glucose load (2g/kg)]		
30 min	137.7±14.6	279.7±39.1†
60 min	131.3±7.5	297.3±9.5†
120 min	102.3±16.8	138±46.8
Plasma triglyceride (mg/dl)	47.7±21.6	133.2±21.9†
Plasma Total cholesterol (mg/dl)	60.8±7.6	72.2±7.0

Data are means ± SD. *$p < 0.05$, †$p < 0.01$ vs. LETO rats.

OLETF rats showed increased ACh receptor expression and ACh-induced contraction in the intestine

ACh-induced cholinergic signaling is mediated by muscarinic ACh receptors, which regulate intestinal tone under physiological conditions (28). As shown in Fig. 4A, the colon ring preparations of the OLETF rats had increased levels of muscarinic M3 ACh receptor (ACh-M3R) protein, one of the major ACh receptors expressed in the intestine. The expression levels for myosin light chain (MLC) protein in the colon were, however, not significantly different between the OLETF and LETO rats (Fig. 4B).

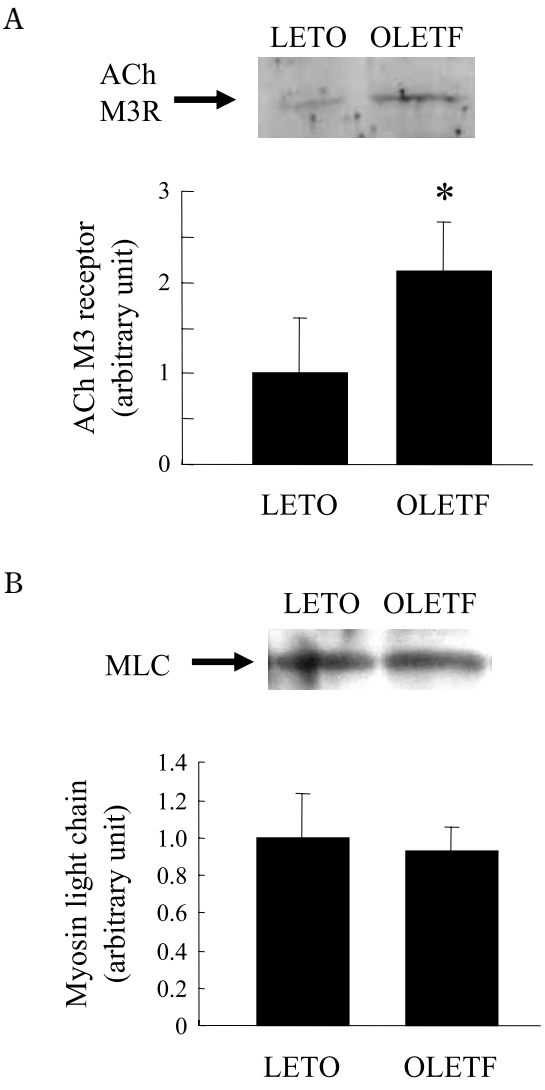

Fig. 4 The protein expression levels of muscarinic M3 ACh receptor (ACh-M3R; A) and myosin light chain (MLC; B) in the colon of OLETF and LETO rats. The intensities of the immunoreactive bands were quantified by scanning densitometry and expressed as arbitrary units (the ordinate axis). The mean values in the LETO group were designated as 1.0. Data are expressed as means ± SD for 4 rats in each group. *$P < 0.01$, compared with LETO group.

We next examined whether elevated ACh-M3R protein expression affected ACh-induced contraction in colon ring preparations of OLETF rats. It should be noted that the ACh-induced contraction of the intestinal rings isolated from normal LETO rats (Fig. 5) were lower than those isolated from the Wistar rats (Fig. 3). Since LETO rats at 17 weeks of age were used in the experiments for Fig. 5 (Wistar rats aging 7-9 weeks were used in the experiments for Fig. 3), observed low contraction in the LETO ring preparations might involve the effect of the aging, or of the difference in rat strain. However, we could not clearly show the relationships between these factors and intestinal contraction in the present study. In our experiments with the Long-Evans rat strain, ACh (100 μM) -induced colonic contraction was significantly higher in the OLETF rats than in the LETO rats (open bar) (Fig. 5).

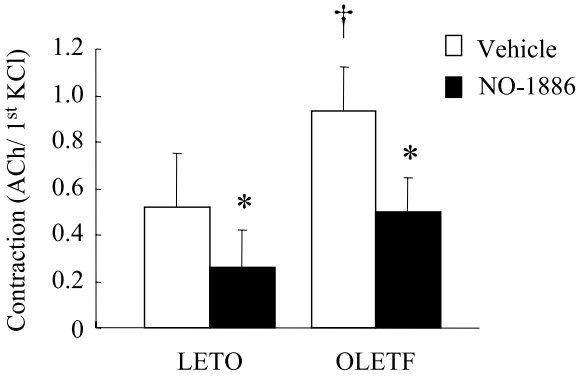

Fig. 5 ACh-induced contraction of colonic ring preparations of the OLETF and the LETO rats. The colon ring preparations from both rat groups were treated with or without NO-1886 (10 μM) for 20 min prior to the ACh (100 μM)-induced contraction. Each contractile value was standardized against the contraction level induced by 50 mM KCl in the absence of pretreatment (1st KCl). Data are expressed as means ± SD for 4 rats in each group. * $P<0.01$, compared with vehicle group. † $P<0.01$, compared with LETO (vehicle) group.

NO-1886 attenuated ACh-induced excessive contraction of the colon of OLETF rats

To examine the effects of NO-1886 on colonic contraction in OLETF and LETO rats, colon ring preparations from both rat groups were treated with or without NO-1886 for 20 min prior to ACh (100 μM) -induced contractions. ACh-induced contraction was attenuated significantly by pretreatment with NO-1886 in both rat groups (closed bars in Fig. 5). It should be noted that ACh-induced contractile levels of OLETF colons were decreased by NO-1886 pretreatment to normal levels seen in the LETO colons without NO-1886 treatment (compare closed bar of OLETF rat group with open bar of LETO rat group in Fig. 5).

DISCUSSION

NO-1886 belongs to the class of lipoprotein lipase activators that have beneficial effects on abnormal lipid metabolism observed in diabetic or obese conditions (14, 15). Many studies on the use of NO-1886 have therefore suggested that this drug may become an effective agent for treating hyperlipidemia, diabetes and obesity (13-15). However, the cellular and molecular mechanisms of these improvements remain to be clarified. Furthermore, only a few reports have demonstrated the direct effects of NO-1886 on the intracellular signaling pathways (17). Therefore, investigating the effect of NO-1886 on cellular functions responsible for the regulation of nutrient metabolism as well as other pathophysiological conditions has the potential to reveal more details of the pharmacological properties of this drug.

In this paper we show that NO-1886 attenuated KCl- and/or ACh-induced contraction in rat intestines. The inhibitory effect of NO-1886 on excess contraction of smooth muscle cells was first demonstrated in vascular smooth muscle cells of rats (17). In the vasculature, NO-1886 inhibited KCl-induced or α1-adrenergic receptor-mediated contractions (17). Although the precise mechanisms underlying NO-1886-induced inhibition of smooth muscle contractions remain unclear, it is possible the mechanism may involve Ca^{2+} influx-dependent contractile components in the vascular smooth muscle cells (17). Inhibition of MLCK, an enzyme involved in the final step of the contractile mechanisms of smooth muscle cells (29), abolished NO-1886-induced vasorelaxation (17), indicating that NO-1886 attenuates smooth muscle contraction via an extracellular Ca^{2+}, and an MLCK-dependent pathway. As extracellular Ca^{2+} and MLCK also participate in gastrointestinal smooth muscle contractile mechanisms (18), we examined the effect of a Ca^{2+}-free environment or inhibitors of signaling molecules including MLCK, on the action of NO-1886. The lack of extracellular Ca^{2+} or inhibition of MLCK by ML-9 blocked the relaxing effect of NO-1886 in the intestines similar to that observed in vascular smooth

muscle cells. We therefore concluded that NO-1886 had the ability to attenuate contractions in both intestinal and aortic smooth muscle cells.

Gastrointestinal motility, including contraction of intestinal smooth muscle cells, regulates intestinal transit of nutrients, thereby affecting their digestion and absorption (12). In this regard, it has been shown that rapid intestinal transit may result in reduced rates of absorption, whereas delayed transit accelerates the rate, leading to accumulation of excess nutrients in the body (12). However, while increased intestinal absorption of nutrients has been suggested to occur in obesity (30), intestinal transit rate itself, may not be altered in this condition (30, 31). Previous *in vivo* studies have demonstrated that NO-1886 decreased accumulation of excess nutrients including blood glucose, lipids and also reduced body fat mass (15, 16). These facts suggest that NO-1886 would not have adverse effects on nutrient metabolism even if it had an inhibitory effect on intestinal contraction.

It is well established that abnormal intestinal motility is associated with various digestive diseases such as irritable bowel syndrome and gastrointestinal symptoms including constipation, diarrhea and bloating (8-10). Some reports have described these alterations in intestinal motility being induced by stressed conditions or by metabolic syndromes including diabetes and obesity (1, 2, 12). Shinbori, *et al.* (11) demonstrated that ileal ring preparations from STZ-induced diabetic rats had hyper-contractile ability, while Xing and Chen speculated that colonic motility may be altered in obese patients (12). The concept that NO-1886 may be applicable for treatment of metabolic disturbances including obesity and diabetes prompted us to examine, 1) whether obesity-based metabolic complications actually alter intestinal contractility, and 2) whether NO-1886 affected intestinal contractility under such conditions.

In this study we used OLETF rats as a model of metabolic disturbances associated with severe obesity (26, 27). The OLETF rats at 17 weeks of age used in the study had visceral fat obesity and higher plasma triacylglycerol concentrations compared with normal control (LETO) rats. Blood glucose levels in the fed condition were also higher in the OLETF rats than in the LETO rats, although these levels were still within the normal range (Table 1). Blood glucose levels did not increase significantly in the OLETF rats until after oral loading of a large amount of glucose, suggesting that this rat model rat at 17 weeks of age had visceral obesity and glucose intolerance (or insulin resistance), but did not yet show hyperglycemia under sedentary conditions. To examine the effect of obesity on intestinal contractility, ACh-induced contractile force was measured using colonic ring preparations isolated from OLETF and LETO rats. ACh receptor-mediated hypercontractility has been reported in intestinal strips from type I diabetic rats (11), a finding that implies a relationship between blood glucose levels and intestinal motility. Although the OLETF rats used in our study did not have excessive hyperglycemia, as seen in type I diabetic rats (11), ACh-induced contraction of the colon was actually increased compared with control LETO rats. In addition, we showed the protein levels of ACh-M3R were elevated in the colon of the OLETF rats.

Two ACh receptor subtypes, namely ACh-M2 receptor (ACh-M2R) and ACh-M3R are expressed in intestinal smooth muscles (25). M3-receptors stimulate intestinal smooth muscle contraction by elevating intracellular Ca^{2+}, while M2 receptors induce contraction indirectly by inhibiting the production of cyclic AMP (25). Despite the predominance of M2-receptors, direct contraction of intestinal and bladder smooth muscle is mediated by the M3-receptor subtype, and only this subtype is involved in contraction *in vitro* (25). Shinbori, *et al.* (11) reported that mRNA levels of ACh-M3R, but not ACh-M2R, were increased in the ileum of type I diabetic rats. On the other hand, Carrier and Aronstam (32) demonstrated that muscarinic responsiveness, but not muscarinic receptor content, was increased in ileal smooth muscle in the same strain of diabetic rats. Only a few reports had described intestinal expression levels of each ACh receptor in the obese condition. In our study we found that colonic contractions were increased in the obese condition with insulin resistance and that this impairment was possibly due to an elevation of ACh-M3R in smooth muscle cells. Further studies are needed to clarify the mechanisms underlying obesity-induced alterations in intestinal ACh receptor expression.

Our results showed that pretreatment with NO-1886 attenuated ACh-induced colonic hypercontractility in OLETF rats to normal levels seen in the LETO rat colon preparations. These results indicate that NO-1886 improves obesity-induced colonic hypercontractility, in addition to its beneficial effects on metabolic abnormalities (14-16). However, in the present study this inhibitory effect of NO-1886 on excessive contraction was assessed only *in vitro* us-

ing intestinal ring preparations. Therefore, whether or not NO-1886 has similar effects *in vivo* was not determined in this study.

In summary, we demonstrated that NO-1886 attenuated KCl- and ACh-induced contraction in rat intestines. This effect of NO-1886 was dose-dependent and required extracellular Ca^{2+} and intracellular MLCK. NO-1886 also attenuated excess intestinal contraction caused by obesity in combination with insulin resistance. Future work involving oral administration of NO-1886 to animals would reveal the more direct effects of this drug on altering intestinal motility associated with metabolic abnormalities.

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