Diabetes-related changes in contractile responses of stomach fundus to endothelin-1 in streptozotocin-induced diabetic rats

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Abstract

The contractile response of the stomach fundus to endothelin-1 (ET-1) was examined in streptozotocin (STZ)-induced diabetic rats. In STZ-diabetic rats (versus age-matched control rats) (a) ET-1 caused a longer-lasting contraction of stomach fundus strips, and (b) in the dose-response curve, the ET-1-induced contraction was significantly greater for a given concentration (3×10^{-7} to 10^{-7} M). Although repeated application of ET-1 led to desensitization, the desensitization was less pronounced in STZ-diabetic rats than in the controls. The density of the binding sites for [¹²⁵I]-ET-1 was increased in the diabetic stomach fundus (versus the controls), but K_d values were similar between the two groups. The ET_B receptor mRNA expression level was significantly increased in the diabetic stomach fundus. These results suggest that the diabetes-related enhancement of the ET-1-induced contraction of the stomach fundus may be due to an increase in the ET_B receptor population.

Key words: contraction, desensitization, diabetes, endothelin, stomach

Introduction

Endothelin-1 (ET-1), originally isolated by Yanagisawa *et al.* (1988) from porcine aortic endothelial cells, is the most powerful endogenous vasoconstrictor agent known, and moreover it has been found to be a powerful mitogen for vascular smooth muscle cells (Goto *et al.*, 1996; Miyauchi and Masaki, 1999; Masaki, 2004). It has been reported that endothelins are produced in the gut (Ota *et al.*, 1991), and that their receptors are present within the stomach and intestine (Takahashi *et al.*, 1990b). In rat isolated stomach strip, ET-1 causes dose-dependent contractions (Wallace *et al.*, 1989b; Fulginiti *et al.*, 1993; Shimomura *et al.*, 1994; Allcock *et al.*, 1995). The functional effects of ET-1 are mediated through at least two distinct subtypes of receptors, the ET_A and ET_B receptors (Goto *et al.*, 1996; Miyauchi and Masaki, 1999; Masaki,

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2004). In isolated smooth muscle cells from guinea pig stomach, two ET receptors are present, but whereas the ET_A receptor mediates contraction, the ET_B receptor is involved in neither contraction nor relaxation (Kitsukawa *et al.*, 1994). Also, functional studies demonstrated that ET-1 and the ET_B receptor-selective agonist sarafotoxin S6c elicit contraction of the rat fundic strip via high affinity ET_B receptors (Gray *et al.*, 1995). The above suggests that ET-1 may play an important role in regulating gastric motility, although the physiological role played by ET-1 in stomach contraction is still not understood.

Diabetes mellitus is a metabolic disease affecting every organ system in the body, including the gastrointestinal tract (Katz and Spiro, 1966). Indeed, diabetic gastroparesis is a well-known complication of long-standing diabetes (Keshavarzian *et al.*, 1987). Diabetic gastroparesis is a condition comprising a decrease in fundic and antral motor activity (Fox and Behar, 1980; Keshavarzian *et al.*, 1987), a reduction in, or lack of, the interdigestive migrating motor complex (Fox and Behar, 1980), gastric dysrhythmias (Koch *et al.*, 1989), and “pylorospasms” (Mearin *et al.*, 1986). The etiology of diabetic gastroparesis is still not fully established, but important factors seem to be autonomic neuropathy (Keshavarzian *et al.*, 1987), acute hyperglycemia (Rayner *et al.*, 2001), and abnormalities of gut hormones and neurotransmitters, such as motilin (Achem-Karam *et al.*, 1985), gastrin (Feldman *et al.*, 1979), and nitric oxide (Bult *et al.*, 1990). Other factors that may modulate gastrointestinal motility include thyroid function (Holdsworth and Besser, 1968), gastric acid secretion (Bortolotti and Barbara, 1988), and *Helicobacter pylori* infection (Perri *et al.*, 1996).

Interestingly, plasma ET-1 levels are known to be increased in the diabetic state (Takahashi *et al.*, 1990a; Makino and Kamata, 1998a,b, 2000, 2002; Makino *et al.*, 2001; Kanie and Kamata, 2002; Kanie *et al.*, 2003), and moreover the plasma concentration of big endothelin-1, the precursor of ET-1, is elevated in patients with diabetes mellitus (Tsunoda *et al.*, 1991). Thus, although ET-1 may also contribute to the normal maintenance of vascular tone, a pathogenetic role is implied by its overproduction in patients with diabetes (Takahashi *et al.*, 1990a). Indeed, the vasomotor response to ET-1 is altered in several vessels in the diabetic state (Makino and Kamata, 1998, 2000; Makino *et al.*, 2001; Khan and Chakrabarti, 2003; Matsumoto *et al.*, 2004a). In contrast, to our knowledge there are no reports concerning any change in the contractile response of the stomach fundus to ET-1 in diabetic rats, although plasma ET-1 is known to be elevated in such animals.

The aims of the present study were to look for evidence of (1) changes in the contractile response of the stomach fundus to ET-1 in STZ-induced diabetic rats and (2) changes in the density of ET-1 receptors in these rats.

Materials and Methods

Animals and experimental design

Male Wistar rats (8 wk old and 180–230 g body weight) received a single injection via the tail vein of STZ 65 mg/kg dissolved in a citrate buffer. Age-matched control rats were injected with the buffer alone. Food and water were given *ad libitum*. This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the

Committee on the Care and Use of Laboratory Animals of Hoshi University (which is accredited by the Ministry of Education, Culture, Sports, Science and Technology, Japan).

Measurement of plasma glucose

Twelve weeks after the injection of STZ or buffer, plasma glucose was determined using a commercially available enzyme kit (Wako Chemical Company, Osaka, Japan), which made use of the *O*-toluidine method.

Preparation of tissues

Male Wistar rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), then killed by decapitation. The stomach was excised, and fundus strip preparations of approximately 20 mm length and 2 mm width were made essentially by our published methods (Kamata *et al.*, 1988, 1993). Each strip was cut out along the line of the longitudinal muscle, and the mucous layer was completely removed to avoid reflex activity. The fundus strips were suspended in a 10 ml organ bath containing modified Krebs Ringer solution at 37°C, aerated with 95% O₂ and 5% CO₂. The tension loaded onto each strip was 1.0 g. Contractions were recorded isotonically using an isotonic transducer connected to an ink-writing recorder (Nihon Kohden, Tokyo, Japan). The composition of the modified Krebs Ringer solution (mM) was as follows: NaCl 120; KCl 4.7; CaCl₂ 2.0; MgCl₂ 1.2; NaHCO₃ 25; KH₂PO₄ 1.2; and glucose 14. The fundus strips were left in the above conditions for 30 min at 37°C. When ET-1 was to be applied to the strip, the tissue was preincubated with 10⁻⁶ M atropine and 10⁻⁶ M guanethidine (to avoid any cholinergic or adrenergic effects being induced throughout the experiment).

Receptor-binding assay

For each group (STZ-diabetic or age-matched control rats) a single membrane preparation was obtained from a total of 70 stomach fundus strips. The strips were homogenized in 10 vol (w/v) of ice-cold buffer A (50 mM Tris-HCl, pH 7.4, containing 120 mM NaCl and 5 mM KCl) using a Polytron (Kinematica) set at position 7 for 10 sec, three times. The homogenate was incubated on ice in the presence of 300 mM KCl and 10 mM EDTA for 30 min with intermittent mixing, then centrifuged at 50,000 g for 15 min at 4°C. The pellet was resuspended in 20 vol of ice-cold buffer B (50 mM Tris-HCl, pH 7.4) using a Polytron at position 7 for 10 sec, then centrifuged again as above. The washing procedure was repeated twice more, and the final pellet was resuspended in 60 vol of buffer B. For the binding experiments, the reaction was initiated by adding 400 μ l of the freshly prepared membrane preparation to 100 μ l of 50 mM Tris-HCl (pH 7.4) containing 0.02% bovine serum albumin, 4 μ g/ml chymostatin, 4 μ g/ml leupeptin, 40 μ g/ml bacitracin, and commercially obtained ¹²⁵I-labeled ET-1 samples. This was left at 20°C for 30 min, and the reaction was then terminated by adding 5 ml of ice-cold buffer A to each tube. The contents of each tube were filtered immediately through Whatman GF/C glass fiber filters (pre-treated with 0.1% polyethyleneimine in water for more than 3 h to reduce the non-specific adsorption of ¹²⁵I-labeled ET-1 to the filters). Each filter was then quickly washed once with 20 ml of ice-cold buffer A, and the radioactivity was determined using a γ counter. Non-specific binding was determined using 1 μ M ET-1. The protein content of the

Table 1 Oligonucleotide primer sequences for ET-1 receptors and GAPDH, together with PCR protocols

Target gene	PCR Primer Sequences	PCR Protocols	Product Size
ET _A receptor	5'-ATCGCTGACAATGCTGAGAG-3'	94°C/60 s	226 bp
	5'-CCACGATGAAAATGGTACAG-3'	54°C/60 s	
ET _B receptor	5'-AGCTGGTGCCCTTCATACA-3' 5'-TCTTGGCCACTTCTCGTCT-3'	72°C/60 s	
		26 cycles	
		94°C/60 s	447 bp
		54°C/60 s	
GAPDH	5'-TCCCTCAAGATTGTCAGCAA-3' 5'-AGATCCACAACGGATACATT-3'	72°C/60 s	
		26 cycles	
		94°C/60 s	308 bp
		54°C/60 s	
		72°C/60 s	
		21 cycles	

membrane suspension was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Measurement of the expressions of the mRNAs for ET receptors (using RT-PCR)

RNA was isolated by the guanidinium method (Chomczynski and Sacchi, 1987). Briefly, rat stomach fundus strips were carefully isolated (as described above). They were then homogenized in RNA buffer, and the RNA was quantified by ultraviolet-absorbance spectrophotometry. For the RT-PCR analysis, first-strand cDNA was synthesized from total RNA using Oligo (dT) 20 and a ThermoScript™ RT-PCR System (Invitrogen Corp., Carlsbad, CA, U.S.A.). All primers were synthesized by Sigma-Genosys (St. Louis, MO, U.S.A.). Individual sequences, PCR conditions, and product sizes are shown in Table 1. To ensure that we were within the exponential phase of the semi-quantitative PCR reaction (Wang *et al.*, 1989), the appropriate number of cycles was newly established for each set of samples. Control PCR reactions, in which the RT was omitted, were run simultaneously to check for successful removal of the genomic DNA. The PCR products so obtained were analyzed on ethidium-bromide-stained agarose (1.5%) gels, and quantified by scanning densitometry, with the amount of each product being normalized with respect to the amount of GAPDH product.

Drugs

The following drugs were used in the present study: bacitracin, chymostatin, bovine serum albumin (Fraction V), leupeptin, and streptozotocin (Sigma St. Louis, MO, U.S.A); endothelin-1 (Peptide Institute Inc., Osaka, Japan); ¹²⁵I-labeled ET-1 (Amersham International plc., Buckinghamshire, U.K.); and polyethyleneimine (Tokyo Kasei Pharmaceuticals, Tokyo, Japan). All concentrations are expressed as the final molar concentration of the base in the organ bath. ¹²⁵I-labeled ET-1 was stored under liquid N₂ until needed for experimentation, when it was aliquoted out and stored at -20°C.

Statistical analysis

The contractile force developed by fundus strips from control and diabetic rats is expressed in g tension/mg tissue, the data being given as the mean $\pm$ S.E.M. When appropriate, statistical differences were assessed using Dunnett's test for multiple comparisons after a one-way analysis of variance, a probability level of $P < 0.05$ being regarded as significant. Statistical comparisons between concentration-response curves were assessed using a two-way ANOVA with a Bonferroni correction performed post-hoc to correct for multiple comparisons; again, $P < 0.05$ was considered significant.

Results

Change in body weight and plasma glucose

In accord with our previous reports (Matsumoto *et al.*, 2003, 2004b), at the time of the experiment (a) all STZ-treated rats exhibited hyperglycemia, their blood glucose concentrations (567.0 ± 13.7 mg/dl, $n=15$) being significantly higher than those of the age-matched nondiabetic control rats [118.8 ± 2.8 mg/dl, $n=15$ ($P < 0.001$)] and (b) the body weights of the diabetic rats (252.7 ± 8.0 g, $n=15$) were significantly lower than those of the age-matched control rats [474.4 ± 6.1 g, $n=15$ ($P < 0.001$)].

Contractile responses to ET-1

The contractile responses (g tension/mg tissue) induced in stomach fundus strips from control and diabetic rats by ET-1 and the calcium-channel activator, BayK 8644, are shown in Fig. 1. Although there was a significant increase in the maximal contractile response to ET-1 in STZ-diabetic rats (versus the control rats) (Fig. 1A), the Bay K 8644-induced contraction was almost the same between the two groups (Fig. 1B). A single administration of ET-1 (10^{-7} M) induced a longer-lasting contraction in STZ-diabetic rats than in the age-matched controls (Fig. 2). The fundus strips were exposed to 10^{-7} M ET-1 for 20 min. After washout, at an interval of 20–30 min, this procedure was repeated three times in the same preparations. As shown in Fig. 3A, repeated application of ET-1 (10^{-7} M) led to a marked desensitization in the age-matched control rats, but the desensitization was much less pronounced in STZ-diabetic rats. Quantitative summary data are shown in Fig. 3B.

Receptor binding assay

The specific binding of 125 I-labeled ET-1 was saturable with increasing concentrations of the ligand, and a Scatchard analysis of the saturation curve indicated a single binding site. After 12 weeks of diabetes (*i.e.*, 12 weeks after the STZ injection): (a) the receptor density ($B_{\max}$) was greater than in the age-matched controls, when expressed as the number of receptors per mg protein (Table 2), but (b) the K_d values were similar between the two groups of rats (Table 2).

Expressions of mRNAs for ET receptors

Using RT-PCR analysis on the total RNA isolated from the stomach fundus, we found the following. The expression of GAPDH mRNA showed no difference between the diabetic group

Fig. 1. Concentration-response curves for ET-1- (A) and Bay K 8644- (B) induced contractions in stomach fundus strips from age-matched controls and STZ-induced diabetic rats. Each data-point represents the mean $\pm$ S.E.M. of 10 (A) or 6 (B) experiments, the SE mean being included only when it exceeds the dimension of the symbol used. ** $P < 0.01$, *** $P < 0.001$, *vs.* control.

Fig. 2. Time-course of changes in contractile response to ET-1 (10^{-7} M) in stomach fundus strips from age-matched control ($n=6$) and diabetic ($n=6$) rats. Each data-point represents the mean $\pm$ S.E.M. of 6 experiments. * $P < 0.05$, *** $P < 0.001$, *vs.* control.

Fig. 3. Changes in ET-1 response upon repeated application in stomach fundus strips from age-matched control and diabetic rats. The fundus strips were exposed to 10^{-7} M ET-1 for 20 min. After washout, at an interval of 20–30 min, this procedure was repeated three times in the same preparations. (A) Representative traces showing the ET-1 (10^{-7} M)-induced contraction in strips from age-matched control (upper) and diabetic (lower) rats. (B) Peak contractile response to repeatedly applied (first, second, third) ET-1 (10^{-7} M) in strips from age-matched control (n=6) and diabetic (n=6) rats. Each column represents the mean $\pm$ S.E.M. of 6 experiments. ** P <0.01 vs. "First" control, ## P <0.01, vs. "First" diabetic.

and the age-matched control group, but the mRNA expression for the ET_B receptor, but not for the ET_A receptor, was significantly greater in the diabetic rats than in the age-matched controls (Fig. 4).

Discussion

The main conclusion to be drawn from the present study is that the contractile response of the stomach fundus to ET-1 was increased in STZ-induced diabetic rats, a change that may be due to an increased density of the ET_B receptors for ET-1.

Table 2 125 I-Endothelin-1 binding assay in stomach fundus strips from controls and streptozotocin-induced diabetic rats

Experimental groups	Bmax (fmol/mg protein)	Kd (nM)
Control	721.4 $\pm$ 60.7	200.1 $\pm$ 17.8
Diabetic	1818.7 $\pm$ 170.8**	164.7 $\pm$ 24.2

Bmax and Kd values were calculated by Scatchard plot analysis of binding data. Values are expressed as mean $\pm$ S.E.M. for 4 separate experiments. ** $P < 0.01$ vs. control.

Fig. 4. RT-PCR assay of expressions of mRNAs for ET receptors in strips of stomach fundus from age-matched controls and STZ-diabetic rats. (A) Expressions of mRNAs for ET_A and ET_B receptors by RT-PCR. (B) Quantitative analysis by scanning densitometry. Values are each the mean $\pm$ S.E.M. of six determinations (target gene/GAPDH). The RT-PCR assay was performed as described in Materials and Methods. Each total RNA preparation (3.0 μ g) was reverse-transcribed, and the cDNA product was PCR-amplified using various primers, several cycles being employed. A portion of the PCR reaction product was electrophoresed on a 1.5% agarose gel containing ethidium bromide.

ET-1 is involved in the contraction of a variety of tissues, including gastric smooth muscle and vascular smooth muscle (Kitsukawa *et al.*, 1994; Allcock *et al.*, 1995; Goto *et al.*, 1996; Miyauchi and Masaki, 1999) and it is thought to act through two receptors, ET_A and ET_B receptors (Goto *et al.*, 1996; Miyauchi and Masaki, 1999). Both of these receptor-subtypes may be coupled to phospholipase C (PLC) via a GTP-binding protein. Activation of PLC induces phosphatidylinositol hydrolysis, rapid formation of 1, 4, 5-inositol triphosphate (IP₃), and accumulation of 1,2-diacylglycerol (DG). IP₃ stimulates the release of Ca²⁺ from the intracellular stores, including the endoplasmic reticulum. For example, application of ET-1 to arterial smooth muscle cells causes a rapid, transient increase in the intracellular Ca²⁺ concentration ([Ca²⁺]_i) followed by a sustained increase in [Ca²⁺]_i (Simonson and Dunn, 1990). The initial transient [Ca²⁺]_i response is not dependent on the presence of extracellular Ca²⁺, and is the result of IP₃-induced mobilization of Ca²⁺ from the intracellular stores. In contrast, the sustained increase in [Ca²⁺]_i appears to be due to an influx of extracellular Ca²⁺ through either dihydropyridine-sensitive voltage-dependent L-type Ca²⁺ channels (LTCC) (Goto *et al.*, 1989) or receptor-operated (nonselective) cation channels that are insensitive to dihydropyridine (Iwamuro *et al.*, 1998). In the present study, ET-1 induced a dose-dependent contraction in stomach fundus strips and this contractile response was much greater in the STZ-diabetic group than in the control group (Fig. 1A). In contrast, we found no diabetes-related change (a) in the contractile

response of the fundus to the LTCC activator, Bay K8644 (Fig. 1B) or (b) in the Ca^{2+} -induced contractile response in strips preincubated with an extracellular bathing solution containing zero calcium (data not shown). This suggests that the increase in the ET-1-induced contractile response in the diabetic group was not due to any alteration in Ca^{2+} influx via Ca^{2+} -channels.

Prolonged and repeated exposure to agonist reduces the responsiveness of G-protein-coupled receptors (Ferguson, 2001). Substantial experimental evidence has divided this reduced function into three separate, but related, receptor events: 1) desensitization, 2) internalization, and 3) downregulation (Bunemann and Hosey, 1999; Ferguson, 2001; Ueda *et al.*, 2001). Like many G protein-coupled receptor signaling systems (Ferguson, 2001), the receptor/Gq/PLC system(s) activated by the ET_A and ET_B receptors display a diminishing responsiveness to prolonged stimulation (Leite *et al.*, 1994; Khac *et al.*, 1994). In the present study, the single administration of ET-1 showed long-lasting contraction in diabetic as compared to the controls (Fig. 2). Moreover, when stomach fundus strips from control rats were repeatedly exposed to ET-1, marked desensitization was observed (Fig. 3). On the other hand, the stomach fundus strips from diabetic rats showed resistance to desensitization upon repeated ET-1 exposure (Fig. 3). This intergroup difference in the degree of desensitization may be particularly important in the regulation of ET receptor-mediated signaling, since the binding of endothelins to their receptors is essentially irreversible under physiological conditions (Sokolovsky, 1995). Moreover, the rate and extent of desensitization to ET-1 signaling reflects the degree of receptor occupancy (Leite *et al.*, 1994). The fact that two different classes of ET receptors (ET_A and ET_B) are present on gastric muscle cells is attested by several findings (Kitsukawa *et al.*, 1994; Allcock *et al.*, 1995; Mizuguchi *et al.*, 1997). In accord with these previous findings, in the present study ^{125}I -labeled ET-1 binding was found to be saturable and specific (Table 2). We showed here that the affinity of ^{125}I -labeled ET-1 binding to stomach fundus smooth muscle was similar between the control and diabetic groups. However, the receptor density (B_{max}) was significantly increased in the diabetic stomach fundus. Furthermore, the mRNA expression level for the ET_B receptor was greatly increased in the diabetic stomach fundus (Fig. 4). These results suggest that the enhanced ET-1 contraction and resistance to ET-1 desensitization seen in the STZ-diabetic stomach fundus are attributable to an upregulation of ET_B receptors. This speculation is in line with the previous finding that ET_B receptors mediate contraction in rat stomach strips (Allcock *et al.*, 1995), and that upregulated ET_B receptors were attributed to increased contraction in several arteries (Adner *et al.*, 1996; Henriksson *et al.*, 2003, 2004; Uddman *et al.*, 2003). In gastrointestinal smooth muscle, ET-1 induced an initial transient relaxation followed by a sustained contraction (Allcock *et al.*, 1995; Irie *et al.*, 1995; Chakder and Rattan, 1999). Imaeda *et al.* (Imaeda *et al.*, 2002) reported that ET-1 hyperpolarizes the membrane by activating apamin-sensitive K^+ channels, mainly as a result of receptor-mediated Ca^{2+} entry and partly by Ca^{2+} release from intracellular stores in guinea-pig isolated lower oesophageal sphincter circular smooth muscle. The hyperpolarization triggers the initial transient relaxation, which acts to oppose the sustained contraction (Imaeda *et al.*, 2002). Although in this study we focus ET-1-induced contractile responses in rat stomach fundus compared with diabetic and control rats, further investigation is required on these points.

The pathological significance of a diabetes-related enhancement of ET-1-induced contractions in the rat stomach fundus (as observed here *in vitro*) is unclear. Several reports exist to show that ET-1 affects gastrointestinal function. For instance selective infusion of ET-1 into the gastric artery induced gastric mucosal erosion in the rat (Whittle and Esplugues, 1988), and intravenous infusion of ET-1 exacerbated the damage induced by intragastric instillation of ethanol or acid (Wallace *et al.*, 1989a). Moreover, when injected submucosally into the rat gastric mucosa, ET-1 causes a potent, long-lasting vasoconstriction of the regional microvasculature and induces gastric ulceration (Lazaratos *et al.*, 1993). Taken together, the present evidence of an enhancement of ET-1-induced contractions and of an augmentation of ET-1 signaling in rat stomach fundus strips in STZ-diabetes may be important since such changes may underlie the disruption of gastric transit and the modulation of gastrointestinal function seen in the diabetic state.

In summary, this study has demonstrated an enhanced contraction to ET-1 in strips of gastric fundus from STZ-induced diabetic rats. The results indicate that such enhanced, prolonged contractile responses to ET-1, and their resistance to desensitization, may be due to an upregulation of ET receptors (probably the ET_B subtype) in the diabetic gastric fundus.

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