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Gene expression, localization, and pharmacological characterization of endothelin receptors in diabetic rat bladder dome

Motoaki Saito, Yoshihiro Wada, Kazuyoshi Ikeda, ZeJing Wang, Shannon D. Smith, Harris E. Foster Jr., Kazuhiko Nishi, Robert M. Weiss, Jamshid Latifpour *

Section of Urology, Yale University School of Medicine, P.O. Box 208041, 333 Cedar Street, New Haven, CT 06520, USA

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Abstract

As there are significant amounts of functional endothelin receptors in the mammalian urinary tract, we examined the effect of experimental diabetes on the expression of endothelin receptors and their mRNAs in the rat bladder dome. The density of endothelin receptors in the rat bladder dome was higher (8 and 16 weeks following the onset of diabetes) than in age-matched controls. Insulin treatment, started 8 weeks after the induction of diabetes, partially reversed the endothelin receptor alterations. The pharmacological profile of the endothelin receptors in the bladder dome was similar in all groups and was consistent with the predominance of the endothelin ET_A receptor subtype (ET_A:ET_B = approximately 4:1). Autoradiographic studies demonstrated that the endothelin receptors were located in all tissue components of the bladder, including epithelial and muscular layers. Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) data indicated that diabetes increased the expression level of gene transcripts for both endothelin receptor subtypes and that insulin treatment reversed the mRNA upregulation.

Keywords: Endothelin receptor; Diabetes; Urinary bladder

1. Introduction

Endothelin-1, which was originally isolated from the culture media of porcine aortic endothelial cells, is the most potent vasoconstrictive peptide identified to date (Yanagisawa et al., 1988). There are three endothelin isoforms, i.e., endothelin-1, endothelin-2 and endothelin-3 (Inoue et al., 1989). Endothelins and sarafotoxins elicit a wide spectrum of vascular and non-vascular actions in a variety of tissues. These actions are mediated by interaction of the endothelins with specific receptors (Rubanyi and Polokoff, 1994; Goto et al., 1996; Douglas and Ohlstein, 1997; Webb and Meek, 1997). Cloning and sequencing studies of cDNAs for these receptors have revealed the existence of three distinct subtypes, i.e., endothelin ET_A, endothelin ET_B and endothelin ET_C receptor subtypes (Arai et al., 1990; Sakurai et al., 1990; Karne et al., 1993; Goto et al., 1996). The endothelin ET_A receptor subtype has a high affinity for endothelin-1 and endothelin-2 and a low affinity for endothelin-3, while the endothelin ET_B receptor subtype has similar affinities for endothelin-1, endothelin-2 and endothelin-3 (Arai et al., 1990; Sakurai et al., 1990). The endothelin ET_C receptor subtype has a higher affinity for endothelin-3 than for endothelin-1 and endothelin-2 (Emori et al., 1990; Karne et al., 1993).

The mammalian urinary tract contains significant amounts of endothelin receptors (Bolger et al., 1990; Traish et al., 1992; Latifpour et al., 1995). Endothelins induce prolonged contractile responses in isolated rabbit (Garcia-Pascual et al., 1990; Traish et al., 1992), pig (Persson et al., 1992), rat (Donoso et al., 1994), and human (Maggi et al., 1989) lower urinary tract smooth muscle. Alterations in endothelin receptor-effector systems have been described in diabetes mellitus. In streptozotosin-induced diabetic rats there is an alteration in the modulatory effects of glucose and insulin on endothelin-1 release from endothelial cells (Hu et al., 1993) and from mesenteric arteries (Takeda et al., 1991), elevated blood and urine levels of endothelin-1 (Morabito et al., 1994), a down regulation of cardiac endothelin receptors (Nayler et al., 1989), and an increase

* Corresponding author. Tel.: +1-203-785-5516; fax: +1-203-737-5912.

E-mail address: jamshid.latifpour@yale.edu (J. Latifpour).

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in the expression of endothelin receptors in the ureter, vas deferens and prostate of experimentally induced diabetic rats (Saito et al., 1996a,b; Nakamura et al., 1997). In the present study, we examined the effects of streptozotosin-induced diabetes and insulin treatment on endothelin receptors in the rat bladder dome utilizing radioligand receptor) binding and light microscopic autoradiography techniques. We also examined the diabetes-induced changes in endothelin ET_A and endothelin ET_B receptors at their mRNA levels in the rat bladders by reverse transcription polymerase chain reaction (RT-PCR).

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (55–56 days old, 230–285 g) were divided into five groups and maintained for 8 or 16 weeks, i.e., 8 weeks diabetic, 8 weeks age-matched control, 16 weeks diabetic, 16 weeks diabetic-insulin-treated, and 16 weeks age-matched control.

Diabetes was induced in the 8 and 16 weeks diabetic and 16 weeks diabetic-insulin-treated groups with a single injection of 65 mg/kg streptozotocin dissolved in 0.1 M citrate buffer (pH 4.5) via the lateral tail vein. The age-matched control groups were injected with the same volume of the citrate buffer. Two days after the injection of streptozotocin, the induction of diabetes was confirmed by measuring urinary glucose. After 8 weeks from induction of diabetes, the 16 weeks diabetic-insulin-treated group started to receive 5–8 units of protamine zinc insulin subcutaneously daily for an 8-week period, while the 16 weeks diabetic group was maintained without insulin treatment. All groups were kept under identical conditions and had free access to food and drinking water. After 8 or 16 weeks from the initial injection of streptozotocin or vehicle, rats were killed by decapitation. Blood samples were collected for measurement of serum glucose levels and insulin levels. The bladders were rapidly removed and cleaned of fat and connective tissue.

Glucose concentrations were measured by the hexokinase method (Glucose H. K., Sigma St. Louis, MO). Serum insulin levels were determined with radioimmunoassay kits (Diagnostic systems Laboratories, Webster, TX).

2.2. Tissue preparation

For biochemical studies, rat bladder domes were rapidly dissected from the bladder base at the level of the ureteral orifices, frozen in liquid nitrogen and stored at −80°C until used. For autoradiographic studies, whole rat bladders were embedded in frozen tissue media (Histo Prep, Fisher Chemical, Orangeberg, NY), frozen on dry ice and stored at −80°C.

2.3. Binding experiments on membrane particulates

Frozen bladder domes were thawed, cut into small pieces on ice and then homogenized in 40–50 volumes of ice-cold 20 mM HEPES buffer (pH 7.4) containing 100 mM NaCl, 3 mM EDTA, 1 mM EGTA and the following protease inhibitors; 0.1 mM phenylmethylsulfonyl fluoride and 10 μg/ml each of aprotinine, leupeptine, pepstatin A and soy bean trypsin inhibitor with a Brinkman Polytron at a speed of 7 for a total of 60 s in four bursts. Each burst was separated by a 30 s interval for cooling of the tissues. The homogenate then was centrifuged at 49,000 × g for 15 min at 4°C. The supernatant was discarded and the resultant pellet was rehomogenized, filtered through a metal sieve with a pore size of 250 μm and recentrifuged under the same conditions as above. The final pellet was suspended in 20 volumes of the same buffer. Protein content was determined by the method of Lowry using bovine serum albumin as standard (Lowry et al., 1951). Binding experiments were performed as previously reported with minor modification (Latifpour et al., 1995). In saturation experiments, the membrane suspensions were diluted in the incubation buffer (50 mM Tris–HCl, pH 7.4 containing 154 mM NaCl, 25 mM $MnCl_2$, 1 mM EDTA, 1 mM *N*-acetyl-DL-methionine, 0.25% bovine serum albumin and 0.14% bacitracin). Aliquots of membrane preparations of the rat bladder dome were incubated, in triplicates, for 120 min with increasing concentrations of [^{125}I]endothelin-1 (4–120 pM) in a total volume of 0.25 ml at 23°C. At the end of the incubation period, the reaction mixtures were filtered rapidly under a vacuum through Whatman) GF/B glass fiber filters, which previously had been treated with a 1% solution of bovine serum albumin to reduce non-specific binding to the filter papers, using a Brandel Cell Harvester (Model M-24R, Brandel Instruments, Gaithersburg, MD). Each filter disc was washed intensively with 80 ml of ice-cold 50 mM Tris–HCl buffer (pH 8.0). The radioactivity on glass fiber discs was counted with a gamma counter (Packard Multi-Prias Analyzer) at an efficiency of 70–75%. Non-specific binding was determined in the presence of 100 nM unlabelled endothelin-1. Specific bindings were calculated by subtracting non-specific bindings from total bindings of the radioligands to the glass fibers.

In inhibition binding studies, membrane suspensions of the bladder dome were prepared in the same manner as with saturation experiments. Aliquots of membrane particulates were incubated with a fixed concentration of [^{125}I]endothelin-1 (approximately 20 pM) in the presence or absence of increasing concentrations of following unlabelled peptides: endothelin-1 (non-selective), endothelin-3 (endothelin ET_B receptor selective), BQ 123, cyclo(D-α-aspartyl-L-prolyl-D-valyl-L-leucyl-D-tryptophyl) (endothelin ET_A selective receptor), BQ 610, *N*-[1-formyl-*N*[*N*- (hexahydro-1*H*-azepin-1-yl)cabonyl]-L-leucyl]-D-tryptophyl]-D-tryptophan (endothelin ET_A receptor selec-

tive), IRL 1620, endothelin-1(8-21), *N*-Suc-[Glu^{9},Ala^{11,15}], (endothelin ET_B receptor selective) and sarafotoxin S6c (endothelin ET_B receptor selective) (Williams et al., 1991; Ihara et al., 1992; Takai et al., 1992; Ishikawa et al., 1993) in a total volume of 0.25 ml for 120 min at 23°C. The inhibition assays were carried out in duplicates. The remainder of the procedure was the same as that of the saturation studies.

2.4. Light microscopy autoradiography

Serial sections of the rat bladder of the 16 weeks control, 16 weeks diabetic and 16 weeks diabetic-insulin-treated groups, 20 μm thick, were cut with a microtome cryostat (2800 Frigocut N, Leica, Malver, PA) at −20°C and mounted on aminoalkysilane-coated microscope slides. Slides were stored in a plastic slide box at −80°C until assayed. Autoradiographic methodology described by Kuhar and Unnerstall (1990) was modified to investigate the localization of endothelin receptors in the rat bladder. Autoradiographic localization of [^{125}I]endothelin-1 binding was performed on adjacent sections obtained from a single bladder from each group. To reduce the effect of endogenous peptide levels, and the non-specific binding of [^{125}I]endothelin-1 to the rat bladder, slide sections were pre-incubated with an autoradiography buffer (20 mM HEPES containing 140 mM NaCl, 4 mM KCl, 1 mM K_2HPO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM D-glucose, 0.5% bovine serum albumin and 0.01% bacitracin) for 30 min at 23°C. After the preincubation period, the rat bladder sections were incubated with the same 20 mM HEPES buffer plus increasing concentrations of [^{125}I]endothelin-1 (20–80 pM) in a total volume of 0.50 ml for 120 min at 23°C. To determine non-specific binding, adjacent sections were incubated with the same concentration(s) of [^{125}I]endothelin-1 in the presence of 100 nM endothelin-1. The sections, then were washed twice in ice-cold Tris-HCl buffer (pH 8.0) for 20 min, quickly dipped in distilled water, and dried in a stream of cold air. The tissue sections were placed in apposition with a high resolution tritium sensitive film (Hyperfilm-^{3}H, Amersham, Arlington, IL) in standard X-ray cassettes for 2.5 days at 4°C to generate autoradiograms. Following the exposure, the film was developed in D19 (Kodak, Rochester, NY), at 23°C for 5 min, fixed for 10 min in the Kodak normal fixer, and washed in tap water for 30 min. Adjacent bladder sections, then were stained with hematoxylin (10 min) and eosin (2 min), dehydrated and coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ).

The localization of endothelin ET_A and ET_B receptor subtypes in the rat bladder was accomplished by incubating tissue sections with a fixed concentration of [^{125}I]endothelin-1 (20 pM) and appropriate concentrations of subtype selective compounds, i.e., 300 nM BQ 123, and 30 nM Sarafotoxin S6c. These concentrations of BQ 123 and Sarafotoxin S6c, that are approximately 100 times greater than that of their high affinity constants, K_{iH}, (Latifpour et al., 1995) occupy virtually all of the endothelin ET_A and ET_B receptor subtypes, respectively, in the rat bladder sections. Non-specific binding was determined in the presence of [^{125}I]endothelin-1 and 100 nM unlabelled endothelin-1.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

2.5.1. RNA isolation

Total RNA was extracted from frozen bladder dome using TRIZOL reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's instruction. One hundred mg of tissue was homogenized in 1 ml of TRIZOL reagent with a Brinkman Polytron (Brinkman Instruments, Westbury, NY) for a total of 60 s at room temperature. The homogenates were transferred to a microcentrifuge tube and 0.2 ml of chloroform was added. The samples were vigorously shaken, incubated for 2 min, centrifuged at 12,000 × g for 15 min at 4°C, and the aqueous phase containing RNA was transferred to a fresh tube. The RNA, from aqueous phase, was precipitated by mixing with 0.5 ml of isopropyl alcohol. Samples were incubated for 10 min, centrifuged at 12,000 × g, and the supernatant was discarded. The RNA pellet was washed with 75% ethanol, air-dried and redissolved in diethylpyrocarbonate-treated water. The quantity and purity of the RNA was determined by optical density at 260 nm to that at 280 nm. RNA samples were aliquoted and stored at −80°C as ethanol precipitates for later use.

2.5.2. Reverse transcription (RT)

RT-PCR was performed in 0.2 ml thin-walled tubes in a Perkin-Elmer DNA thermocycler (Branchburg, NJ). The cDNA template for PCR was generated from total RNA using reverse transcriptase according to the manufacturer's recommendations (Life Technologies, Gaithersburg, MD). The RT reaction mixture contained 5 μg of total RNA, 500 ng of oligo (dT), 1 × first strand buffer, 10 mM dithiothreitol, 0.5 mM each deoxynucleotide 5′-triphosphate (dNTP), and 200 units of Superscript II in a total volume of 20 μl. The mixture was incubated at 42°C for 50 min, heated to 70°C for 15 min, to denature the reverse transcriptase, and then cooled to 4°C.

2.5.3. Polymerase chain reaction (PCR)

Oligonucleotide primer pairs for RT-PCR were selected from the previous reports based on the published rat cDNA sequences (Table 1). PCR in exponential phase was performed to allow comparative analysis of numerous cDNA samples. The PCR mixture contained 1 μl of cDNA, 1 × PCR buffer, 1.5 mM $MgCl_2$, 200 μM each dNTP, 0.4 μM primer pairs, and 5 units of *Taq* DNA polymerase in a

Table 1
Design of oligonucleotide for RT-PCR

Gene	PCR Primer	Nucleotides	Product	References
Endothelin ET_A receptor				
sense	GTGTTTAAGCTGTT GGCGGG	455–474	780 bp	Terada et al., 1992 Lin et al., 1991
antisense	CGAGGTCATGAGGC TTTTGG	1215–1234		
Endothelin ET_B receptor				
sense	AGCTGGTGCCCTTCATACA GAAGGC	724–748	919 bp	Terada et al., 1992 Sakurai et al., 1990
antisense	TGCACACCTTTCCGC AAGCACG	1621–1642		
β-Actin				
sense	ACCTTCAACACCCCAGCC ATGTACG	2170–2194	698 bp	Nudel et al., 1983 Shigematsu et al., 1996
antisense	CTGATCCACATCTGCTGG AAGGTGG	3055–3079		

total volume of 100 μl for each reaction (Life Technologies, Gaithersburg, MD). The following PCR profile was used: cDNA was denatured initially for 3 min at 94°C and then cycled starting with denaturing at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1.5 min. The last cycle included a prolonged extension at 72°C for 10 min. For these experiments, the optimal PCR cycling length was used for each of the primer pairs, such that the PCR product-RNA relationship was kept in the log-linear phase. The number of cycles chosen were 25 for β-actin and 35 for endothelin ET_A and endothelin ET_B receptors. The linear range for RNA was 0.1 to 0.5 μg. All RT-PCR experiments were routinely controlled by conducting PCR omitting the reverse transcription.

PCR products were separated by electrophoresis on 1.2% agarose gels together with 1.0 μg of 100 bp DNA ladder (Life Technologies, Gaithersburg, MD) as size markers, and stained with ethidium bromide. Stained bands for endothelin ET_A, endothelin ET_B and β-actin were digitized and analyzed with a digital camera. The results were expressed as the relative endothelin receptor mRNA levels normalized with that of β-actin expression. The RT-PCR products were extracted and purified using QI-Aquick Gel Extraction Kit (Qiagen, Valencia, CA) and their nucleotide sequences were determined by the fluorescent dideoxynucleotide chain termination method with RT-PCR primers and with Taq FS DNA polymerase using an automated sequencer (W.M. Keck Biotechnology Resource Lab., Yale University, New Haven, CT).

2.6. Analysis of data

Saturation data were analyzed according to Rosenthal (1967) using linear regression of bound/free vs. bound in order to calculate the maximum number of binding sites, B_{max}, and equilibrium dissociation constant, K_D. Inhibition data were analyzed by an iterative non-linear least square curve-fitting procedure on the basis of a one or a two bind site model (Munson and Rodbard, 1980). The equation used for the two binding site model is as follows:

$$Y = A + (B - A)/100\{C/[1 + (10^X/10^D)] + (100 - C)/[1 + (10^X/10^E)]\}$$

where A and B are the binding in the presence of zero and maximum concentrations of a competing agent, respectively; C is the percentage of high affinity receptor binding sites; and D and E are the log EC_{50} for the high and low affinity binding sites, respectively. The significance of the fit to the one-site or two-site model was determined by comparing the residual sum of the squares for the respective fits.

The autoradiograms and stained bladder sections were examined and analyzed using a computer assisted image processing and analysis programs (NIH Image, Bethesda, MD; Adobe Photoshop, San Jose, CA).

PCR products for endothelin ET_A and ET_B receptors and β-actin were digitized with a DC120 Zoom Digital Kodak Camera and the images were analyzed with 1D Image Analysis Software (Kodak, Rochester, NY).

Statistical analyses between groups were performed using analysis of variance and the multiple comparison Fisher's test. $P \leq 0.05$ was regarded as the level of significance.

2.7. Drugs and chemicals

[^{125}I]Endothelin-1 (2200 Ci/mM) was purchased from New England Nuclear (Boston, MA). Endothelin-1, endothelin-3, BQ 123, BQ 610, Sarafotoxin S6c and IRL 1620 were obtained from Peninsula Laboratory (Belmont, CA). phenylmethylsulfonyl fluoride, aprotinine, leupeptine, pepstatin A, *N*-acetyl-DL-methionine, soy bean trypsin

inhibitor, bovine serum albumin and bacitracin were obtained from Sigma (St. Louis, MO).

3. Results

3.1. Animals general features

The general features of experimental animals are shown in Table 2. The age-matched control groups (8 and 16 weeks) showed an increase in their body weights throughout the 16 week study period, while the final body weights of the 8 and 16 week diabetic groups were only 45% and 52% of those of the age-matched control groups, respectively. The 8 and 16 weeks diabetic rats had hyperglycemia, hypoinsulinemia, glucosuria (more than 1000 mg/ml), polyuria and polydipsia compared to the age-matched control rats (data not shown). The 8 and 16 weeks diabetic rats demonstrated a significant increase in the weights of their bladder domes compared with those of age-matched controls (Table 2). Although insulin treatment did not normalize the serum glucose levels of the diabetic rats, the insulin treatment did increase the body weight of the diabetic animals (75% of the 16 weeks control animals), decreased their water intake, and partially inhibited the diabetes-induced increase in the weight of the bladder dome (155% of that of the 16 weeks control bladder dome).

Table 2
General features of experimental animals
Diabetes was induced in diabetic and insulin-treated diabetic groups with a single i.v. injection of 65 mg/kg streptozotocin. Beginning 8 weeks after the induction of diabetes, the insulin-treated diabetic group received 5–8 units of protamine zinc insulin, subcutaneously daily for 8 weeks whereas the diabetic group was left untreated for the same period of time. Data are shown as mean ± S.E.M. of 12 to 15 determinations in each group.

	Control	Diabetic	Diabetic-Ins
8 Weeks			
Body wt., g			
Initial	283 ± 3	275 ± 2	–
Final	581 ± 24	263 ± 12[a]	–
Bladder dome, wt., mg	144 ± 7	290 ± 26[a]	–
S. glucose, mM	8.6 ± 0.3	30.7 ± 1.5[a]	–
S. insulin, μU/ml	19.6 ± 2.6	2.2 ± 0.5[a]	–
16 Weeks			
Body wt., g			
Initial	287 ± 5	283 ± 4	290 ± 5
Final	645 ± 24	336 ± 19[b]	482 ± 26[c]
Bladder dome, wt., mg	141 ± 5	277 ± 20[b]	218 ± 18[c]
S. glucose, mM	8.3 ± 0.3	29.0 ± 0.8[a]	26.3 ± 2.3[a]
S. insulin, μU/ml	20.7 ± 2.0	4.4 ± 0.9[b]	45.5 ± 18.0

[a] Significantly different from control group.
[b] Significantly different from control and diabetic-insulin-treated groups.
[c] Significantly different from control and diabetic groups. $P \leq 0.05$ is level of significance.

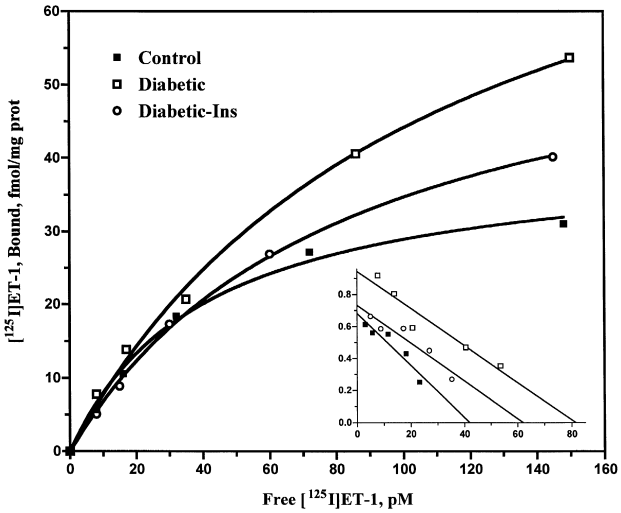

Fig. 1. Saturation of [^{125}I]endothelin-1 binding to the rat bladder dome. Aliquots of membrane particulates of the bladder dome were incubated with increasing concentrations of [^{125}I]endothelin-1 for 2 h at 23°C. Specific binding was defined in the presence and absence of 100 nM endothelin-1. Data are plotted according to Rosenthal (1967). Each curve represents the mean of a single experiment performed in triplicate.

3.2. Binding data for characterization of [^{125}I]endothelin-1 binding to membrane particulates

[^{125}I]endothelin-1 was bound to a single class of specific, saturable, high affinity binding sites, i.e., the endothelin receptors, in the bladder dome membrane particulates from each group (Fig. 1). The maximum number of the binding sites (B_{max} values) and the equilibrium dissociation constant (K_D values) of the rat bladder dome are summarized Table 3. The density of endothelin receptors

Table 3
Saturation of [^{125}I]endothelin-1 binding in rat bladder dome
Aliquots of membrane particulates were incubated with increasing concentrations of [^{125}I]endothelin-1 for 2 h at 23°C. Specific binding was defined in the presence and absence of 100 nM endothelin-1. Data are analyzed by the linear regression of bound vs. bound/free according to Rosenthal (1967). B_{max}, maximum number of binding sites; K_D, equilibrium dissociation constant. Data are shown as mean ± S.E.M. of 5 to 6 experiments performed in triplicate.

	Control	Diabetic	Diabetic-Ins
8 Weeks			
B_{max}			
fmol/mg protein	49 ± 4	90 ± 10[a]	–
fmol/g tissue	1200 ± 136	2413 ± 427[a]	–
K_D, pM	63 ± 5	50 ± 3	–
16 Weeks			
B_{max}			
fmol/mg protein	41 ± 3	75 ± 3[b]	55 ± 3[c]
fmol/g tissue	1400 ± 89	2710 ± 362[b]	2280 ± 295[c]
K_D, pM	61 ± 9	66 ± 8	62 ± 9

[a] Significantly different from control group.
[b] Significantly different from control and diabetic-insulin-treated groups.
[c] Significantly different from control and diabetic groups. $P \leq 0.05$ is level of significance.

in the bladder dome of the D_8 and D_{16} rats on the basis of fmol per milligram of protein or fmol per gram wet weight of tissue was significantly increased compared with that of the age-matched controls. The B_{max} values of endothelin receptors in the bladder dome of the insulin-treated diabetic group were significantly smaller than those of the 16 weeks diabetic bladder domes, but still significantly greater than those of the 16 weeks control bladder domes, whether normalized to protein or wet tissue concentration. No significant differences were found in the K_D values for [^{125}I]endothelin-1 binding sites to the bladder dome between any of the groups studied. These data indicate that streptozotosin-induced diabetes increases the densities of endothelin receptors in the rat bladder dome and that insulin) treatment partially reverses the diabetes-induced endothelin receptor upregulation of the rat bladder dome.

To investigate possible diabetes- and/or insulin treatment-induced alterations in the pharmacological properties of endothelin receptors, inhibition experiments were performed in which [^{125}I]endothelin-1 binding to the rat bladder dome of 16-weeks groups was inhibited competitively by selective and non-selective endothelin agonists or antagonists: endothelin-1 (non-selective), endothelin-3 (endothelin ET_B receptor selective), BQ 123 (endothelin ET_A receptor selective), BQ 610 (endothelin ET_A receptor selective), Sarafotoxin S6c (endothelin ET_B receptor selective) and IRL 1620 (endothelin ET_B receptor selective) (Figs. 2–4, Table 4). Endothelin-1 produced monophasic inhibition curves, which were better fitted to a one-site than to a two-site mode, in the 16 weeks control, diabetic and insulin-treated diabetic groups, suggesting the presence of a single population of high affinity endothelin-1 binding sites in these tissues. In contrast to the findings with endothelin-1, the selective ligands, BQ 123, BQ 610, Sarafotoxin S6c and IRL 1620, and endothelin-3 generated biphasic inhibition curves with Hill coefficients considerably less than unity which were fitted to a two-site model significantly better than to a one-site model suggesting the presence of multiple endothelin receptor subtypes in the rat bladder dome. The K_i values of high and low affinity [^{125}I]endothelin-1 binding sites for endothelin-3, BQ-123, BQ 610, Sarafotoxin S6c and IRL 1620 and the proportions of high and low affinity binding sites for these compounds are shown in Table 4. Endothelin-3, Sarafotoxin S6c and IRL 1620, selective agonists for endothelin ET_B receptors, inhibited 14% to 35% of high affinity and 65% to 86% of low affinity [^{125}I]endothelin-1 binding sites in the bladder dome. In contrast to these findings, BQ 123 and BQ 610, selective endothelin ET_A receptor antagonists,

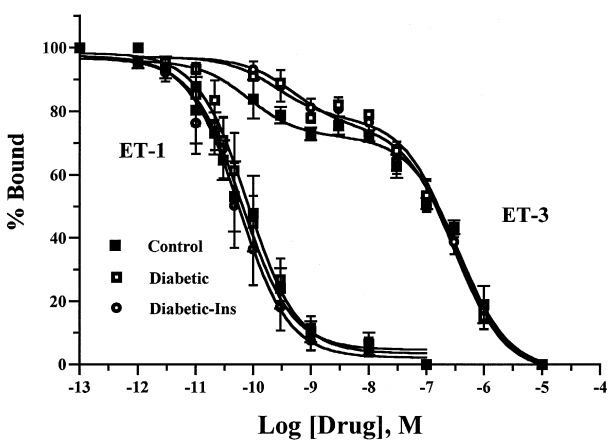

Fig. 2. Inhibition of [^{125}I]endothelin-1 binding to the rat bladder dome by endothelin-1 and endothelin-3. Aliquots of membrane particulates were incubated with increasing concentrations of unlabelled compound and a fixed concentration of [^{125}I]endothelin-1 for 2 h at 23°C. Each curve represents the mean of 3–4 separate experiments performed in duplicate. The curves are the computer fits of data points according to a one binding site model (endothelin-1) and a two-binding site model (endothelin-3).

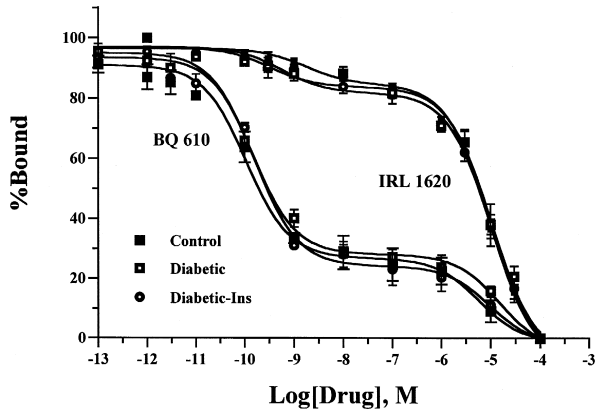

Fig. 3. Inhibition of [^{125}I]endothelin-1 binding to the rat bladder dome by BQ 610 and IRL 1620. Aliquots of membrane particulates were incubated with increasing concentrations of an unlabelled compound and a fixed concentration of [^{125}I]endothelin-1 for 2 h at 23°C. Each curve represents the mean of 3–4 separate experiments performed in duplicate. The curves are the computer fits of data points according to a two binding site model.

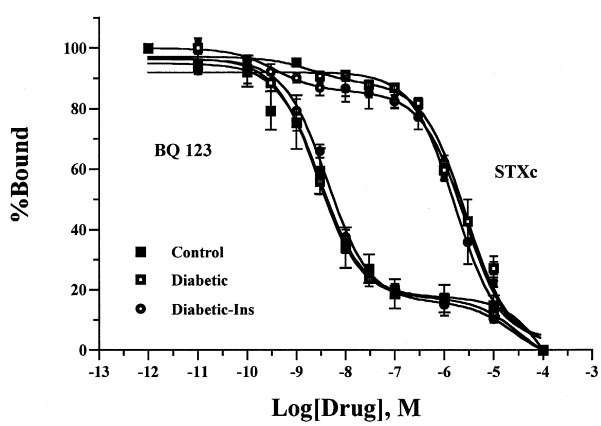

Fig. 4. Inhibition of [^{125}I]endothelin-1 binding to the rat bladder dome by BQ 123 and STXc. Aliquots of membrane particulates were incubated with increasing concentrations of an unlabelled compound and a fixed concentration of [^{125}I]endothelin-1 for 2 h at 23°C. Each curve represents the mean of 3–4 separate experiments performed in duplicate. The curves are the computer fits of data points according to a two binding site model.

Table 4
Inhibition of [^{125}I]endothelin-1 binding to rat bladder dome by various compounds
Aliquots of membrane particulates were incubated with increasing concentrations of endothelin-1, endothelin-3, BQ 123, BQ 610, Sarafotoxin S6c (STXc) or IRL 1620 and with a fixed concentration of [^{125}I]endothelin-1 for 2 h at 23°C. K_i, inhibition constant; K_{iH} and K_{iL}, inhibition constants for high and low affinity binding sites, respectively. $\%R_H/R_T$ is the proportion of high affinity to total binding sites. Values are mean ± S.E.M. of 4 to 5 separate experiments performed in duplicate.

	Control	Diabetic	Diabetic-Ins
ET-T			
K_i (pM)	76.1 ± 30	88.3 ± 27	53 ± 15
ET-3			
K_{iH} (pM)	167 ± 85	437 ± 310	149 ± 30
K_{iL} (nM)	40.1 ± 5.6	39.2 ± 7.1	41.5 ± 19
$\%R_H/R_T$	35.4 ± 3	32.9 ± 5.6	28.2 ± 5.4
BQ 123			
K_{iH} (pM)	2117 ± 661	1760 ± 77	3118 ± 533
K_{iL} (nM)	7650 ± 3460	18,275 ± 8642	5546 ± 4833
$\%R_H/R_T$	79.2 ± 4.1	77.8 ± 3.5	80.9 ± 0.6
BQ 610			
K_{iH} (pM)	28.6 ± 16.0	39.9 ± 16.7	49.8 ± 22.9
K_{iL} (nM)	673 ± 112	1603 ± 1117	1932 ± 1243
$\%R_H/R_T$	65.5 ± 5.6	65.7 ± 4.4	62.8 ± 4.8
Sarafotoxin S6c			
K_{iH} (pM)	304 ± 202	273 ± 195	296 ± 52
K_{iL} (nM)	1891 ± 349	2262 ± 943	2114 ± 691
$\%R_H/R_T$	22.2 ± 3.1	18.1 ± 6.8	14.2 ± 2.6
IRL 1620			
K_{iH} (pM)	253 ± 155	334 ± 141	296 ± 79
K_{iL} (nM)	6859 ± 1855	8227 ± 1480	8380 ± 3584
$\%R_H/R_T$	15.1 ± 1.6	15.5 ± 2.1	18.4 ± 3.6

inhibited approximately 63% to 81% of high affinity [^{125}I]endothelin-1 binding sites and 19% to 37% of low affinity [^{125}I]endothelin-1 binding sites in the diabetic, diabetic-insulin-treated and age-matched control bladder domes. High affinity constants (K_{iH} values) and low affinity constants (K_{iL} values) for subtype selective compounds and the proportion of heterogeneity of endothelin receptor subtypes, determined from $\%R_H/R_T$, in the rat bladder dome were similar between the diabetic, diabetic-insulin-treated and age-matched control groups Table 4.

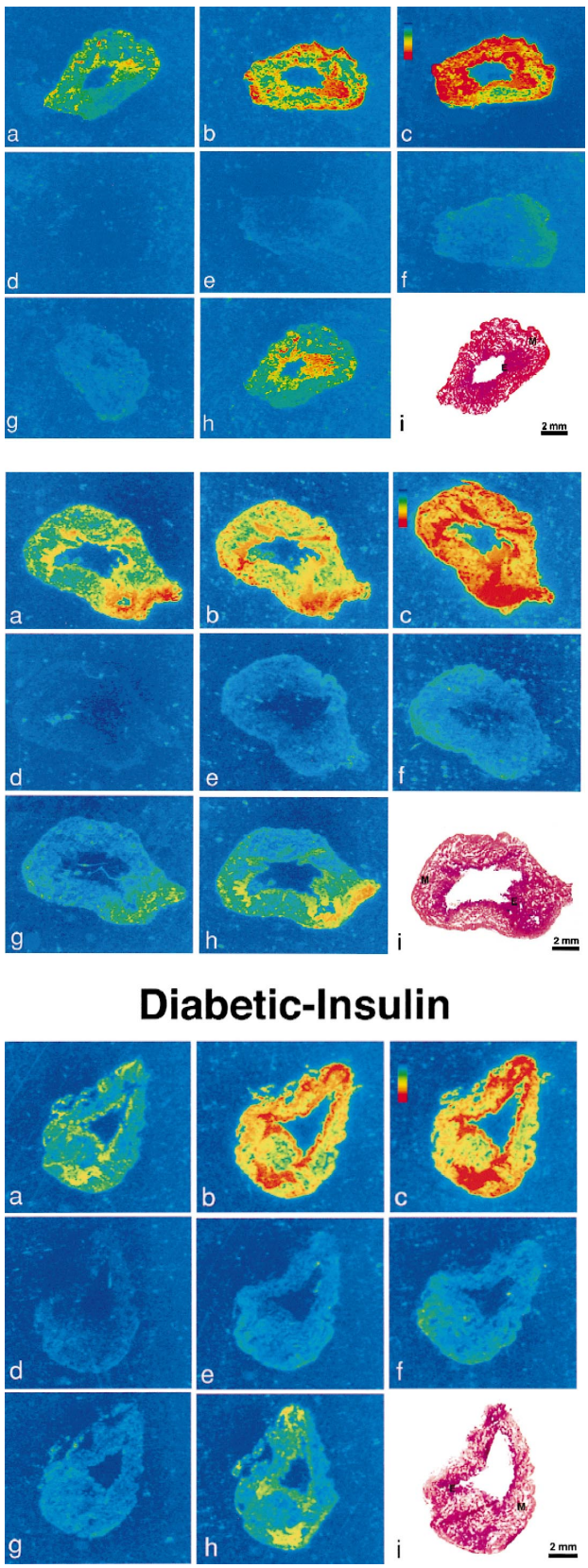

Fig. 5. Autoradiograms of [^{125}I]endothelin-1 binding to rat urinary bladder, and hematoxylin-eosin staining in adjacent bladder sections from 16 week control (A), diabetic (B) and insulin treated diabetic (C) rats. Autoradiograms are presented as computer generated pseudocolor images. The color key shown in panels a in each group demonstrates the color coding spectrum from the red (highest) to the blue (lowest) concentration of [^{125}I]endothelin-1 binding to the bladder sections. Panels a, b and c demonstrate the levels of total binding with 20, 40 and 80 pM [^{125}I]endothelin-1, respectively. Panels d, e and f are corresponding nonspecific binding levels obtained in the presence of 100 nM endothelin-1. Images g and h show the levels of 20 pM [^{125}I]endothelin-1 binding in the presence of 300 nM BQ 123 and 30 nM Sarafotoxin S6c showing the expression of endothelin ET_B and ET_A receptors, respectively. Panel i, is an H-E stained photomicrograph. E and M demonstrate epithelial and muscularis regions.

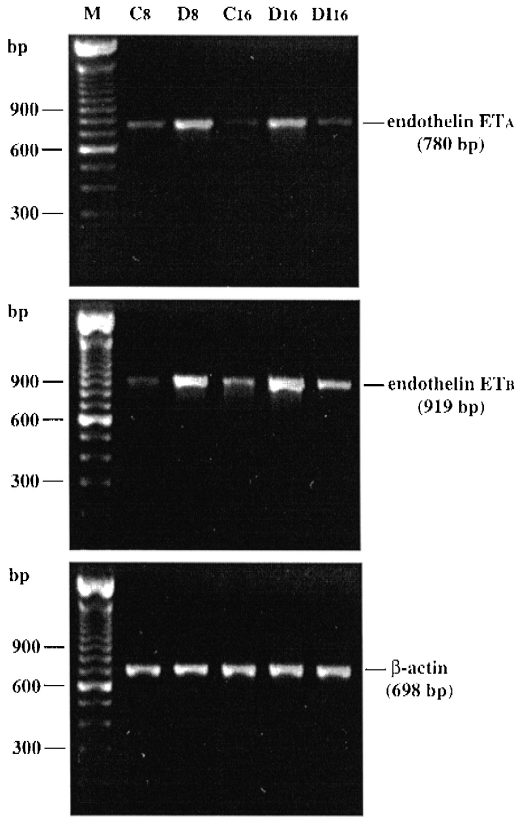

Fig. 6. RT-PCR detection of mRNAs for endothelin ET_A, ET_B receptors and β-actin in the rat bladder dome. Using specific oligonucleotide primers, the expected PCR products of 780 bp (ET_A) , 919 bp (ET_B) and 698 bp (β-actin) were detected. Lane M contains a 100 bp DNA ladder as DNA-size marker. C8, 8 week control; D8, 8 week diabetic; C16, 16 week control; D16, 16 week diabetic; DI16, diabetic insulin treated.

Our inhibition data suggest that the predominant endothelin receptor in the rat bladder dome is the endothelin ET_A receptor subtype and that the induction of diabetes or insulin treatment did not significantly affect the pharmacological properties of the endothelin receptor subtypes in the rat bladder dome.

3.3. Light microscopy autoradiography data

The autoradiograms of the bladder sections revealed a heterogenous distribution of [^{125}I]endothelin-1 binding sites in the rat bladder dome in all groups (Fig. 5). To localize endothelin ET_A and ET_B binding sites in the rat bladder, we incubated the sections in buffer containing 20 pM of [^{125}I]endothelin-1 in the presence of 30 nM of Sarafotoxin S6c (endothelin ET_B receptor selective), and 300 nM of BQ 123 (endothelin ETA receptor selective), respectively (Fig. 5). The results suggest that the predominant endothelin receptor subtype in the epithelium is of the ET_A subtype, whereas similar amounts of endothelin ET_A and ET_B receptor subtypes are present in the muscle of the rat bladder.

3.4. RT-PCR data

The mRNA expression of endothelin ET_A and ET_B receptors in the rat bladder dome was assessed by RT-PCR. The PCR products separated on agarose-gel electrophoresis are shown in Fig. 6. Amplified cDNA fragments for endothelin ET_A and ET_B receptors were detected in the bladder dome from each group of rats. Omission of the reverse transcriptase from the reaction mixture resulted in no PCR products (data not shown). PCR products were extracted from the agarose gel, sequenced and confirmed to be the rat endothelin ET_A and ET_B receptors and β-actin. The size and base pair alignments and coding sequences of the PCR products were as following: 780 bp size from 455–1234 bp for endothelin ET_A, 919 bp size from 724–1642 bp for endothelin ET_B, and 698 bp size from 2170–3079 bp for β-actin. The relative expression of endothelin receptor isoforms was normalized against β-actin expression. The induction of diabetes increased significantly the expression of both endothelin ET_A and ET_B gene transcripts compared to the age-matched controls (Figs. 6, 7). The insulin treatment, started 8 weeks after the induction of diabetes, reversed the expressions of both

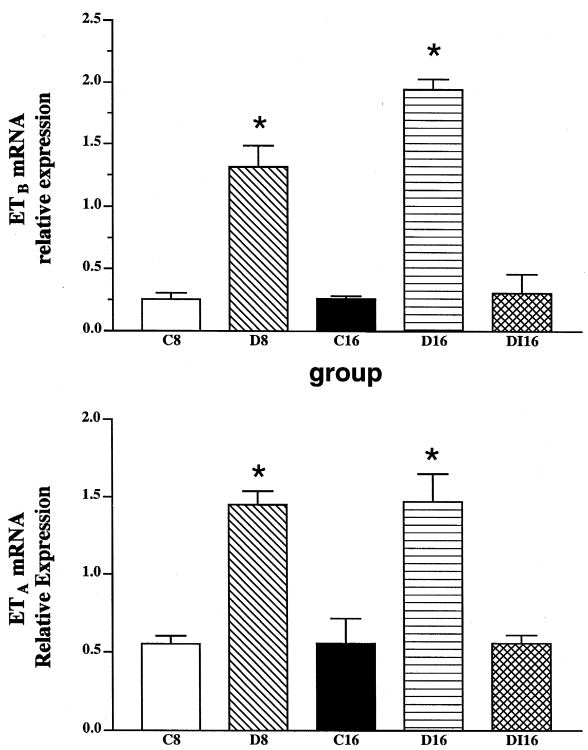

Fig. 7. Relative expressions of endothelin ET_A and ET_B receptor mRNAs to that of β-actin in the rat bladder dome. The results were expressed as the relative mRNA levels normalized with β-actin. C8, 8 week control; D8, 8 week diabetic; C16, 16 week control; D16, 16 week diabetic; DI16, diabetic insulin treated. Each bar represents the mean ± SEM of three separate experiments in each group. *, Significantly different from controls and insulin treated group ($P < 0.05$).

endothelin ET_A and ET_B receptor mRNAs to the control levels.

4. Discussion

The present study provides evidence that experimental diabetes alters the level of the expression of endothelin receptors and their mRNA in the rat bladder.

Diabetes-induced bladder dysfunctions in humans, i.e., loss of sensation, atony, urinary retention and overflow incontinence, are well recognized (Faerman et al., 1971; Ellenberg, 1980). These abnormalities have been attributed in part to peripheral autonomic neuropathy (Faerman et al., 1971; Buck et al., 1976). Streptozotosin-induced diabetes in animals has proved to be a useful model for studying diabetes-induced abnormalities that occur in a number of organ systems including the urinary bladder (Lincoln et al., 1984; Longhurst and Belis, 1986; Latifpour et al., 1989; Ozturk et al., 1996).

Endothelins are potent contractile agents in vascular and non-vascular smooth muscles (Rubanyi and Polokoff, 1994; Goto et al., 1996; Douglas and Ohlstein, 1997). Endothelin-1 activates a variety of signal transduction pathways to produce a diverse range of biologic responses (Douglas and Ohlstein, 1997). Endothelin-1-induced contraction of the urinary bladder are accompanied by stimulation of phosphotidylinositol hydrolysis with resultant elevation of intracellular Ca^{2+} (Garcia-Pascual et al., 1990; Persson et al., 1992). Saenz de Tejada et al. (1992) suggest that endothelins may act as an autocrine hormone in the regulation of the bladder smooth muscle tone by interacting with their specific receptors.

In the present communication, we demonstrate that streptozotosin-induced diabetes increased the density of endothelin receptors in the rat bladder dome and that late insulin treatment partially reversed this upregulation of endothelin receptors. Because both sugar-induced diuresis and diabetes cause an enlarged and hypertrophied bladder and an increase in the density of muscarinic receptors in the rat bladder dome, we considered the possibility that the upregulation of endothelin receptors in the diabetic rat bladder dome may be related to diabetes-associated diuresis (Latifpour et al., 1991; Fukumoto et al., 1994). Our preliminary data, however, indicate that sugar-induced diuresis does not increase the expression of endothelin receptors in the rat bladder dome (Latifpour et al., 1993). The absence of an upregulation in the density of endothelin receptors in the bladder dome of diuretic rats indicates that diuresis alone is not responsible for the endothelin receptor upregulation in the diabetic rat bladder dome. The pattern of regulation of endothelin receptors in diabetic rat bladder dome is similar to that in diabetic rat ureter in which both streptozotosin-induced diabetes and sugar-induced diuresis cause hypertrophy, but only diabetes is associated with an increase in the ureteral endothelin receptors (Nakamura et al., 1997).

To examine the possible diabetic- and/or insulin treatment-induced alterations in the pharmacological properties of endothelin receptors in the rat bladder dome, inhibition experiments with [^{125}I]endothelin-1 were performed using selective and non-selective endothelin agonists and antagonists: endothelin-1 (non-selective) and endothelin-3, Sarafotoxin S6c and IRL 1620 (endothelin ET_B receptor selective), and BQ 123 and BQ 610 (endothelin ET_A receptor selective). According to analyses with a non-linear curve fitting program, the data derived from the non-selective agonist, ET-1, were better fitted to a one-site than to a two-site model, whereas the data from endothelin-3, BQ 123, BQ 610, Sarafotoxin S6c and IRL 1620 were better fitted to a two-site model than to a one-site model in the rat bladder dome. Approximately 70–80% of the total endothelin receptors are of the endothelin ET_A receptor subtype, and 20–30% are of the endothelin ET_B receptor subtype in the rat bladder dome, and neither) the induction of diabetes nor insulin treatment of the diabetes significantly alters the pharmacological profile of endothelin receptors in this tissue.

In autoradiographic studies, [^{125}I]endothelin-1 binding sites had a heterogenous pattern of distribution with significant amounts of endothelin receptors being observed in transitional epithelium, submucosa, all muscle layers of the bladder, serosa and blood vessels. The density, proportion of the receptor subtypes, and pharmacological properties of the endothelin receptors in rat bladder dome are similar to that in the rabbit bladder (Latifpour et al., 1995). Saenz de Tejada et al. (1992) reported that endothelin-like immunoreactivity and its mRNA are localized to the transitional epithelium, serosa, and smooth muscle of the bladder, and to the vascular endothelium and smooth muscle of the blood vessels of the bladder and suggested that endothelins may act as autocrine hormones and play a role in the regulation of the bladder wall structure and smooth muscle tone. Garcia-Pascual et al. (1990) in autoradiographic studies of the rabbit bladder reported that the density of [^{125}I]endothelin-1 binding sites was high in blood vessels and higher in longitudinally than in circularly oriented bladder muscle and that the urothelium had few [^{125}I]endothelin-1 binding sites. Although a high density of endothelin receptors also is shown in the longitudinal muscle of the rat bladder, the rat urothelium has significant amounts of [^{125}I]endothelin-1 binding sites, as well.

The contractile response in the rat bladder has been suggested to be mediated by only the endothelin ET_A receptor subtype (Donoso et al., 1994). Endothelins have been shown to stimulate the release of nitric oxide (NO) from mammalian endothelial cells by activation of the endothelin ET_B receptor subtype (Warner et al., 1992; Rubanyi and Polokoff, 1994), with resultant relaxation of vascular tissues (Karaki et al., 1993). In addition, endothelin-1 has been reported to induce mucosal secretion in the

colon (Kiyohara et al., 1993). The functional role of the endothelin ET_A receptor subtype in the urothelium of the rat bladder is still unclear and warrants further studies.

The results of our RT-PCR studies demonstrate that the gene transcripts for endothelin receptor mRNAs are present in the bladder tissues from all experimental groups. Furthermore, similar to the increase in the expression of endothelin receptors, the levels of mRNAs for both endothelin receptors are increased by the induction of diabetes and insulin treatment reverses this upregulation.

In summary, experimental diabetes causes an upregulation of expression of the endothelin receptors and their gene transcript mRNAs in the rat bladder dome and insulin treatment, started 8 weeks after the induction of diabetes, decreases this upregulation. Neither the induction of diabetes nor the insulin treatment of the diabetes affects the subtype specificity and composition of endothelin receptor subtypes in the rat bladder dome. Furthermore autoradiographic studies reveal that the endothelin ET_A receptor subtype is the predominant receptor subtype in the epithelium and that both endothelin ET_A and ET_B receptors are located in the muscle layers of the rat bladder.

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References

Arai, H., Hori, S., Aramori, I., Ohkubo, H., Nakanishi, S., 1990. Cloning and expression of cDNA encoding an endothelin receptor. Nature 348, 730–732.

Bolger, G.T., Liard, F., Krogsrud, R., Thibeault, D., Jaramillo, J., 1990. Tissue specificity of endothelin binding sites. J. Cardiovasc. Pharmacol. 16, 367–375.

Buck, A.C., Reed, P.I., Siddiq, Y.K., Chisholm, G.D., Russell Fraser, T., 1976. Bladder dysfunction and neuropathy in diabetes. Diabetologia 12, 251–258.

Donoso, M.V., Salas, C., Sepulveda, G., Lewin, J., Fournier, A., Huidobro, J.P., 1994. Involvement of ET_A receptors in the facilitation by endothelin-1 of non-adrenergic non-cholinergic transmission in the rat urinary bladder. Br. J. Pharmacol. 111, 473–479.

Douglas, S.A., Ohlstein, E.H., 1997. Signal transduction mechanisms mediating the vascular actions of endothelin. J. Vasc. Res. 34, 152–164.

Ellenberg, M., 1980. Development of urinary bladder dysfunction in diabetes mellitus. Ann. Intern. Med. 92, 32123.

Emori, T., Hirata, Y., Marumo, F., 1990. Specific receptors for endothelin-3 in cultured bovine endothelial cells and its cellular mechanism of action. FEBS Lett. 263, 261–264.

Faerman, I., Mayer, M., Jaddzinsky, M., Alvarez, E., Fox, D., Zilbervarg, G.J., Cibeira, J.B., Colinas, R., 1971. Asymptomatic neurogenic bladder in juvenile diabetes. Diabetologia 7, 168–172.

Fukumoto, Y., Yoshida, M., Weiss, R.M., Latifpour, J., 1994. Reversibility of diabetes- and diuresis-induced alterations in rat bladder dome muscarinic receptors. Diabetes 43, 819–826.

Garcia-Pascual, A., Larsson, B., Andersson, K.-E., 1990. Contractile effects of endothelin-1 and localization of endothelin binding sites in rabbit lower urinary tract smooth muscle. Acta Physiol. Scand. 140, 545–555.

Goto, K., Hama, H., Kasuya, Y., 1996. Molecular and pathophysiological significance of endothelin. Jpn. J. Pharmacol. 72, 261–290.

Hu, R.-M., Levin, E.R., Pedram, A., Frank, H.J.L., 1993. Insulin stimulates production and secretion of endothelin from bovine endothelial cells. Diabetes 42, 351–358.

Ihara, M., Noguchi, K., Saeki, T., Fukuroda, T., Tsuchida, S., Kimura, S., Fukami, T., Ishikawa, K., Nishikibe, M., Yano, M., 1992. Biological profiles of highly potent novel endothelin antagonists selective for ET_A receptor. Life Sci. 50, 247–255.

Inoue, A., Yanagisawa, M., Kimura, S., Kasuya, Y., Miyauchi, T., Goto, K., Masaki, T., 1989. The human endothelin family: three structurally and pharmacologicaly distinct isopeptides predicted by three separate genes. Proc. Natl. Acad. Sci., U.S.A. 86, 2863–2867.

Ishikawa, K., Fukami, T., Nagase, T., Mase, T., Hayama, K., Niiyama, K., Fujita, K., Urakawa, Y., Kumagai, U., Fukuroda, T., Ihara, M., Yano, M., 1993. Endothelin antagonistic peptide derivatives with high selectivity for ET_A receptors. In: Schneider, C.H., Eberle, A.N. (Eds.), Peptide. ESCOM, Leiden, Netherlands, pp. 685–712.

Karaki, H., Sudjarwo, S.A., Hori, M., Sakata, K., Urade, Y., Takai, M., Okada, T., 1993. ET_B receptor antagonist, IRL 1038, selectively inhibited the endothelin induced endothelium dependent vascular relaxation. Eur. J. Pharmacol. 231, 371–374.

Karne, S., Jayawickreme, C.K., Lerner, M.R., 1993. Cloning and characterization of an endothelin-3 specific receptor (ET_C receptor) from *Xenopus laevis* dermal melanophores. J. Biol. Chem. 268, 19126–19133.

Kiyohara, T., Okuno, M., Nakanishi, T., Shimomura, Y., Matsuzawa, Y., 1993. Effect of endothelin 1 on ion transport in isolated rat colon. Gastroenterology 104, 1328–1336.

Kuhar, M.J., Unnerstall, J.R., 1990. Methods in neurotransmitter receptor analysis. In: Yamamura, H., Enna, S.J., Kuhar, M.J. (Eds.), Receptor Autoradiography. Raven Press, New York, pp. 177–218.

Latifpour, J., Davenport, T.A., Kamai, T., Fukumoto, Y., Yoshida, M., Weiss, R.M., 1993. Effect of experimental diabetes and diuresis on bladder dome endothelin receptors. J. Urol. 149, 489A.

Latifpour, J., Fukumoto, Y., Weiss, R.M., 1995. Regional differences in the density and subtype specificity of endothelin receptors in rabbit urinary tract. Naunyn-Schmiedeberg's Arch. Pharmacol. 352, 459–768.

Latifpour, J., Gousse, A., Kondo, S., Morita, T., Weiss, R.M., 1989. Effects of experimental diabetes on biochemical and functional characteristics of bladder muscarinic receptors. J. Pharmacol. Exp. Ther. 248, 81–88.

Latifpour, J., Nishimoto, T., Marian, M.J., Yoshida, M., Weiss, R.M., 1991. Differential regulation of bladder beta adrenergic and muscarinic cholinergic receptors in experimental diabetes. Diabetes 40, 1150–1156.

Lin, H.Y., Kaji, E.H., Winkel, G.K., Ives, H.E., Lodish, H.F., 1991. Cloning and functional expression of a vascular smooth muscle endothelin receptor. Proc. Natl. Acad. Sci. U.S.A. 88, 3185–3189.

Lincoln, J., Haven, A.J., Sawyer, M., Burnstock, G., 1984. The smooth muscle of rat bladder in the early stages of streptozotocin-induced diabetes. Br. J. Urol. 56, 24–30.

Longhurst, P.A., Belis, J.A., 1986. Abnormalities of rat bladder contractility in streptozotocin-induced diabetes mellitus. J. Pharmacol. Exp. Ther. 238, 773–777.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with folin phenol reagent. J. Biol. Chem. 193, 265–274.

Maggi, C.A., Giuliani, S., Patacchini, R., Santicoli, P., Turini, D., Barbanti, G., Meli, A., 1989. A potent contractile activity of endothelin on the human isolated urinary bladder. Br. J. Pharmacol. 96, 755–757.

Morabito, E., Corsico, N., Serafini, S., Martelli, E.A., 1994. Elevated

urinary excretion of endothelins in streptozotocin diabetic rats. Life Sci. 54, 197–200.

Munson, P., Rodbard, D., 1980. Ligand: a versatile computerized approach for ligand-binding systems. Anal. Biochem. 107, 220–239.

Nakamura, I., Saito, M., Fukumoto, Y., Yoshida, M., Nishi, K., Weiss, R.M., Latifpour, J., 1997. Experimental diabetes upregulates the expression of ureteral endothelin receptors. Peptides 18, 1091–1093.

Nayler, W.G., Liu, J., Panagiotopouios, S., Casley, D.J., 1989. Streptozotocin-induced diabetes reduced the density of [^{125}I]endothelin-binding sites in rat cardiac membranes. Br. J. Pharmacol. 97, 993.

Nudel, U., Zakut, R., Shani, M., Neuman, S., Levy, Z., Yaffe, D., 1983. The nucleotide sequence of the rat cytoplasmic beta-actin gene. Nucleic Acids Res. 11, 1759–1771.

Ozturk, Y., Altan, M., Yildizoglu-Ari, N., 1996. Effects of experimental diabetes and insulin on smooth muscle functions. Pharmacol. Rev. 48, 69–112.

Persson, K., Gracia-Pascual, A., Holmquist, F., Andersson, K.-E., 1992. Endothelin 1 induced contractions of isolated pig detrusor and vesical arterial smooth muscle: calcium dependence and phosphoinositide hydrolysis. Gen. Pharmacol. 23, 445–453.

Rosenthal, H.E., 1967. Graphic methods for the determination and presentation of binding parameters in a complex system. Anal. Biochem. 20, 525–532.

Rubanyi, G.M., Polokoff, M.A., 1994. Endothelins: molecular biology, biochemistry, pharmacology, physiology, and pathophysiology. Pharmacol. Rev. 46, 325–415.

Saenz de Tejada, I.S., Mueller, J.D., de Las Morenas, A., Michado, M., Moreland, R.B., Krane, R.J., Wolfe, H.J., Traish, A.M., 1992. Endothelin in the urinary bladder: I. Synthesis of endothelin-1 by epithelia, smooth muscle and fibroblasts suggests autocrine and paracrine cellular regulation. J. Urol. 148, 1290–1298.

Saito, M., Foster, H.E. Jr., Weiss, R.M., Latifpour, J., 1996a. Effect of experimental diabetes on rat prostate endothelin receptors. Eur. J. Pharmacol. 310, 197–200.

Saito, M., Nishi, K., Fukumoto, Y., Weiss, R.M., Latifpour, J., 1996b. Characterization of endothelin receptors in streptozotocin induced diabetic rat vas deferens. Biochem. Pharmacol. 52, 1593.

Sakurai, T., Yanagisawa, M., Takuwa, Y., Miyazaki, H., Kimura, S., Goto, K., Masaki, T., 1990. Cloning of a cDNA encoding a non-isopeptide selective subtype of the endothelin receptor. Nature 348, 732–735.

Shigematsu, K., Nakatani, A., Kawai, K., Moriuchi, R., Katamine, S., Miyamoto, T., Niwa, M., 1996. Two subtypes of endothelin receptors and endothelin peptides are expressed in differential cell types of the rat placenta: in vitro receptor autoradiographic and in situ hybridization studies. Endocrinology 137, 738–748.

Takai, M., Umehara, I., Yamasaki, K., Watakabe, T., Fujitani, Y., Oda, K., Urade, Y., Inui, T., Yamamura, T., Okada, T., 1992. A potent and specific agonist, Suc-[Glu^{9},Ala^{11,15}]-endothelin-1(8-21), IRL 1620, for the ET_B receptor. Biochem. Biophys. Res. Commun. 184, 953–959.

Takeda, Y., Miyamori, I., Yoneda, T., Takeda, R., 1991. Production of endothelin-1 from the mesenteric arteries of streptozotocin induced diabetic rats. Life Sci. 48, 2553–2556.

Terada, Y., Tomita, K., Nonoguchi, H., Marumo, F., 1992. Different localization of two types of endothelin receptor mRNA in microdissected rat nephron segments using reverse transcription and polymerase chain reaction assay. J. Clin. Invest. 90, 107–112.

Traish, A.M., Moran, E., Krane, R.J., Saenz de Tejada, I., 1992. Endothelin in the urinary bladder: II. Characterization of endothelin receptor subtypes. J. Urol. 148, 1299–1306.

Warner, T.D., Schmidt, H.H., Murad, F., 1992. Interactions of endothelins and EDRF in bovine native endothelial cells: selective effect of endothelin-3. Am. J. Physiol. 262, H1600–H1605.

Webb, M.L., Meek, T.D., 1997. Inhibitors of endothelin. Med. Res. Rev. 17, 17–67.

Williams, D.L., Jones, K.L., Pettibone, D.J., Lis, E.V., Clineschimidt, B.V., 1991. Sarafotoxin S6c: an agonist which distinguishes between endothelin receptor subtypes. Biochem. Biophys. Res. Commun. 175, 556–561.

Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K., Masaki, T., 1988. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 332, 411–415.