

## N-hexacosanol prevents diabetes-induced rat ileal dysfunction without qualitative alteration of the muscarinic receptor system

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### ABSTRACT

We evaluated the effects of N-hexacosanol, a cyclohexenonic long-chain fatty alcohol, on muscarinic receptors in diabetic rat ileal dysfunction. Eight-week-old male SD rats were divided into four groups. After induction of diabetes (streptozotocin 50 mg/kg, i.p.), three groups were maintained for eight weeks with treatment by N-hexacosanol (0, 2 or 8 mg/kg, s.c. every day). Ileum function was investigated by organ bath studies using carbachol and KCl, and the expression levels of muscarinic M<sub>2</sub> and M<sub>3</sub> receptors were investigated by real-time polymerase chain reaction. Various concentrations of subtype-selective muscarinic antagonists, *i.e.*, atropine (non-selective), pirenzepine (M<sub>1</sub> selective), methoctramine (M<sub>2</sub> selective), and 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP, M<sub>1</sub>/M<sub>3</sub> selective), were used in this study. In the presence and absence of these antagonists, contractile response curves to increasing concentrations of carbachol were investigated. Treatment with N-hexacosanol did not alter the diabetic status of the rats, but did significantly prevent the carbachol-induced hypercontractility in diabetic rat ileum. Estimation of the pA<sub>2</sub> values for atropine, pirenzepine, methoctramine, and 4-DAMP indicated that the carbachol-induced contractile response in the ileum is mainly mediated through the muscarinic M<sub>3</sub> receptor subtype in all groups. Furthermore, N-hexacosanol significantly prevented the diabetes-induced up-regulation of intestinal muscarinic M<sub>2</sub> and M<sub>3</sub> receptor mRNAs in streptozotocin-diabetic rats. Our data indicated that N-hexacosanol exerts preventive effects with respect to carbachol-induced hypercontractility in the diabetic rat ileum without qualitative alteration of the muscarinic receptor system.

Gastrointestinal smooth muscles receive a variety of excitatory and inhibitory inputs from the enteric nervous system. As cholinergic nerves are activated, the neurotransmitter acetylcholine (ACh) is released from their terminals. Subsequently, ACh acts on

smooth muscle cells to activate cell-surface muscarinic receptors, thus activating various intracellular signaling pathways, and in turn inducing smooth muscle contraction (6, 7). To date, five muscarinic receptor subtypes (M<sub>1</sub>–M<sub>5</sub>) have been identified (5, 20). A recent reverse transcriptase-polymerase chain reaction (RT-PCR) study reported the possible expression of all five subtypes in gastrointestinal smooth muscle (5). The muscarinic M<sub>2</sub> and M<sub>3</sub> receptor subtypes are preferentially expressed, with a preponderance of the former subtype. In general, the muscarinic M<sub>3</sub> receptor subtype in smooth muscle is coupled to the G protein Gq/G11 and mediates the

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contraction of smooth muscle via the activation of the phospholipase C pathway, while the muscarinic  $M_2$  receptor subtype is coupled to the G protein  $G_i/G_o$  and inhibits adenylylase, which leads to relaxation of the smooth muscle (6, 7). Nonetheless, the recent use of mutant mice lacking certain muscarinic receptor subtypes has suggested that not only muscarinic  $M_3$  but also  $M_2$  receptor subtypes may play a direct role in inducing contraction in gastric and ileal smooth muscle (11, 19).

Diabetic neuropathy is a major complication of diabetes mellitus that affects various sites of the entire body, including the gastrointestinal tract. Gastrointestinal motility disorders such as vomiting, nausea, abdominal distension, and diarrhea are frequently reported in patients with diabetes (8, 18). These dysfunctions are primarily induced by diabetes-associated neuropathy (14).

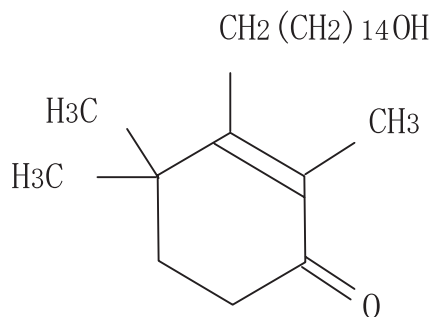
The tropical plant *Hygrophilia erecta* Hochr. has been shown to contain some cyclohexenonic long-chain fatty alcohols that exert neurotrophic effects on neurons cultured from the cerebral cortex (2, 3). The C26-alcohol N-hexacosanol has been found to directly increase neurite extension as well as the biochemical differentiation of these neurons. Our previous reports indicated that N-hexacosanol has a beneficial effect not only on disorders of the central nervous system, but also on peripheral neuropathy (10, 13, 15, 16). For example, we described the therapeutic effects of N-hexacosanol on streptozotocin induced-diabetic neuropathy in the trachea, urinary bladder, and ileum of the rat (13, 15, 16). Recently, we reported that N-hexacosanol ameliorates diabetes-induced hypercontractility of the rat ileum induced by carbachol, and we found that treatment with N-hexacosanol partially reverses the overexpression of muscarinic  $M_2$  and  $M_3$  receptor mRNAs (16). However, it remains important to investigate possible pathological changes in the pharmacological profiles of these muscarinic receptors and their mRNAs induced by diabetes in ileal smooth muscle tissue.

In order to gain a better understanding of the detailed mechanisms of action of N-hexacosanol on peripheral neurons and ileal smooth muscle, we investigated ileal contractions using KCl and carbachol with or without subtype-selective muscarinic antagonists, *i.e.*, atropine (ATR, non-selective), pirenzepine (PRZ,  $M_1$  selective), methoctramine (MTR,  $M_2$  selective), and 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP;  $M_1/M_3$  selective). Furthermore, we utilized a quantitative PCR-based method to identify possible diabetes-induced altera-

tions in the levels of expression of muscarinic  $M_2$  and  $M_3$  receptor mRNAs.

## MATERIALS AND METHODS

**Animal model.** All animal experiments were performed in accordance with the guidelines established by the Tottori University Committee for Animal Experimentation. Diabetes was induced in eight-week-old male Sprague–Dawley rats (SLC, Shizuoka, Japan) by an intraperitoneal injection of streptozotocin (STZ) (50 mg/kg) dissolved in 0.1 M citrate-phosphate buffer (pH 4.2), according to a method described in our previous reports (10, 13, 15, 16). Rats were divided randomly into four age-matched groups as follows: non-diabetic control rats (group A), untreated diabetic rats (group B), and diabetic rats treated with N-hexacosanol at a daily dose of 2 or 8 mg/kg (groups C and D, respectively) ( $n = 6-8$ ). The chemical structure of N-hexacosanol used in this study is shown in Fig. 1. N-hexacosanol was dissolved in ethanol, and then a mixture of physiological saline/Tween 80 was added to obtain an ethanol : saline : Tween 80 volume ratio of 5 : 92.15 : 2.85 (total volume: approximately 0.3 mL). Two days after the injection of STZ or vehicle, the induction of diabetes was confirmed by measuring urinary glucose with Pretest 3all (Wako Pure Chemical, Osaka, Japan), and the diabetic rats were defined as those with a urinary glucose result in excess of the baseline value (+++). N-hexacosanol (groups C and D) or vehicle (groups A and B) was injected subcutaneously into each rat daily, starting on the day when diabetes had been confirmed, according to a method used in previous reports (9). All groups were otherwise kept under identical conditions, and had access to food and drinking water *ad libitum*. Eight weeks after the induction of diabetes, the rats were sacrificed with an overdose of pentobarbital (60 mg, *i.p.*). Blood sam-



**Fig. 1** The chemical structure of N-hexacosanol.

ples were collected from the vena cava, and the ileum was removed from each animal and was placed in Krebs–Henseleit solution with the following composition (mM): NaCl 118.0, KCl 4.7, MgSO<sub>4</sub> 1.2, CaCl<sub>2</sub> 2.5, KH<sub>2</sub>PO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 24.9, glucose 5.6 and sodium pyruvate 2.0; these samples of blood and ileum were used for functional, biochemical, and molecular biological studies.

*Serum glucose and insulin measurement.* Serum glucose concentrations in the experimental rats were measured by the hexokinase method (Glucose C II; Wako Pure Chemical), which was carried out according to the kit manufacturer's instructions. Insulin concentrations were also measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Rat Insulin ELISA, Mercodia AB, Uppsala, Sweden).

*Measurement of contractile force in isolated ileum.* Functional studies were performed according to a method described in our previous report (16). The rat ileum was removed in order to obtain the longitudinal muscles. The ileum was rinsed with Krebs–Henseleit solution, and the most distal 10 cm were discarded; the remaining ileum was cut into about 1 cm segments. Ileal segments were mounted longitudinally between a hook in the bottom of the muscle chamber and a force displacement transducer in an organ bath (30 mL) containing Krebs–Henseleit solution, and the segments treated in this manner were bubbled with 5% CO<sub>2</sub> and 95% O<sub>2</sub> (37°C). One hook was suspended from a transducer (type 45196A; San-ei Instruments, Tokyo, Japan), and the lower hook was fixed to a plastic support leg attached to a micrometer (Mitutoyo, Tokyo, Japan). The unstretched ileal preparations were equilibrated for 30 min. A load of 1.0 g was applied to each strip by micrometer adjustment, and the load was readjusted to this level 30 min later. Changes in the tone of the strips were measured with a force transducer, and recorded on a personal computer (Macintosh G3; Apple Computer, Cupertino, CA) by use of Chart v 3.6.9 software and a PowerLab/16sp data acquisition system (AD Instruments, Castle Hill, Australia). The contractile responses to 100 mM KCl and cumulative concentration-response curves to carbachol ( $10^{-8}$ – $3 \times 10^{-5}$  M) were recorded.

Carbachol-induced contractile responses were measured in the presence or absence of various concentrations of muscarinic antagonists: PRZ (M<sub>1</sub> selective,  $10^{-7}$ ,  $10^{-6}$ ,  $10^{-5}$  M), MTR (M<sub>2</sub> selective,  $10^{-8}$ ,  $10^{-7}$ ,  $10^{-6}$  M), 4-DAMP (M<sub>1</sub>/M<sub>3</sub> selective,  $10^{-9}$ ,  $10^{-8}$ ,

$10^{-7}$  M) and ATR (nonselective,  $10^{-10}$ ,  $10^{-9}$ ,  $10^{-8}$  M). Antagonists were added 30 min prior to the administration of carbachol. After completion of concentration-response curve, the tissue was washed until base-line force returned to the resting level; the samples were then equilibrated for 30 min, and the next contractile concentration-response curve was constructed.

*Real-time polymerase chain reaction (PCR) (quantification of muscarinic M<sub>2</sub> and M<sub>3</sub> receptor mRNAs).* Measurement of muscarinic receptor mRNAs was performed according to a method outlined in our previous reports (9, 13, 16). Muscarinic M<sub>2</sub> and M<sub>3</sub> receptor mRNAs in the experimental ileum were measured by real-time PCR. The mRNAs were purified using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The reverse transcriptase (RT) mixture (28 µL) containing 2 µg of total RNA was prepared and incubated at 37°C for 60 min according to a previously reported method (9, 13, 16). Fifteen microliters of the mixture were used for real-time PCR, which was carried out using a LightCycler thermal cycler system with a LightCycler-FastStart Hybridization Probe kit according to the manufacturer's instructions (Roche Diagnostics, Tokyo, Japan). The primers and probes were synthesized commercially by Nihon Gene Research Laboratories, Inc. (Sendai, Japan), and these sequences are shown in Table 1. The predicted product sizes of muscarinic M<sub>2</sub> and M<sub>3</sub> receptors were 148 bp and 149 bp, respectively. The primer and probe of the β-actin used were from the LightCycler-Primer/Probe Set (rat). β-actin was used as the internal standard. A total of 5 µL of solution was used for the sample. The specificity of the reaction was confirmed by 2% agarose gel electrophoresis.

*Data analysis.* The data for the contractions induced by carbachol were normalized by those obtained by induction with 100 mM KCl. The EC<sub>50</sub> and E<sub>max</sub> values were obtained using a Macintosh computer (G3) loaded with Chart v3.6.9 software and a PowerLab/16sp data acquisition system. The dose ratio was obtained from the ratio of EC<sub>50</sub> values for carbachol in the presence or absence of an antagonist. Antagonist dissociation constants (K<sub>B</sub>) were determined from the following equation:

$$K_B = \text{antagonist (M)} / (\text{dose ratio} - 1)$$

pA<sub>2</sub> values were obtained from the intercept on the

x axis of the Schild plots (1). Schild plots were constructed by plotting the log of (dose ratio - 1) against the log of the molar concentration of antagonist. The  $EC_{50}$  values were calculated as geometric means, whereas the  $E_{max}$  values were calculated as arithmetic means. A statistical comparison of differences between groups was performed using analysis of variance and Fisher's multiple comparison tests.  $P < 0.05$  was regarded as the level of significance.

**Drugs and chemicals.** N-hexacosanol was obtained from Meiji Milk Products Co., Ltd., Tokyo, Japan. Carbachol, 4-DAMP, PRZ, and MTR were purchased from Sigma (St. Louis, Japan). STZ and ATR were purchased from Wako Pure Chemical Co. All other chemicals were available commercially and of reagent grade.

## RESULTS

### *General features of the experimental animals*

The general features of the experimental animals are shown in Table 2. The untreated diabetic rats showed no weight gain, significantly higher serum glucose, and lower insulin levels. Treatment with N-hexacosanol (2 mg or 8 mg/kg) did not change the body weight, serum glucose and insulin levels compared to those of untreated diabetic rats. These data are consistent with the findings in our previous

reports (9, 10, 13, 15, 16).

### *Measurement of contractile response*

The  $E_{max}$  and  $EC_{50}$  values for the contractile responses of the longitudinal ileal muscles to carbachol, and the contractile responses of the longitudinal ileal muscles to KCl (100 mM) were determined in the absence of muscarinic antagonists (Fig. 2). Diabetes-induced ileal hyperreactivity to carbachol was prevented by treatment with both low and high doses of N-hexacosanol. Both doses of N-hexacosanol treatment significantly inhibited ileal hyperreactivity. The  $EC_{50}$  values obtained in response to carbachol indicated no significant difference between any of the four groups. The  $EC_{50}$  values in groups A, B, C, and D were  $0.83 \pm 0.16$ ,  $0.82 \pm 0.19$ ,  $0.46 \pm 0.09$ , and  $0.51 \pm 0.06 \mu\text{M}$ , respectively. The  $pA_2$  values and slopes for four subtype-selective and non-selective muscarinic antagonists were calculated from the Schild plots (Table 3). For all four antagonists, there was no significant difference in the  $pA_2$  values between groups. Whether diabetic rats were treated with or without N-hexacosanol (Groups B–D), the  $pA_2$  values for each antagonist were similar to those of the control group (Group A). The rank order of  $pA_2$  values for these muscarinic antagonists in the rat ileal smooth muscle was  $\text{ATR} \geq 4\text{-DAMP} > \text{MTR} > \text{PRZ}$  in all groups.

**Table 1** Oligonucleotide primers and probes used for amplification of  $M_2$  and  $M_3$  muscarinic receptors

Gene	Position	Oligonucleotide sequence 5'-3'
$M_2$	(primer) forward (1390–1409)	5'-CCACTCCAGAGATGACAAC-3'
$M_2$	(primer) reverse (1519–1537)	5'-GGCTACAACGTTCTGCTT-3'
$M_3$	(primer) forward (1227–1245)	5'-GGACTGTGGATGTGGAGAG-3'
$M_3$	(primer) reverse (1358–1375)	5'-CGAGGAGTTGGTGTCAGA
$M_2$	(probe) (1433–1460)-Flu	5'-CCAACTAGTTCTACAGTGGTACTCGTTGGGGTGT-3'
$M_2$	(probe) LC-(1462–1494)	5'-XACACATCACCTTTTTGGGCCTTGGTGACT-3'
$M_3$	(probe) (1267–1284)-Flu	5'-CCAGAAGAGCATGGGTGATGGTGACAAC-3'
$M_3$	(probe) LC-(1286–1325)	5'-XGTCAGAAGGATTTCCACCAAGCTTCCCATCCT-3'

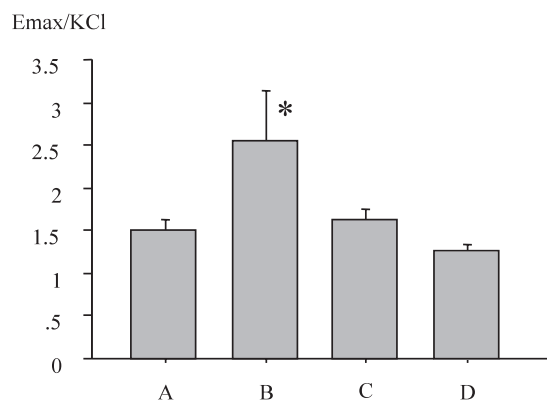
**Table 2** General features of the experimental rats

	Body Weight (g)			Serum glucose (mg/dL)	Serum insulin ( $\mu\text{g/L}$ )
	8 weeks old	12 weeks old	16 weeks old		
A	$305.6 \pm 16.0$	$418.8 \pm 10.9$	$464.3 \pm 18.1$	$148.5 \pm 9.8$	$2.92 \pm 0.80$
B	$269.0 \pm 7.7$	$289.7 \pm 15.6^*$	$246.7 \pm 18.0^*$	$460.9 \pm 60.0^*$	$0.19 \pm 0.05^*$
C	$277.1 \pm 6.3$	$290.9 \pm 11.8^*$	$265.0 \pm 9.9^*$	$454.1 \pm 36.3^*$	$0.12 \pm 0.01^*$
D	$288.8 \pm 8.3$	$291.3 \pm 9.9^*$	$241.4 \pm 16.0^*$	$481.9 \pm 22.8^*$	$0.14 \pm 0.02^*$

A: control rats, B: diabetic rats treated with vehicle, C: diabetic rats treated with N-hexacosanol (2 mg/kg), and D: diabetic rats treated with N-hexacosanol (8 mg/kg). Data are shown as mean  $\pm$  SEM of six to eight separated determinations in each group. \*significantly different from A group.

### Measurement of muscarinic $M_2$ and $M_3$ receptor mRNAs in the ileum

We also investigated the expression of muscarinic  $M_2$  and  $M_3$  receptor mRNAs using real-time PCR (Fig. 3). As regards the muscarinic  $M_3$  receptor



**Fig. 2** Contractile responses in experimental rat ileum.  $E_{max}$  values induced by carbachol were normalized by contractions induced by 100 mM KCl in the rat ileum smooth muscle. A: control rats, B: diabetic rats treated with vehicle, C: diabetic rats treated with N-hexacosanol (2 mg/kg), and D: diabetic rats treated with N-hexacosanol (8 mg/kg). Data are shown as mean  $\pm$  S.E.M. of 4–6 separate determinations in each group. \*significantly different from A and D groups.

**Table 3**  $pA_2$  values and slopes of Schild plots for muscarinic antagonists in rat ileum

Antagonist	Group	$pA_2$	slope
Pirenzepine	A	6.84 ( 6.64 ~ 7.21)	1.32 $\pm$ 0.28
	B	7.32 ( 7.31 ~ 7.33)	0.85 $\pm$ 0.04
	C	7.44 ( 7.38 ~ 7.52)	0.89 $\pm$ 0.06
	D	7.25 ( 7.16 ~ 7.36)	1.02 $\pm$ 0.04
Methoctramine	A	8.38 ( 8.22 ~ 8.65)	0.86 $\pm$ 0.05
	B	7.94 ( 7.73 ~ 8.34)	1.05 $\pm$ 0.34
	C	7.45 ( 7.18 ~ 8.27)	1.26 $\pm$ 0.09
	D	8.59 ( 8.52 ~ 8.69)	0.80 $\pm$ 0.04
4-DAMP	A	9.41 ( 9.25 ~ 9.67)	0.91 $\pm$ 0.06
	B	9.41 ( 9.28 ~ 9.60)	1.31 $\pm$ 0.16
	C	9.35 ( 9.15 ~ 9.72)	1.42 $\pm$ 0.71
	D	9.56 ( 9.41 ~ 9.78)	1.10 $\pm$ 0.03
Atropine	A	10.33 (10.22 ~ 10.48)	0.77 $\pm$ 0.09
	B	9.46 ( 9.44 ~ 9.48)	1.09 $\pm$ 0.01
	C	10.59 (10.52 ~ 10.68)	0.83 $\pm$ 0.05
	D	9.98 ( 9.89 ~ 10.09)	1.23 $\pm$ 0.30

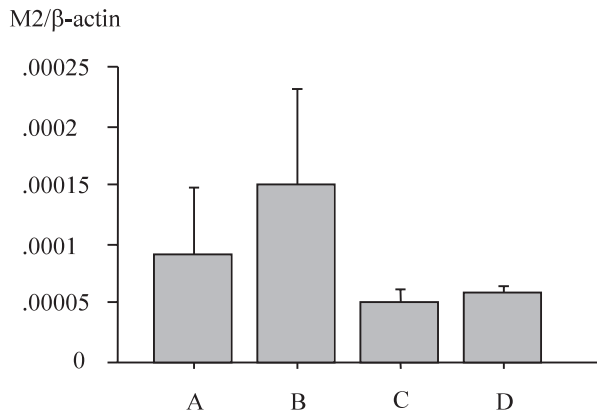
$pA_2$  values are presented as mean (mean + S.E.M. ~ mean - S.E.M.), whereas slope was presented as mean  $\pm$  S.E.M. In each experiment,  $n = 4$ –5.

mRNA (Fig. 3A), the levels of mRNA expression in the untreated diabetic rats (group B,  $3.79 \pm 1.83 \times 10^{-2}$ ) were significantly higher than those of the control group (group A,  $0.26 \pm 0.03 \times 10^{-2}$ ). This increase in mRNA expression tended to be reversed by treatment with N-hexacosanol in a dose-dependent manner (group C,  $1.64 \pm 0.64 \times 10^{-2}$ ; group D,  $0.65 \pm 0.45 \times 10^{-2}$ ). The quantification of muscarinic  $M_2$  receptor mRNAs in groups A, B, C, and D were  $0.92 \pm 0.55 \times 10^{-4}$ ,  $1.52 \pm 0.79 \times 10^{-4}$ ,  $0.50 \pm 0.12 \times 10^{-4}$ , and  $0.58 \pm 0.05 \times 10^{-4}$ , respectively, and there was no significant difference between any groups (Fig. 3A). It should be noted that changes in muscarinic  $M_2$  receptor mRNA levels tended to parallel those of muscarinic  $M_3$  mRNA. However, the level of expression of muscarinic  $M_3$  receptor mRNAs was significantly higher than that of muscarinic  $M_2$  receptor mRNAs.

### DISCUSSION

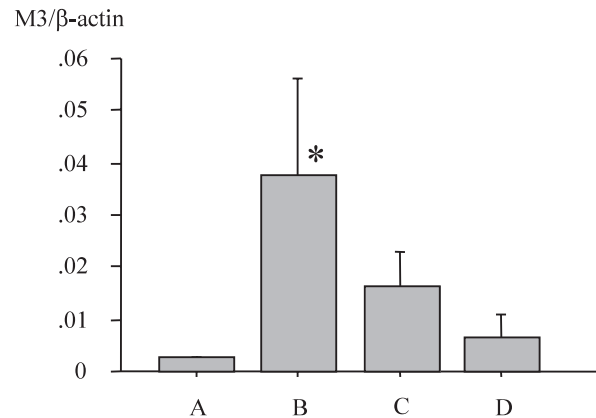
In the present study, we demonstrated that N-hexacosanol prevented diabetes-induced hypercontractility of the rat ileum caused by carbachol, and it also prevented the up-regulation of the expression of both muscarinic  $M_2$  and  $M_3$  receptor mRNAs. In the present study, although the  $EC_{50}$  values obtained in the presence of carbachol were not significantly different between any of the groups examined, the  $E_{max}$  values in the diabetic rat ileal smooth muscle were significantly higher than those of the control rats. Our data regarding contractile responses to carbachol indicated that the muscarinic receptors on ileal smooth muscles, as well as the downstream signal pathway, might be altered by the onset of diabetes. Subsequently, we calculated the  $pA_2$  values and their slopes using four subtype-selective and non-selective muscarinic antagonists in order to investigate the affinity of the receptors. Interestingly, the rank order of the series of muscarinic antagonists was  $ATR \geq 4$ -DAMP > MTR > PRZ in all groups studied.

Previously, our group and others have demonstrated that the contractile responses of ileal smooth muscle to acetylcholine or cholinergic agonists are enhanced in the context of diabetes (4, 16). In one past report (16), although the  $EC_{50}$  values obtained with carbachol were not significantly different between any groups studied, the  $E_{max}$  values in the diabetic rat ileal smooth muscle were significantly higher than those in control rats. As regards the effects of carbachol, our contractile response data indicated that the muscarinic receptors on the ileal



**Fig. 3A** Muscarinic  $M_2$  receptor mRNAs normalized by  $\beta$ -actin. A: control rats, B: diabetic rats treated with vehicle, C: diabetic rats treated with N-hexacosanol (2 mg/kg), and D: diabetic rats treated with N-hexacosanol (8 mg/kg). Data are shown as mean  $\pm$  S.E.M. of 4–6 separated determinations in each group.

smooth muscles, as well as the signal pathway downstream of these receptors, might be altered by the induction of diabetes. It remains important to obtain data when N-hexacosanol is administered simultaneously with the induction of diabetes, as in such cases, diabetes-induced-hypercontractility is prevented either partially or even entirely. In the present study, not only high but also low doses of treatment with this compound prevented diabetes-induced hypercontractility of the rat ileum. Recently, we reported that N-hexacosanol prevented the diabetes-induced hypercontractility caused by carbachol in the smooth muscle of the diabetic rat bladder (9). In order to confirm these putative changes in the muscarinic receptor system, we calculated the  $pA_2$  values and their slopes using four subtype non-selective and selective muscarinic antagonists in order to investigate the affinity of the receptors for these agents. There were no significant differences in the  $pA_2$  values and slopes between any of the groups with any of the muscarinic antagonists used. The results of the  $pA_2$  value calculations for this series of muscarinic antagonists were similar in all groups, and the rank order of the values was:  $ATR \geq 4\text{-DAMP} > MTR > PRZ$ . These findings indicate that alteration of the contractile response via muscarinic  $M_2$  and  $M_3$  receptors is not due to changes in muscarinic receptor affinity in diabetic rat ileal smooth muscles; such changes instead appear to be the result of quantitative rather than qualitative changes in the muscarinic receptor system. Mutoh and his associates previously reported that the rank order of  $pA_2$  values for these muscarinic antagonists



**Fig. 3B** Muscarinic  $M_3$  receptor mRNAs normalized by  $\beta$ -actin. A: control rats, B: diabetic rats treated with vehicle, C: diabetic rats treated with N-hexacosanol (2 mg/kg), and D: diabetic rats treated with N-hexacosanol (8 mg/kg). Data are shown as mean  $\pm$  S.E.M. of 4–6 separate determinations in each group. \*significantly different from A and D groups.

was  $ATR \geq 4\text{-DAMP} > PRZ > MTR$  in the rabbit bladder dome (12), and we also previously reported that the rank order of  $pA_2$  values for these muscarinic antagonists in the rat bladder smooth muscle was  $ATR \geq 4\text{-DAMP} > MTR > PRZ$  (9). As regards the role of muscarinic receptors, Mutoh *et al.* suggested that the rabbit bladder dome undergoes contractions via the muscarinic  $M_3$  receptor subtype (12). Taken together, the findings to date suggest that rat ileal smooth muscle primarily contracts via the muscarinic  $M_3$  receptor subtype, and muscarinic contractile systems are not altered by the induction of diabetes. However, based on the results of this study, we can not rule out the possibility that the muscarinic  $M_2$  receptor subtype does not directly contribute to cholinergic contractions. In addition, Unno *et al.* reported results for muscarinic  $M_2$ -knockout, muscarinic  $M_3$ -knockout, muscarinic  $M_2/M_3$ -double knockout, and wild-type mice, demonstrating that muscarinic  $M_2$  and  $M_3$  receptors participate in mediating cholinergic contractions in the mouse ileum, with the latter subtype of receptors assuming a substantial role in this regard (19).

In order to elucidate the mechanisms of diabetes-induced hypercontractility of the rat ileum, we measured the expression levels of muscarinic  $M_2$  and  $M_3$  receptor mRNAs using real-time PCR. We demonstrated here that the mRNA levels of both muscarinic  $M_2$  and  $M_3$  receptors were increased under diabetic conditions, as compared to control levels, and N-hexacosanol prevented the up-regulation of expression of these mRNAs. The present study also

demonstrated that muscarinic M<sub>3</sub> receptor mRNA levels were significantly higher in 8-week-old diabetic rats than in controls, and that both low and high doses of N-hexacosanol treatment entirely prevented increases in muscarinic M<sub>3</sub> receptor mRNAs. The same tendencies were observed for muscarinic M<sub>2</sub> receptor mRNAs. Previously, we reported that N-hexacosanol exerts therapeutic (not preventive) effects on hypercontractility in the diabetic ileum by ameliorating the overexpression of muscarinic M<sub>2</sub> and M<sub>3</sub> receptor mRNAs (16). Based on our previous and present data, it appears likely that the overexpression of muscarinic M<sub>2</sub> and M<sub>3</sub> receptor mRNAs is related to the hypercontractility of ileal smooth muscle in diabetic rats. The diabetes-associated neuropathy may inhibit the release of acetylcholine from cholinergic nerves, in turn inducing an overexpression of muscarinic receptors in the diabetic ileum. As a result of such an overexpression of muscarinic receptors, signaling downstream of muscarinic M<sub>2</sub> and M<sub>3</sub> receptors may be enhanced, and ileal smooth muscle contraction may be increased, according to the results of the present organ bath study.

Present and previous reports indicate that the preventive effects of N-hexacosanol in diabetic neuropathy occur according to a mechanism that differs from that of insulin therapy (9, 10, 13, 15). Treatment with both insulin and N-hexacosanol may prevent or reverse diabetic peripheral neuropathy. However, the detailed mechanism of this preventive effect remains unclear, and clearly warrants further study.

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