

Cyclohexenonic long-chain fatty alcohol has therapeutic effects on diabetes-induced angiopathy in the rat aorta

Chiko Shinbori, Motoaki Saito, Yukako Kinoshita, Itaru Satoh, Tomoharu Kono, Takuya Hanada, Eiji Nanba, Kaori Adachi, Hiroto Suzuki, Masashi Yamada and Keisuke Satoh

Department of Pathophysiological and Therapeutic Science, Division of Molecular Pharmacology, Tottori University Faculty of Medicine, Yonago, Japan (C.S., M.S., Y.K., I.S., T.K., T.H., K.S.), Division of Functional Genomics, Research Center for Bioscience and Technology, Tottori University, Yonago, Japan (E.N., K.A.), Meiji Dairies Corporation Pharmaceuticals Department, Tokyo, Japan (H.S., M.Y.)

Correspondence:

Motoaki Saito, MD, Ph.D

Department of Pathophysiological and Therapeutic Science, Division of Molecular Pharmacology, Tottori University Faculty of Medicine, 86 Nishimachi, Yonago, 683-8503, Japan

Telephone : +81-859-38-6163

FAX : +81-859-38-6160

e-mail address : saitomo@grape.med.tottori-u.ac.jp

Abstract

We studied the effects of cyclohexenonic long-chain fatty alcohol (N-hexacosanol) on diabetes-induced angiopathy in the rat aorta. Male Sprague-Dawley rats were divided into 4 groups, a control group and 3 other groups in which diabetes was induced by streptozotocin (50 mg/kg i.p.). Four weeks after the induction of diabetes, the 3 groups received treatment with either vehicle or N-hexacosanol (2 or 8 mg/kg, i.p. every day) for another 4 weeks. To determine the mechanisms of diabetic vascular dysfunction and the effects of N-hexacosanol, we conducted organ bath studies and real-time polymerase chain reaction on muscarinic M₃ receptor, and endothelial and inducible nitric oxide synthase (eNOS and iNOS) mRNAs in the rat aorta. Treatment with N-hexacosanol did not alter the diabetic status, but improved the diabetes-induced hypercontraction produced by norepinephrine and the damaged endothelium-dependent relaxation of the rat aorta induced by acetylcholine. Furthermore, in the diabetic rats, both muscarinic M₃ receptor and iNOS mRNAs were significantly increased, and N-hexacosanol reversed these upregulations. However, the expression of eNOS mRNA showed no change in all groups. These results indicate that N-hexacosanol has

beneficial effects on functional dysfunction and reverses the upregulation of muscarinic

M₃ receptor and iNOS mRNAs in the diabetic rat aorta.

Key Words: N-hexacosanol, NOS, muscarinic M₃ receptor, aorta, endothelium, diabetes

1. Introduction

Cardiovascular disease is one of the leading causes of death in diabetes mellitus, which alters vascular responsiveness to several vasoconstrictors and vasodilators and is a major factor underlying the development of this disease (Senses et al., 2001). Most of the complications in diabetes are caused by increased serum glucose and the increased generation of oxygen-derived free radicals, which lead to endothelium dysfunction and neuropathy. Although strict glycemic control delays the onset and slows down the progression of diabetic vascular complications (The Diabetic Control and Complications Trial Research Group, 1993), this strategy is not successful in all patients. There have been many reports demonstrating functional changes in various smooth muscle cells from diabetic animals (Ozturk et al., 1996). It has been shown that vessels from diabetic animals exhibit abnormal endothelium-dependent vascular relaxation to acetylcholine (Oyama et al., 1986). These functional changes may be associated with endothelium dysfunction in diabetes. Impaired endothelium-dependent vasodilatation may arise from several mechanisms: decreased production of one of the endothelium-derived relaxing factors (EDRFs), including nitric

oxide (NO) and prostacyclin (PGI₂), enhanced inactivation of EDRF, impaired diffusion of EDRF to the underlying smooth muscle, and enhanced generation of endothelium-derived constricting factors (EDCF) (De Vriese et al., 2000). In particular, NO plays an important role in vasodilation. In 1980, Furchgott and Zawadzki described a mediator that is released by the vascular endothelium in response to acetylcholine and causes vascular smooth muscle relaxation (Furchgott and Zawadzki, 1980). NO is synthesized by three different NO-synthase (NOS) isoforms: inducible NOS (iNOS), brain-type (bNOS), and endothelial constitutive NOS (eNOS) (Forstmann et al., 1994). It is generally assumed that these three NOS enzyme isotypes play an important role in maintaining proper functions in homeostasis (Bode-Boger et al., 1996; Cooke and Tsao, 1997). However, under chronic proinflammatory conditions, such as those at work during arteriosclerosis, local expression of the iNOS isotype is seen in endothelia and other cell types (Wilcox et al., 1997). Furthermore, previous studies using pharmacological approaches with muscarinic receptor agonists and antagonists suggest that the muscarinic M₃ receptor mainly mediates vasodilation via the actions of eNOS in the rat aorta (Boulanger et al., 1994; Khurana et al., 2004). It is well known

that the muscarinic M₃ receptor is mainly expressed in the endothelium in the aorta.

However, the extent of the contribution of the muscarinic M₃ receptor to diabetic aortic dysfunction is not well known.

Cyclohexenonic long-chain fatty alcohol (N-hexacosanol) exhibits a wide variety of biological actions. A previous study showed that N-hexacosanol has neurotrophic activation on cultured neurons from the cerebral cortex (Borg et al., 1990). We have reported the ameliorative effects of N-hexacosanol on diabetes-induced hyperreactivity of the bladder and trachea in the rat (Satoh et al., 2005; Suzuki et al., 2006; Saito et al., 2007). We have also reported that diabetes-induced hypercontraction of the aorta was ameliorated by treatment with N-hexacosanol (Kinoshita et al., 2006). However, the effect on hyporelaxation in the aorta is still unknown. From the above results, we hypothesized that N-hexacosanol may have a beneficial effect on the muscarinic M₃ receptor and on eNOS and iNOS in the diabetes-induced rat aorta.

In order to investigate the therapeutic effects of N-hexacosanol on diabetes-induced vascular dysfunction and reveal its molecular mechanisms, we conducted organ bath studies and quantification of the muscarinic M₃ receptor and eNOS and iNOS mRNA in

the rat aorta.

2. Materials and Methods

2.1. 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 8-week-male Sprague-Dawley rats (SLC, Shizuoka, Japan) by administering an intraperitoneal injection of streptozotocin (50 mg/kg) dissolved in 0.1 M citrate-phosphate buffer (pH 4.2), according to the method of our previous reports (Sato et al., 2005; Suzuki et al., 2006; Kinoshita et al., 2006; Saito et al., 2007). The rats were divided randomly into 4 age-matched groups (n=6-8). One group was the non-diabetic control (group A), and was administered the vehicle; diabetes was induced in the other three groups. Four weeks after the induction of diabetes, the three groups were treated for another 4 weeks with either vehicle (group B) or with N-hexacosanol at a daily dose of 2 or 8 mg/kg (groups C and D, respectively). N-hexacosanol was dissolved in ethanol, then a mixture of physiological saline/ Tween 80 was added to obtain an ethanol: saline: Tween 80 ratio of 5: 92.15: 2.85 (total volume approximately 0.3 ml). The structure of the N-hexacosanol used in this study is shown in Fig. 1.

Two days after the injection of streptozotocin or vehicle, the induction of diabetes was confirmed by measuring urinary glucose with Pretest 3a II (Wako Pure Chemical, Osaka, Japan). Either Nhexacosanol (groups C and D) or vehicle (groups A and B) was injected i.p. every day beginning 4 weeks after the induction of diabetes, according to the method used in our previous reports (Sato et al., 2005; Suzuki et al., 2006; Kinoshita et al., 2006; Saito et al., 2007). All groups were kept under identical conditions, with 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 / animal, i.p.). Blood samples were collected from the vena cava, and the aorta was removed from each animal and placed in Krebs-Henseleit solution comprised of (mM): NaCl 118.0, KCl 4.7, MgSO₄ 1.2, CaCl₂ 2.5, KH₂PO₄ 1.2, NaHCO₃ 24.9, glucose 5.6 and sodium pyruvate 2.0, for use in functional and biochemical studies.

2.2. Serum glucose and insulin measurement

Serum glucose concentrations in the experimental rats were measured by the hexokinase method (Glucose C II, Wako Pure Chemical, Osaka, Japan), which was carried out

according to the kit manufacturer's instructions. Insulin concentrations were also measured by ELISA according to the manufacturer's instructions (Rat Insulin ELISA, Merckodia AB, Uppsala, Sweden).

2.3. In vitro organ bath experiments

Functional studies were performed according to our previous report (Kinoshita et al., 2006). The thoracic aortas were cut into approximately 3-mm-long ring segments. Each ring was suspended on a wire hook in an organ bath (25 ml) containing Krebs-Henseleit solution, and bubbled with 5% CO₂ and 95% O₂ (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 to a micrometer (Mitutoyo, Tokyo, Japan). Each ring was equilibrated unstretched for 30 minutes. A load of 0.5 g was applied to each ring by micrometer adjustment, and the load was readjusted to this level 30 minutes later. Changes in the tone were recorded by a force transducer 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). Following a 30-minute period of equilibration, the rings were exposed to 100 mM KCl. In the aorta rings, the contractile response to norepinephrine (1×10^{-9} - 3×10^{-6} M) was determined cumulatively. After a 30-minute washout period, propranolol (1×10^{-6} M) was added to prevent the involvement of β -adrenoceptors. Endothelium-mediated relaxation was measured as a concentration-response curve to acetylcholine (1×10^{-8} - 3×10^{-5} M) in rings precontracted with the submaximal dose of norepinephrine (3×10^{-7} M). Endothelium-independent aortic relaxation in response to nitroglycerin (1×10^{-6} M) was also measured in the rings.

2.4. Real-time polymerase chain reaction (quantification of muscarinic M₃ receptor, eNOS and iNOS mRNAs)

Expressions of muscarinic M₃ receptor, eNOS and iNOS mRNAs in the experimental aorta were measured by real-time polymerase chain reaction (PCR) methods. The mRNAs were purified by the RNeasy Mini Kit (Quiagen, Valencia, CA) according to the manufacturer's instructions. A reverse transcriptase mixture (28 μ l) containing 2 μ g of total RNA was made and incubated at 37 °C for 60 minutes. Fifteen μ l of the

master mix was used for the 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 muscarinic M₃ receptor (GeneBank Accession: NM_012527) primers and probe sequences were the following: primer forward (1227-1245: 5'GGACTGTGGATGTGGAGAG-3'), primer reverse (1358-1375: 5'-CGAGGAGTTGGTGTGTCAGA-3') and probe forward (1267-1284: 5'-CCA GAAGAGCATGGGTGATGGTGACAAC-3'), probe reverse (1286-1325: 5'-XGTCAGAAGGATTCACCAAGC-TTCCCATCCT-3'). The primer and probe of the β -actin (GeneBank Accession: NM_031144) and eNOS (GeneBank Accession: AJ011116) and iNOS (GeneBank Accession: D44591) used were from the LightCycler-Primer/Probe Set (rat) (Roche Diagnostics, Tokyo, Japan). A total of 5 μ l of template was used for the sample. The specificity of the reaction was confirmed by 2 % agarose gel electrophoresis. The primers for the β -actin gene were used as the internal standard and gene levels were analyzed by real-time PCR using the same reverse transcriptase mixture.

2.5. Data analysis

The data for the contractions induced by norepinephrine were normalized by the contractions induced by 100 mM KCl. The relaxation responses with submaximal contraction caused by 3×10^{-7} M norepinephrine were expressed as percentages of nitroglycerin. The ED_{50} and E_{max} values were obtained using a Macintosh computer (G3) loaded with Chart v3.6.9 software and a PowerLab/16sp data acquisition system. The ED_{50} values were calculated as geometric means, whereas the E_{max} values were calculated as arithmetic means. The expressions of muscarinic M_3 receptor, eNOS and iNOS mRNAs were quantified according to the expression of β -actin mRNA in the experimental rat aorta. 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.

2.6. Drugs and chemicals

3-(15 Hydroxypentadecyl)-2,4,4-trimethyl-2-cyclohexen 1-one (N-hexacosanol) was obtained from Meiji Milk Products Co., Ltd., Tokyo, Japan. Propranolol and

norepinephrine were purchased from Sigma (St. Louis, MO), and nitroglycerin

(millisrol[®]) was purchased from Nihonkayaku Co., Ltd. (Tokyo, Japan).

Streptozotocin was purchased from Wako Pure Chemical Co. (Osaka, Japan). All

other chemicals were available commercially and of reagent grade.

3. Results

3.1. General features of the experimental animals

The data obtained for the general features and serum concentrations of insulin and glucose in the experimental animals are shown in Table 1. The diabetic rats that were not treated with N-hexacosanol showed no weight gain during the experimental period. The diabetic rats displayed significantly higher serum glucose and lower serum insulin levels than the control rats. Treatment with N-hexacosanol (at either 2 or 8 mg/kg) neither increased body weight and insulin levels nor reduced serum glucose in the diabetic animals.

3.2. Measurement of contraction (A) and relaxation (B)

A: In the diabetic rat aortic rings, the contraction produced by norepinephrine was augmented (Fig. 2A). With treatment of N-hexacosanol (8 mg/kg), the maximum contraction was significantly attenuated toward that obtained using control rat aortic rings (Fig. 2A). The maximum contraction (E_{\max}) and EC_{50} values are shown in Table 2.

B: The maximum relaxation (E_{\max}) and EC_{50} values are shown in Table 2. In the aortic rings precontracted by norepinephrine with intact endothelium obtained from all groups, the relaxation was produced in a dose-dependent manner (Fig. 2B). The relaxation was markedly reduced in the diabetic aorta (Fig. 2B). With treatment of N-hexacosanol, the attenuated relaxation was recovered; data are shown in Fig. 2B. Induction of diabetes significantly increased EC_{50} values for acetylcholine-induced relaxation. Treatment with either dose of N-hexacosanol, however, did not ameliorate these increased EC_{50} values. The nitroglycerin-induced relaxation was not changed in any groups (Table 2).

3.3. Measurement of muscarinic M_3 receptor, eNOS and iNOS mRNAs in the aorta

Fig. 3 shows the expression of muscarinic M_3 receptor, eNOS and iNOS mRNAs in the aorta. No significant difference in the eNOS mRNA levels was found between any groups while the M_3 receptor and iNOS mRNA levels were significantly higher in the diabetes than in the control group. In addition, N-hexacosanol reversed these increases

in a dose-dependent manner (Fig. 3).

4. Discussion

In the diabetic state, the rat aorta exhibited enhanced vascular reactivity to norepinephrine and impairment of endothelium-dependent-relaxation by acetylcholine. While the eNOS mRNA level was not changed, the muscarinic M₃ receptor and iNOS mRNA levels were significantly increased in the diabetic aorta. Although N-hexacosanol did not improve the general features or levels of serum glucose and insulin in the diabetic rats, it significantly improved the enhanced contraction produced by the α_1 -agonist norepinephrine and the impairment of endothelium-dependent relaxation induced by acetylcholine in a dose-dependent manner.

Previous reports demonstrated that aortic rings prepared from rats with streptozotocin-induced diabetes showed increased contraction in response to vasoconstrictor agents and reduced relaxation in response to endothelium-dependent relaxant agents, but not to endothelium-independent relaxant agents (Oyama et al., 1986; Pieper et al., 1998; Kinoshita et al., 2006). Previously, we demonstrated that maximum relaxation in response to nitroglycerin was only around 50 % in Kinoshita's report, while maximum relaxation in response to nitroglycerin exceeded 90 % in this

study (Kinoshita et al., 2006). In Kinoshita's reports, submaximal contraction was induced by the thromboxane A₂ analogue U46619 at a molar dose of 3×10^{-8} , while in this experiment, we chose the physiological agonist norepinephrine. Submaximal contraction was induced by norepinephrine at a molar dose of 3×10^{-7} . The extents of contraction produced with agonists were distinct. The contraction produced by U46619 was stronger than that by norepinephrine. The extent of relaxation by nitroglycerin might be smaller than that by norepinephrine (Satoh et al., 1993). In this study, we carefully observed the contractile responses induced by norepinephrine, and decided that submaximal contraction was induced by norepinephrine at a molar dose of 3×10^{-7} . This may be the reason we obtained maximum relaxation in response to nitroglycerin exceeding 90 % in this study. In this study, although the degree of relaxation induced by acetylcholine was significantly improved by the high dose of N-hexacosanol, the EC₅₀ values for acetylcholine were not reversed by this dose. These data suggest that the affinity of the endothelium for acetylcholine was not reversed by treatment with N-hexacosanol.

Diabetes mellitus has been reported to induce hyperglycemia and injured vascular

endothelial cells and peripheral nerve cells (Oyama et al., 1986; Ozturk et al., 1996; Shinozaki et al., 2003). Previous studies have shown that N-hexacosanol treatment has no effect on serum glucose and insulin levels of diabetic rats (Satoh et al., 2005; Suzuki et al., 2006; Kinoshita et al., 2006; saito et al., 2007). Therefore, the finding that this drug does not influence the serum glucose levels indicates that the mechanisms of its reversible effect on the diabetic rat aorta do not occur by reducing serum glucose.

To clarify the mechanism of the ameliorative effect of N-hexacosanol, we performed quantification of muscarinic M₃ receptor, eNOS and iNOS mRNAs by real-time PCR. In the PCR studies, while the eNOS mRNA level was not changed, the muscarinic M₃ receptor and iNOS mRNA levels exhibited significant increases in the induced diabetic aorta. Previous studies suggested that arterial endothelium cells released acetylcholine (Kawashima et al., 1990; Ikeda et al., 1994), and the endothelium dysfunction-induced diabetic states might bring about a reduction in acetylcholine release. Therefore, muscarinic M₃ receptor mRNA might be upregulated in diabetes. And, because eNOS is expressed in endothelial cells, endothelium dysfunctions reduce the eNOS expression. In fact, it has been reported that hyperglycemia and glucose-modified proteins reduce

constitutive NOS expression (Chakravarthy et al., 1998). Bojunga et al reported that eNOS mRNA levels were decreased in the diabetic rat aorta (Bojunga et al., 2004). As competitive reverse-transcriptase PCR is more sensitive than real-time PCR, the reasons for these different results may be due to the small differences detected by real-time PCR. The diabetic status of the animals in other experiments was different from that in ours. Furthermore, the muscarinic M₃ receptor activation on endothelial cells produces NO via eNOS and PGI₂ via phospholipase A2 and arachidonic acid (Jaiswal et al., 1991; Kan et al., 1995; Maguire and Davenport, 2005; Triggle et al., 2003). The PGI₂ pathway may be related to this event. The result obtained for the iNOS mRNA was in agreement with that of a previous study (Bojunga et al., 2004). The results of the present study demonstrate that eNOS and iNOS are regulated differentially in experimental diabetes mellitus. Hyperglycemia and glucose-modified proteins may act to reduce eNOS activity and at the same time increase iNOS gene expression with enhanced NO release. It is also suggested that hyperglycemia markedly activates the β II isoform of protein kinase C (PKC) in endothelial cells by promoting *de novo* synthesis of diacylglycerol (DAG) and increasing mitochondrial superoxide production.

Activated PKC can indeed negatively influence transcription of eNOS while acting positively on the iNOS gene (Bojunga et al., 2004). It is known that iNOS is induced by inflammation, and that iNOS-derived NO exhibits a powerful protective activity towards cellular stress conditions (Buttery et al., 1996). Moreover, other studies have shown that iNOS-derived NO serves to modulate the expression of many different genes that also affect protective responses during stress conditions (Ehrt et al., 2001; Hemmrich et al., 2003). It is possible that iNOS does not contribute directly to the induction of relaxation, but has other responses protecting the vascular endothelium. Previous experimental evidence has suggested that tetrahydrobiopterin (BH₄), the natural and essential cofactor of NOS, plays a crucial role not only in increasing the rate of NO generation by NOS, but also in controlling the formation of superoxide anions (O₂⁻) in endothelial cells (Baek et al., 1993). In fact, oral supplementation with BH₄ restored endothelial function and relieved oxidative tissue damage through the activation of eNOS in the aortas of insulin-resistant rats (Pieper et al., 1997; Shinozaki et al., 2003). It is possible that the influence of eNOS-derived NO production on hyporelaxation is mediated via other factors or stages.

Jover et al. reported that N-hexacosanol may act directly upon nerve terminals inducing Ca^{2+} entry and hence promote neurohormone release (Jover et al., 2005). As muscarinic M_3 receptor subtypes, eNOS and iNOS are expressed in the aortic smooth muscle (Ehlert, 2003). In order to reveal more details and the precise mechanisms of this effect, further studies are needed. Taken together, our data demonstrate that N-hexacosanol normalized the dysfunction of relaxation, and reversed diabetes-induced upregulation of muscarinic M_3 receptor and iNOS mRNAs.

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Figure legends

Fig. 1. The chemical structure of N-hexacosanol used in this study.

Fig. 2. a) Contractile response of rat aortic rings to norepinephrine. The data of contraction induced norepinephrine were normalized by the KCl (100 mM). NE: norepinephrine. b) Endothelium-dependent relaxation of rat aortic rings to acetylcholine. The data of relaxation were normalized by the nitroglycerine. Ach: acetylcholine. A: control rats, B: diabetic rats, 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 six to eight separated determinations in each group. *) significantly different from the other groups. **) Significantly different from B group. ***)Significantly different from B and C groups. ($p < 0.05$)

Fig. 3. a) Muscarinic M_3 receptor mRNA normalized by β -actin. b) eNOS mRNA normalized by β -actin. c) iNOS mRNA normalized by β -actin. A: control rats, B: diabetic rats, 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 six to eight separated determinations in each group. *) Significantly different from A group.

Table 1. General features of the experimental rats

	Body Weight (g)			Serum glucose (mg/dl)	Serum insulin (μ g/l)
	8 weeks old	12 weeks old	16 weeks old		
A	242.9 \pm 3.0	423.6 \pm 5.9	498.8 \pm 10.1	172.8 \pm 13.9	2.39 \pm 0.660
B	246.8 \pm 6.4	259.5 \pm 17.2 ^a	231.8 \pm 23.5 ^a	405.0 \pm 54.3 ^a	0.17 \pm 0.009 ^a
C	250.4 \pm 4.7	251.0 \pm 17.0 ^a	229.0 \pm 25.4 ^a	314.4 \pm 46.9 ^a	0.17 \pm 0.010 ^a
D	253.3 \pm 5.1	257.0 \pm 16.1 ^a	237.0 \pm 17.1 ^a	364.8 \pm 34.3 ^a	0.16 \pm 0.004 ^a

A: control rats, B: diabetic rats treated with sham, 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 six to eight separated determinations in each group. ^a) Significantly different from the A group.

Table 2. Data of functional studies in the rat aorta

	Contraction		Relaxation		
	Norepinephrine		Acetylcholine	Nitroglycerin	
	Emax /KCl (100 mM)	EC ₅₀ (e-8 M)	Maximum relaxation (%)	EC ₅₀ (e-7 M)	Maximum relaxation (%)
A	1.67 ± 0.03	3.58 ± 0.70	68.26 ± 3.14	0.54 ± 0.07	91.76 ± 2.54
B	2.15 ± 0.08 ^a	2.74 ± 0.81	29.81 ± 6.15 ^a	2.28 ± 0.67 ^a	96.46 ± 2.03
C	2.04 ± 0.13 ^a	3.09 ± 0.77	27.36 ± 4.89 ^a	2.10 ± 0.44 ^a	95.92 ± 2.42
D	1.86 ± 0.10 ^{a, b}	3.64 ± 1.22	53.52 ± 4.84 ^{a, c}	2.68 ± 0.49 ^a	97.17 ± 1.18

A: control rat, B:diabetic rat, C: diabetic rat with treatment with N-hexacasanol (2mg/kg), and D: diabetic rat with treatment with N-hexacosanol (8mg/kg). The relaxation responses with submaximal contraction caused by 3×10^{-7} M norepinephrine were expressed as percentages of nitroglycerin. Emax and EC₅₀ values under contraction are for norepinephrine. Maximum relaxation and EC₅₀ values are for acetylcholine in aorta precontracted with norepinephrine. The relaxation responses to nitroglycerine as the percentage of the submaximum contraction obtained with norepinephrine. Data are shown as mean ± S.E.M. of six to eight separated determinations in each group. ^a)Significantly different from A group. ^b)Significantly different from B group. ^c)Significantly different from B and C groups. (p < 0.05)

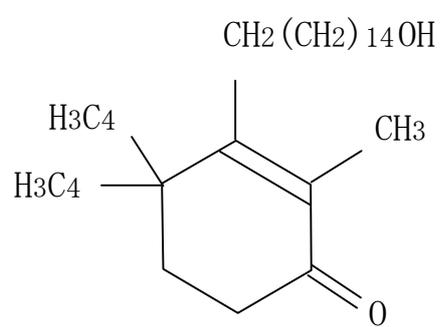


Figure 2. Measurement of contraction and relaxation

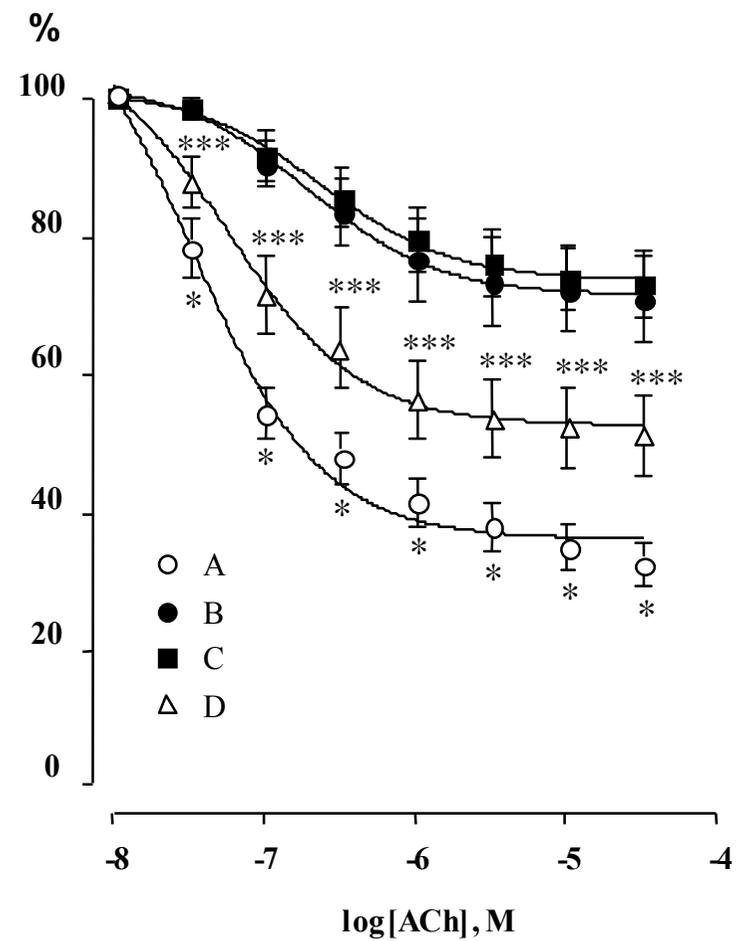
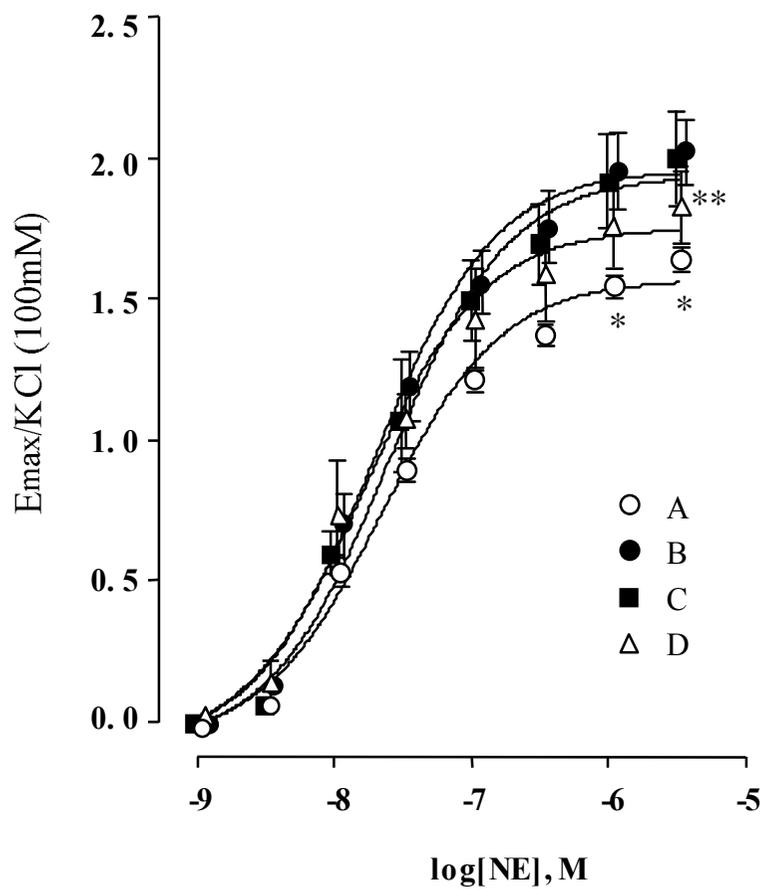


Figure 3. Expression of mRNAs in the rat

