Regulatory effect of experimental diabetes on the expression of endothelin receptor subtypes and their gene transcripts in the rat adrenal gland

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Abstract

Endothelins (ETs) mediate paracrine control of vascular tone and secretion of steroids and catecholamines in the adrenal gland through two ET receptor subtypes, ETA and ETB. The differential distribution and function of these subtypes are responsible for the multiplicity of endothelin actions in this tissue. This study examines the regulatory effects of experimental diabetes on the gene expression, subtype specificity and localization of ET receptor subtypes, ET isopeptides, and endothelin-converting enzyme-1 (ECE-1) in the rat adrenal gland. The densities, pharmacological properties and distribution of ET receptor subtypes ETA and ETB in adrenal glands from streptozotocin-induced diabetic, insulin-treated diabetic and age-matched control rats were investigated, using radioligand receptor binding and autoradiographic techniques. The gene expression of ETA and ETB receptors ET-1, ET-3 and ECE-1 was evaluated using relative multiplex reverse transcription/PCR. The induction of

diabetes caused a marked reduction in body weight but no significant change in adrenal gland size. The density of ET receptors was significantly increased in the diabetic rat adrenal gland, mainly because of an increase in the expression of ETB receptors. Insulin treatment normalized the diabetes-induced changes in the expression levels of ET receptor subtypes to control levels. The expression level of ET-1 mRNA was up-regulated, whereas ET-3 mRNA was down-regulated in the diabetic adrenal gland compared with the controls. The ECE-1 mRNA level in the adrenal gland was not altered by the induction of diabetes. Autoradiographic studies showed that ETA and ETB are the predominant receptor subtypes in the adrenal medulla and cortex respectively. These results suggest that ETA and ETB receptors are differentially distributed and regulated in the diabetic rat adrenal gland.

Journal of Endocrinology (2001) **168**, 163–175

Introduction

Endothelins (ETs), a family of potent vasoactive and growth-regulatory peptides, are involved in the physiological and/or pathophysiological control of adrenocortical functions (Masaki 1993, Pollock 1998). ETs mediate the paracrine control of vascular tone and secretion of steroids and catecholamines in the adrenal gland through two ET receptor subtypes, i.e. ETA and ETB (Nussdorfer *et al.* 1997, Hinojosa-Laborde & Lange 1999). ETs regulate vascular tone in *in situ* perfused rat adrenal glands via both protein kinase C (PKC)-coupled ETA and nitric oxide synthase-coupled ETB receptors, whose activation evokes vasoconstriction and vasodilation respectively (Mazzocchi *et al.* 1998). It has been shown, in the rat adrenal gland, that the zona glomerulosa and the adrenal medulla contain both ETA and ETB, whereas the zona fasciculata/reticularis contains the ETB receptor subtype exclusively

(Belloni *et al.* 1997). As expected from their distribution, the corticosteroid secretagogue effect of ETs is exclusively mediated by the ETB receptor subtype, whereas the catecholamine secretagogue action of ETs is mediated by both the ETA and the ETB subtype (Cozza *et al.* 1992, Belloni *et al.* 1997, Pecci *et al.* 1998). Furthermore, ETs stimulate the proliferation of zona glomerulosa cells via ETA receptor-coupled PKC and tyrosine kinase signaling pathways (Mazzocchi *et al.* 1997). Thus, the differential distribution of these subtypes appears to be responsible for the multiplicity of endothelin actions in this tissue. ETs are expressed at high levels in adrenal glands (Imai *et al.* 1992, Masaki 1993, Goto 1999) and the adrenals contain endothelin-converting enzyme-1 (ECE-1), a key enzyme in the biosynthesis of active ET-1 and ET-3 *in vivo* (Shimada *et al.* 1994, Xu *et al.* 1994, Yanagisawa *et al.* 1998, Korth *et al.* 1999).

The development of cardiovascular abnormalities in diabetes mellitus is accompanied by changes in peripheral blood flow (Lucas & Foy 1977, Lucas 1985, Cohen 1993, Öztürk *et al.* 1996). The adrenal gland receives extremely high levels of blood flow, compared with other organs, through uniquely evolved vasoregulatory mechanisms (Breslow 1992, Kemp *et al.* 1999). However, under the chronic stress of diabetes, adrenal blood flow decreases significantly. An approximately 35% decrease in adrenal blood flow is noted 2 months after the induction of diabetes by streptozotocin (STZ) in the rat (Lucas & Foy 1977). It is conceivable that ETs, as potent vasoactive regulators, may regulate the diabetes-induced changes in adrenal blood flow.

Diabetes mellitus has profound effects on the neuro-endocrine axis, including the hypothalamic–pituitary–adrenal axis (Steger & Rabe 1997). Adrenocortical function is enhanced in STZ-induced diabetes, as evidenced by an increase in plasma corticosterone that correlates positively with plasma adrenocorticotrophic hormone (ACTH) levels and adrenal gland weights (Scribner *et al.* 1991, 1993, Schwartz *et al.* 1997). Diabetic patients, even those with good glycemic control, demonstrate elevated levels of plasma cortisol and ACTH hormone both before and after dexamethasone suppression tests (Cameron *et al.* 1984, Roy *et al.* 1990). These data demonstrate that both experimental and human diabetes mellitus are accompanied by adrenocortical hyperactivity (De Nicola *et al.* 1976). There have been few studies of adrenal medullary function in diabetic animals. However, elevated levels of adrenal and plasma catecholamines and the activities of catecholamine-producing enzymes have been reported in STZ-induced diabetic rats (Fushimi *et al.* 1984, Bitar *et al.* 1987).

This study examines the regulatory effects of STZ-induced diabetes on gene expression, pharmacological properties and the localization of ET receptor subtypes in the rat adrenal gland.

Materials and Methods

Animals

Male Sprague–Dawley rats (55–56 days old) weighing 250–300 g were divided into the following five groups: 8-week control (C8); 8-week diabetic (D8); 16-week control (C16), 16-week diabetic (D16); and diabetics treated with insulin (DI16). Diabetes was induced by an intravenous injection of 65 mg STZ/kg dissolved in 0.1 M citrate (pH 4.5) (Fukumoto *et al.* 1993). Control rats were injected with a vehicle. Eight weeks after STZ-administration, the DI16 group received 5–8 units of protamine zinc insulin subcutaneously daily for an additional eight weeks. The rats were sacrificed by decapitation under anesthesia induced with an intraperitoneal injection of sodium pentobarbital, 8 weeks (groups C8 and

D8) or 16 weeks (groups C16, D16 and DI16) after STZ-administration. The adrenal glands were immediately dissected and frozen in liquid nitrogen for subsequent RNA and membrane-particulate preparations, or embedded in OCT compound (Sakura Finetek, Torrance, CA, USA) for autoradiography.

Preparation of adrenal membrane particulates

The membrane particulates from adrenal glands were prepared as described previously (Latifpour *et al.* 1995, Saito *et al.* 2000a). In brief, the adrenal specimens were homogenized on ice in 20 mM Hepes (pH 7.4) containing 100 mM NaCl, 3 mM EDTA, 1 mM EGTA and the following protease inhibitors: 0.1 mM phenyl methyl sulfonyl fluoride and 10 µg/ml each of aprotinin, leupeptin, pepstatin A, and soybean trypsin inhibitor. The homogenate was centrifuged at $49\,000 \times g$ for 15 min at 4 °C. The pellet was re-homogenized, filtered through a metal sieve (250 µm pore size) and re-centrifuged. The final pellet was suspended in 20 volumes of the same buffer.

Binding experiments on membrane particulates

The density and pharmacological properties of ET receptors in rat adrenal glands were examined by saturation and inhibition experiments (Latifpour *et al.* 1995, Saito *et al.* 2000a, b). In the saturation experiments, the membrane suspensions of the rat adrenal glands prepared in 50 mM Tris–HCl (pH 7.4) containing 154 mM NaCl, 25 mM MnCl₂, 1 mM EDTA, 1 mM *N*-acetyl-DL-methionine, 0.25% bovine serum albumin and 0.14% bacitracin were incubated, in triplicate, for 2 h with increasing concentrations (10–300 pM) of ¹²⁵I-labeled ET-1 (2200 Ci/mmol; New England Nuclear, Boston, MA, USA) in a total volume of 0.25 ml at 23 °C. At the end of the incubation period, using a Brandel Cell Harvester (Model M-24R; Brandel Instruments, Gaithersburg, MD, USA), the reaction mixtures were filtered rapidly under a vacuum through Whatman GF/B glass-fiber filters that had been treated previously with a 1% solution of bovine serum albumin (to reduce non-specific binding to the filter papers). Each filter disc was washed intensively with 80 ml ice-cold 50 mM Tris–HCl (pH 8.0). The radioactivity on the glass-fiber discs was counted with a gamma-counter (Multi-Prias Analyzer; Packard, Meriden, CT, USA) at an efficiency of 70–75%. Non-specific binding was determined in the presence of 100 nM unlabeled ET-1. Specific binding was calculated by subtracting non-specific binding from total binding of radioligand to the glass fiber.

In the inhibition studies, membrane suspensions of the adrenal glands were prepared in the same manner as for the saturation experiments. Aliquots of membrane particulates (2–3 µg protein) were incubated with a fixed concentration of ¹²⁵I-ET-1 (30 pM) for 2 h at 23 °C in the

presence or absence of increasing concentrations of the following unlabeled peptides: BQ 123 (ETA-selective), and sarafotoxin S6c (STXc; ETB-selective) (Williams *et al.* 1991, Ihara *et al.* 1992). The remainder of the procedure was the same as that used for the saturation studies.

Autoradiography

Autoradiographic studies were performed as described previously (Saito *et al.* 2000b). Serial sections (20 µm thick) of the rat adrenal glands of the 16-week control, the 16-week diabetic and the insulin-treated diabetics are cut with a microtome cryostat (2800 Frigocut N; Leica Inc., Malver, PA, USA) at -22°C and mounted on aminoalkylsilane-coated microscope slides. Autoradiographic localization of ^{125}I -ET-1 binding was performed on adjacent sections obtained from a single adrenal gland from each group. To reduce the effect of endogenous peptide levels, and the non-specific binding of ^{125}I -ET-1 to the adrenal glands, slide sections were pre-incubated with an autoradiographic buffer (20 mM Hepes, pH 7.4, containing 140 mM NaCl, 4 mM KCl, 1 mM KH_2PO_4 , 1 mM MgCl_2 , 1 mM CaCl_2 , 10 mM glucose, 25 mg/ml bovine serum albumin and 100 µg/ml bacitracin) for 30 min at 23°C . After the preincubation period, sections were incubated with the increasing concentrations of ^{125}I -ET-1 (30–120 pM) in a total volume of 0.5 ml for 2 h at 23°C . To determine non-specific binding, adjacent sections were incubated with the same concentration of ^{125}I -ET-1 in the presence of unlabeled 100 nM ET-1. The sections were then washed twice in ice-cold 50 mM Tris-HCl (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- ^3H ; Amersham, Arlington, IL, USA) 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, USA) at 23°C for 5 min, fixed for 10 min in the Kodak normal fixer, and washed in tap water for 30 min. Adjacent adrenal sections were then stained with hematoxylin (10 min) and eosin (2 min), dehydrated, and coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ, USA).

The localization of the ETA and ETB receptor subtypes in the rat adrenal gland was determined by incubating tissue sections with a fixed concentration of ^{125}I -ET-1 (30 pM) and appropriate concentrations of subtype-selective compounds, i.e. 300 nM BQ 123, and 30 nM STXc. These concentrations of BQ 123 and STXc, which are approximately 100 times greater than that of their high-affinity constants, K_{IH} , (Latifpour *et al.* 1995) occupy virtually all of the endothelin ETA and ETB receptor subtypes, respectively, in the rat adrenal sections.

In liquid emulsion autoradiography, after radiolabeling was accomplished, the labeled ligand was irreversibly linked to the receptors by fixation with paraformaldehyde

vapor, as previously described (Herkenham & Pert 1982). Briefly, sections were placed in an airtight chamber containing paraformaldehyde crystals and incubated at 80°C for 2 h. After fixation, they were dehydrated in ascending concentrations of alcohol, stained with eosin (2 min), placed in a series of alcohol and xylene washes, and dried. Under a safe light, the dried sections were dipped into LM-1 Hypercoat emulsion (Amersham, Arlington, IL, USA), which had been melted at 43°C , and immediately placed upon a flat, chilled steel plate to gel the emulsion uniformly. After drying, slides were placed in light-tight boxes with Drierite and stored at 4°C for 4 days. Development was performed for 5 min in D19 (Kodak, Rochester, NY, USA), followed by a stop bath and fixation in Kodak normal fixer. The sections were then stained with hematoxylin for 10 min, cleared, dehydrated, and coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ, USA). This technique, in conjunction with film autoradiography, permitted us to localize more accurately the distribution of ET receptor subtypes in the rat adrenal gland.

RNA isolation

Total RNA was extracted from frozen adrenal glands by using TRIZOL reagent (Life Technologies, Gaithersburg, MD, USA) (Simms *et al.* 1993). RNA yields were determined by absorbance at 260 nm. RNA samples were stored at -80°C as ethanol precipitates for later use.

Reverse transcription/polymerase chain reaction

The reverse transcription/polymerase chain reaction (RT-PCR) was performed in 0.2-ml thin-walled tubes in a DNA thermocycler (Perkin-Elmer, Branchburg, NJ, USA) as described previously (Ikeda *et al.* 2000, Saito *et al.* 2000a, b). First-strand cDNA was synthesized in a 20 µl volume at 42°C for 50 min with reverse transcriptase (Superscript II; Life Technologies). The RT reaction mixture contained 5 µg total RNA, 5 mM random decamer, $1 \times$ first-strand buffer, 10 mM dithiothreitol, 0.5 mM each deoxynucleotide 5'-triphosphate (dNTP) and 200 U reverse transcriptase. The reverse transcriptase was inactivated at 70°C for 15 min. Parallel reactions without reverse transcriptase were performed to check for trace genomic DNA contamination. No contamination was detected.

The oligonucleotide primer pairs used for RT-PCR assays are shown in Table 1 (Sakurai *et al.* 1990, 1991, Lin *et al.* 1991, Shiba *et al.* 1992, Shimada *et al.* 1994). In order to eliminate possible interference of genomic DNA in the PCR, the primers were designed to flank an intron. A PCR in exponential phase was performed to allow comparative analysis of numerous cDNA samples. An aliquot of cDNA was amplified in a 50 µl volume containing appropriate PCR buffer, 1.5 mM MgCl_2 , 200 µM

Table 1 Specific primers for RT-PCR

	Sequence		Size (bp)	References
Product				
ECE-1	Sense	5'-CGTAGCGATAGTCTTAGCAC-3'	529	Shimada <i>et al.</i> 1994
	Antisense	5'-GTGCCACACCAAACTACAG-3'		
ET-1	Sense	5'-GCTCCTGCTCCTCTTGATG-3'	500	Sakurai <i>et al.</i> 1991
	Antisense	5'-CTCGCTCTATGTAAGTCATGG-3'		
ET-3	Sense	5'-GCACTTGCTTCACTTATAAGG-3'	384	Shiba <i>et al.</i> 1992
	Antisense	5'-CAGAAGCAAGAAGCATCAGTTG-3'		
ETA	Sense	5'-GTGTTTAAGCTGTTGGCGGG-3'	780	Lin <i>et al.</i> 1991
	Antisense	5'-CGAGGTCATGAGGCTTTGG-3'		
ETB	Sense	5'-AGCTGGTGCCCTTCATACAGAAGGC-3'	919	Sakurai <i>et al.</i> 1990
	Antisense	5'-TGCACACCTTTCCGAAGCACG-3'		

each dNTP, 0.4 μM primer pairs and 5 U *Taq* DNA polymerase (Life Technologies). To determine the optimum conditions for the PCR, the following cycling conditions were used: 3 min at 94 °C, followed by 20–40 cycles of 94 °C for 45 s, 51–55 °C for 30 s and 72 °C for 90 s. The last cycle included a prolonged extension at 72 °C for 5 min. Relative multiplex RT-PCR was performed to measure the expression of mRNAs for ET receptor subtypes and ET isopeptides relative to those of 18S rRNAs (18S) as an internal control. The classic 18S internal standard with 495 bp was used to normalize PCR products of ET-3 and ET receptor subtypes, and plant 18S internal standard with 315 bp was used to normalize the PCR products of ET-1 and ECE-1. This technique compares transcript abundance across multiple samples, using a co-amplified internal control for sample normalization. Primers and Competimers for 18S were purchased from Ambion (Austin, TX, USA). The 18S Competimers were modified at their 3'-ends to block extension by DNA polymerase. By mixing 18S primers with increasing amounts of 18S Competimers, the overall PCR amplification efficiency of 18S cDNA can be reduced without the primers becoming a limiting factor.

Analysis of data

Saturation data were analyzed according to Rosenthal (1967) using linear regression of bound/free versus bound in order to calculate the maximum number of binding sites, B_{max} , and the 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 two-binding-site model (Munson & Rodbard 1980), using a computer-assisted program (GRAPHPAD PRISM; GraphPad Software Inc., San Diego, CA, USA). 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.

PCR products were digitized and analyzed with a DC120 Zoom digital camera and 1D Image Analysis Software (Eastman Kodak Co., Rochester, NY, USA).

Relative expression levels of mRNA were normalized against 18S and are shown as means ± standard error of the mean (S.E.M.). Statistical analyses of general features and receptor binding data were performed using analysis of variance and the multiple-comparison Fisher's test. The RT-PCR data were analyzed by means of unpaired Student's *t*-tests. Differences were regarded as statistically significant at values of $P \leq 0.05$.

Results

As shown previously (Saito *et al.* 2000a), STZ induction of diabetes causes a marked reduction in body weight, an increase in serum glucose levels and a decrease in serum insulin levels relative to age-matched controls. STZ-induced diabetes is not accompanied by changes in adrenal size. Insulin treatment significantly improves the diabetes-induced alterations in serum glucose levels and body weight (data not shown).

The binding assay revealed a single class of specific, saturable, high-affinity and non-interacting binding sites for ^{125}I -ET-1 on the rat adrenal glands (Fig. 1). The maximum number of the binding sites (B_{max}) and the equilibrium dissociation constant (K_d) values for ^{125}I -ET-1 binding to the adrenal glands are shown in Table 2. The density of binding sites for ^{125}I -ET-1 was significantly higher in the diabetic adrenal gland than in the age-matched controls (Fig. 1; Table 2). K_d values for ^{125}I -ET-1 binding were similar in all groups studied. In order to investigate the subtype specificity of ET receptors in the rat adrenal glands, inhibition binding studies were performed in which ^{125}I -ET-1 binding was inhibited by BQ 123 (a selective ETA antagonist) and STXc (a selective ETB agonist) (Figs 2 and 3). Inhibition curves with these ligands display biphasic patterns for all five groups studied. According to analysis with a non-linear curve-fitting program, the data for BQ 123 and STXc are significantly better fitted to a two-site model than to a one-site model in all five experimental groups. The affinity constants and the proportion of high-affinity binding sites to total

Table 2 Maximal binding capacities and dissociation constants of ET receptors in rat adrenal gland

	Control	Diabetic	Diabetic–Insulin
8 weeks			
$B_{\max}$			
fmol/mg protein	84.3 ± 5.6	129.8 ± 5.8 ^a	—
fmol/g tissue	1893 ± 125	2912 ± 131 ^a	—
K_d (pM)	95.5 ± 11.2	102.0 ± 7.2	—
16 weeks			
$B_{\max}$			
fmol/mg protein	90.0 ± 7.9	136.3 ± 4.7 ^a	89.4 ± 6.0
fmol/g tissue	2020 ± 178	3005 ± 524 ^a	1924 ± 228
K_d (pM)	101.8 ± 10.4	104.0 ± 9.5	113.3 ± 13.0

* $B_{\max}$ is the maximal number of binding sites. K_d is the equilibrium dissociation constant. Values are means ± S.E.M. five separate experiments using five animals from each group.

^aSignificantly different from the age-matched control group ($P \leq 0.05$).

Figure 1 Saturable binding of ^{125}I -ET-1 to rat adrenal membrane particulates. Adrenal membrane suspensions were incubated with ^{125}I -ET-1 for 2 h at 23 °C. Specific binding was determined in the presence of 100 nM unlabelled ET-1. Each curve represents the mean of a single experiment performed in triplicate. C8; 8-week control, D8; 8-week diabetic, C16; 16-week control, D16; 16-week diabetic, DI16; insulin-treated diabetes. Inset: Rosenthal plots for ^{125}I -ET-1 binding to each group.

Figure 2 Competitive inhibition of ^{125}I -ET-1 binding to the rat adrenal gland by BQ 123. Adrenal membrane suspensions from each experimental group were incubated with 30 pM ^{125}I -ET-1 in the presence of a designated concentration of BQ 123, an ETA-selective antagonist. Each curve represents the mean of a single experiment performed in triplicate. C8; 8-week control, D8; 8-week diabetic, C16; 16-week control, D16; 16-week diabetic, DI16; insulin-treated diabetics. The curves are the computer fits of data points according to a two-binding-site model.

binding are shown in Table 3. The proportion of high-affinity to total binding sites ($\%R_{H/T}$) for BQ 123 was significantly lower in both the 8-week and the 16-week diabetic glands than in age-matched control adrenal glands, suggesting that the relative contribution of the ETA receptor subtype to the total ET receptor populations was reduced in the diabetic adrenal gland. However, the receptor density of the ETA receptor subtype, calculated from total binding sites ($B_{\max}$) and $\%R_{H/T}$ for BQ 123, did not differ significantly between the experimental groups (data not shown). These data are consistent with the decrease in the relative contribution of the ETA subtype in the diabetic adrenal gland, being due to an increase in the relative contribution of the ETB receptor subtype in this tissue. The $\%R_{H/T}$ for STXc was significantly higher in both the 8-week and the 16-week diabetic glands than in age-matched control adrenal glands, and the density of the ETB receptor subtype, calculated from total binding sites ($B_{\max}$) and $\%R_{H/T}$ for STXc, was significantly raised in diabetic compared with age-matched control adrenal glands (data not shown). These data also are consistent with the expression of the ETB receptor subtype being up-regulated in the diabetic adrenal gland. Thus, STZ-induced diabetes increased the density of ET receptors in the rat adrenal gland, this being

mainly due to an increase in the expression of the ETB receptor subtype. Insulin treatment normalized the diabetes-induced changes in the expression levels of the ET receptor subtypes to control levels (Figs 1–3; Tables 2 and 3).

For further investigation of the regulation of ET receptors in diabetic adrenal glands, mRNA levels for each receptor subtype were determined by means of relative multiplex RT-PCR. The expression of ETA subtype mRNA was not significantly different between each group (data not shown). In contrast, mRNA expression for ETB was significantly higher in the diabetic adrenal gland than in age-matched controls (Fig. 4). These mRNA expression levels are in accord with receptor densities at protein levels determined by radioligand receptor binding techniques.

The expression of ET-1 and ET-3 mRNA was also evaluated with RT-PCR (Figs 5 and 6). After both 8 and 16 weeks of diabetes, ET-1 mRNA increased, whereas ET-3 mRNA decreased. Insulin treatment suppressed ET-1 mRNA relative to that in diabetic animals, whereas insulin increased the ET-3 mRNA expression levels. The expression of ECE-1 mRNA in the adrenal gland was not significantly different between experimental groups (data not shown).

Figure 3 Competitive inhibition of ^{125}I -ET-1 binding to rat adrenal gland by STXc. Adrenal membrane suspensions from each group were incubated with 30 pM ^{125}I -ET-1 in the presence of a designated concentration of STXc, an ETB-selective agonist. Each curve represents the mean of a single experiment performed in triplicate. C8; 8-week control, D8; 8-week diabetic, C16; 16-week control, D16; 16-week diabetic, DI16; insulin-treated diabetics. The curves are the computer fits of data points according to a two-binding-site model.

Autoradiograms obtained from the binding of ^{125}I -ET-1 to adrenal sections demonstrate the specificity, saturability and distribution of ET receptors in the rat adrenal gland (Fig. 7). Increasing concentrations of ^{125}I -ET-1 resulted in higher levels of binding of the labeled peptide to the adrenal sections in a dose-dependent manner (Fig. 7, A–C, panels a, b and c). The addition of an excess of unlabeled ET-1 completely eliminated the ^{125}I -ET-1 binding to the sections, indicating that all radioligand binding observed was to specific binding sites (Fig. 7, A–C, panels d, e and f). Differences between total levels of ^{125}I -ET-1 binding and levels of ^{125}I -ET-1 binding in the presence of selective ET receptor subtype compounds in the various groups were not as apparent in the autoradiographic images of adrenal gland sections as in the analysis of saturation experiments performed on membrane particulates. This is probably because of individual variation and/or a lower level of sensitivity in the autoradiographic studies. The main purpose of the autoradiographic studies was to determine the distribution and localization of the endothelin receptor subtypes and not the differences between experimental groups. To localize ETA and ETB binding sites in the rat adrenal gland, we incubated the tissue sections with a fixed concentration of ^{125}I -ET-1 in the presence of 30 nM STXc (ETB-selective) or 300 nM

BQ 123 (ETA-selective) respectively (Fig. 7, A–C, panels g and h). The data indicated that both ET receptor subtypes were present in all regions of the adrenal gland and that ETA and ETB were the predominant receptor subtypes in the medulla and cortex respectively. The subtype specificity and the detailed localization of ET receptor subtypes are shown in images obtained from liquid emulsion autoradiography (Fig. 8). Figure 8 was prepared from the cortical and medullary areas of the adrenal gland of a control rat, as shown in Fig. 7A (panel g). In the adrenal cortex, ETA receptors were localized densely in the zona glomerulosa, whereas they were almost negligible in the zona fasciculata (Fig. 8D, left panel). In contrast, ETB receptors were found at a higher level than ETA receptors in the cortex and were uniformly distributed throughout the layers of the cortex (Fig. 8E, left panel). At the cortico-medullary junction, the ETA receptor subtype was highly expressed in the zona reticularis and in the medulla (Fig. 8D, right panel). In the zona reticularis, ETB receptors were expressed to a lesser extent than ETA receptors. Significant levels of the ETB receptor subtype were detected in the medulla (Fig. 8E, right panel). Similar autoradiographic data were obtained from experiments in three to four adrenal glands from each experimental group.

Table 3 Competitive inhibition of specific ¹²⁵I-ET-1 binding to the rat adrenal membrane particulates by BQ 123 and STX

	Control	Diabetic	Diabetic–Insulin
8 weeks			
BQ 123			
K _{iH} (nM)	1.8 ± 0.3	2.5 ± 1.0	–
K _{iL} (μM)	0.61 ± 0.11	0.66 ± 0.17	–
%R _{H/T}	37.0 ± 1.1	18.5 ± 0.3 ^a	–
STXc			
K _{iH} (nM)	8.7 ± 1.0	8.2 ± 1.1	–
K _{iL} (μM)	0.17 ± 0.03	0.11 ± 0.01	–
%R _{H/T}	47.3 ± 1.8	61.8 ± 2.1 ^a	–
16 weeks			
BQ 123			
K _{iH} (nM)	3.0 ± 0.6	3.3 ± 0.6	1.8 ± 0.3
K _{iL} (μM)	0.60 ± 0.15	0.71 ± 0.38	0.72 ± 0.17
%R _{H/T}	32.7 ± 1.7	22.0 ± 0.8 ^a	32.9 ± 2.2
STXc			
K _{iH} (nM)	17.2 ± 6.7	16.7 ± 6.4	9.6 ± 1.9
K _{iL} (μM)	0.19 ± 0.04	0.24 ± 0.05	0.14 ± 0.02
%R _{H/T}	46.7 ± 2.0	58.3 ± 1.7 ^a	45.4 ± 3.2

K_{iH} and K_{iL} are estimated dissociation constants for high- and low-affinity binding sites respectively. %R_{H/T} is the proportion of high-affinity binding sites to total binding sites. Values are means ± S.E.M. of five separate experiments using five animals from each group. ^aSignificantly different from the age-matched control group (*P* ≤ 0.05).

Discussion

The present study demonstrates that the induction of diabetes increases ET receptor density in the rat adrenal gland. Characterization of ET receptor subtypes showed that diabetes up-regulates ETB, but not ETA, receptors at both protein and mRNA levels. ET-1 mRNA is increased, whereas ET-3 mRNA is reduced in the diabetic adrenal gland. The ETA and ETB receptor subtypes are predominantly localized in the adrenal medulla and cortex respectively.

The weight of the adrenal gland was not significantly altered in diabetic rats compared with controls, although total body weight was severely reduced. These data are consistent with previous studies which demonstrate adrenal hypersecretion and hypertrophy associated with diabetes (Scribner *et al.* 1991, 1993, Schwartz *et al.* 1997). In STZ-diabetic animal models, adrenalectomy partially restores serum glucose levels and improves diabetes-induced metabolic symptoms (Rodgers *et al.* 1994). Overproduction of adrenal corticosterone is responsible for exacerbating the hyperglycemia in insulin-dependent diabetes mellitus (IDDM) and for causing diabetes-associated growth retardation (De Nicola *et al.* 1976, Rodgers *et al.* 1994, Strack *et al.* 1995). As exogenous corticosterone stimulates food intake and inhibits body-weight gain in adrenalectomized diabetic rats, it has been suggested that glucocorticoids and insulin are interacting reciprocal regu-

lators of energy balance (Strack *et al.* 1995). Thus, complications resulting from IDDM may be due to the actions of counter-regulatory hormones, i.e. adrenal corticosteroids and insulin, rather than to insulin deficiency alone. It is unclear as to how adrenal cortical function is activated in diabetes, but ETs may be involved in adrenal hypersecretion, as *in vivo* and *in vitro* studies show that ETs regulate adrenal functions via multiple pathways.

In recent years, the existence of cross-talk between insulin and ETs has received growing attention because of their effects on adrenal blood flow and the resultant modulation of glucose uptake (Baron *et al.* 1995, Cardillo *et al.* 1999, Gregersen *et al.* 2000). Insulin stimulates ET-1 synthesis and secretion in cultured aortic endothelial cells (Hu *et al.* 1993), and also increases serum ET-1 levels as well as the expression of ET receptors in a variety of smooth-muscle cells, both in control and STZ-induced diabetic rats (Frank *et al.* 1993). The increase in the circulating ET-1, however, may not necessarily be involved in changes in vascular tone, as ET-1 is believed to act in an autocrine/paracrine fashion (Rubanyi & Polokoff 1994). Recent studies that showed that the release of catecholamines in response to hypoglycemia is attenuated in rats pretreated with a selective ETA-receptor antagonist suggest that ETA is also important in the functional response of the adrenal medulla (Lange *et al.* 1999). As ETs are involved in the release of adrenal corticosteroids, it is conceivable that other forms of stress such as

Figure 4 Effects of diabetes and insulin treatment on gene expression of the ETB receptor subtype. (A) Detection of mRNAs for ETB and 18S in the rat adrenal gland by relative multiplex RT-PCR. Representative data from each group are shown. The expected PCR products of 919 bp (ETB) and 495 bp (18S) were detected. Lane M contains a DNA size marker. (B) Relative expression of ETB mRNA normalized against 18S. Each bar represents the mean $\pm$ S.E.M. of six rat adrenal glands in each group. C8; 8-week control, D8; 8-week diabetic, C16; 16-week control, D16; 16-week diabetic, DI16; insulin-treated diabetics. *, Significantly different from C8 ($P \leq 0.05$). **, Significantly different from C16 and D16 ($P \leq 0.05$).

nutritional disorders and fluid and electrolyte imbalances, which might be related to changes in corticosteroid metabolism, may be associated with adrenal ET receptor changes.

Endothelial dysfunction in diabetic vessels may play a role in the diabetes-induced decrease in adrenal blood flow and/or in adrenal hyperfunction. Adrenal blood flow decreases significantly in STZ-induced diabetic rats (Lucas & Foy 1977). In the present study, ETB, but not ETA, receptors were elevated in the diabetic adrenal gland. The activation of ETB in *in situ* perfused rat adrenals causes vasodilation, which lowers intra-adrenal vascular resistance and increases adrenal blood flow (Mazzocchi *et al.* 1998). It has been shown that vasoconstrictile and vasorelaxant responses to ET isopeptides are mediated primarily by ETA and ETB receptors respectively (Masaki *et al.* 1994). Vasodilation resulting from ET-1-induced activation of ETB receptors is modulated by endothelium-derived relaxing substances, such as nitric oxide and prostaglandin I_2 , that are released basally through the activation of ETB receptors (de Nucci *et al.* 1988, Tsukahara *et al.* 1994, Zellers *et al.* 1994, Schilling *et al.* 1995, Gellai *et al.* 1996). It is conceivable that the up-regulation of the ETB

Figure 5 Expression of ET-1 mRNA in diabetic rat adrenal gland. (A) Detection of mRNAs for ET-1 and 18S in the rat adrenal gland by relative multiplex RT-PCR. Representative data from each group are shown. The expected PCR products of 500 bp (ET-1) and 315 bp (18S) were detected. Lane M contains a DNA-size marker. (B) Relative expression of ET-1 mRNA normalized against 18S. Each bar represents the mean $\pm$ S.E.M. of six rat adrenal glands in each group. C8; 8-week control, D8; 8-week diabetic, C16; 16-week control, D16; 16-week diabetic, DI16; insulin-treated diabetics. *, Significantly different from C8 ($P \leq 0.05$); **, Significantly different from C16 and D16 ($P \leq 0.05$).

receptors observed in the diabetic adrenal gland may be a compensatory mechanism for maintaining adrenal blood flow.

Glucocorticoids are hypersecreted in diabetes (Scribner *et al.* 1993, Schwartz *et al.* 1997). The mechanisms underlying this phenomenon are unclear. It is possible that the diabetes-induced ETB receptor and ET-1 isopeptide up-regulation shown in the present study stimulates adrenocortical function, since ETs have been shown to exert corticosteroid secretagogue effects, which are mediated by the ETB receptor subtype (Cozza *et al.* 1992, Belloni *et al.* 1997, Pecci *et al.* 1998). It is interesting to note that in our autographographic studies ETB was the predominant ET receptor subtype in the zona fasciculata, where corticosterone is produced. We speculate that endothelin mediates corticosterone secretion through the activation of ETB receptors in diabetic rats. It is also known that the catecholamine secretagogue action of ET-1 is mediated by both the ETA and the ETB receptors (Belloni *et al.* 1997). Thus, up-regulation of the ET isopeptide, ET-1, and the ET receptors might be involved in hypersecretion of both the adrenal cortex and the adrenal medulla.

Figure 6 Expression of ET-3 mRNA in diabetic rat adrenal gland. (A) Detection of mRNAs for ET-3 and 18S in the rat adrenal gland by relative multiplex RT-PCR. Representative data from each group are shown. The expected PCR products of 384 bp (ET-3) and 495 bp (18S) were detected. Lane M contains a DNA-size marker. (B) Relative expression of and ET-3 mRNA normalized against 18S. Each bar represents the mean ± S.E.M. of six rat adrenal glands in each group. C8; 8-week control, D8; 8-week diabetic, C16; 16-week control, D16; 16-week diabetic, DI16; insulin-treated diabetics. *, Significantly different from C8 ($P \leq 0.05$); **, Significantly different from C16 and DI16 ($P \leq 0.05$).

It has been suggested that ET-1 exerts a marked mitogenic effect on the zona glomerulosa of rat adrenals by acting through ETA receptors (Belloni *et al.* 1996, Mazzocchi *et al.* 1997). This is in accord with the cell-migration theory of adrenocortical cytogenesis, which indicates that the zona glomerulosa is the proliferative layer involved in the maintenance of growth of the entire adrenal cortex (Nussdorfer *et al.* 1997). In our autoradiographic studies, ETA receptors were detected densely in the zona glomerulosa. ETA receptors in this region might be involved in the adrenocortical growth stimulated by ETs. The adrenal hypertrophy observed in STZ-induced

Figure 7 Autoradiograms of ¹²⁵I-ET-1 binding to rat adrenal gland, and hematoxylin-eosin staining in adjacent adrenal sections from 16-week control (A), diabetic (B) and insulin-treated diabetic (C) rats. Panels a, b and c demonstrate the levels of total binding with 80, 40 and 20 pM ¹²⁵I-ET-1 respectively. Panels d, e and f are corresponding non-specific binding levels obtained in the presence of 100 nM unlabeled ET-1. Images g and h show the levels of 20 pM ¹²⁵I-ET-1 binding in the presence of 30 nM STXc and 300 nM BQ 123, showing the expression of ETA and ETB receptor subtypes respectively. Panel i is a hematoxylin-eosin-stained photomicrograph. 'C' and 'M' indicate the cortex and medulla, respectively, of the adrenal gland. The designated cortical and medullary areas in (A), panel g, are shown in Fig. 8.

Figure 8 Liquid emulsion autoradiographic images of ^{125}I -ET-1 binding and hematoxylin-eosin staining of cortical and medullary areas in adjacent sections from control rat adrenal glands. (A), (B) and (C) show the levels of total binding with 80, 40 and 20 pM ^{125}I -ET-1, respectively, in 16-week control rat adrenal glands. (D) and (E) show the levels of 20 pM ^{125}I -ET-1 binding in the presence of 30 nM STXc and 300 nM BQ 123, showing the expression of ETA and ETB receptor subtypes respectively. (F) shows hematoxylin-eosin staining of adjacent adrenal sections (magnification, $400\times$). ZG, zona glomerulosa; ZF, zona fasciculata; ZR, zona reticularis; M, medulla. This figure was prepared from the cortical and medullary areas of an adrenal gland of a control rat, as shown in Fig. 7A, panel g.

diabetes (Lucas & Foy 1977, Scribner *et al.* 1991, 1993, Schwartz *et al.* 1997) might be derived from the growth-promoting effects of ETs.

The present study shows differential regulation of ET isopeptides, i.e. ET-1 mRNA was increased, whereas ET-3 mRNA was reduced following the induction of STZ-induced diabetes. The differential regulation of ET isopeptides and ET receptor subtypes suggests that these factors are regulated by different mechanisms, and that their contribution could compete with, or support, each other. Insulin treatment started 8 weeks after the induction of diabetes effectively reversed the alterations in the expression of ET isopeptides. These findings suggest a direct regulation of the ET system by insulin (Oliver *et al.* 1991). Diabetes-induced changes in ET receptor-

mediated mechanisms may play a role in the development of diabetic complications related to adrenal hyperfunction, and appear to be reversible. The ET system could be a suitable target for pharmacological intervention, and the use of ET- receptor antagonists at an early stage, as postulated by Clozel and co-workers (1993), might prevent or retard the development of diabetic complications related to adrenal hypersecretion.

Acknowledgements

This study was supported, in part, by NIH grants DK 42530 and DK 38311 and by an Edwin Beer Grant from the New York Academy of Medicine.

References

- Baron AD, Steinberg HO, Chaker H, Leaming R, Johnson A & Brechtel G 1995 Insulin-mediated skeletal muscle vasodilation contributes to both insulin sensitivity and responsiveness in lean humans. *Journal of Clinical Investigation* **96** 786–792.
- Belloni AS, Malendowicz LK, Gottardo G & Nussdorfer GG 1996 Endothelin-1 stimulates the proliferation of rat adrenal zona glomerulosa cells, acting via the ETA receptor subtype. *Medical Science Research* **24** 393–394.
- Belloni AS, Pacheco YG, Markowska A, Andreis PG, Meneghelli V, Malendowicz LK & Nussdorfer GG 1997 Distribution and functional significance of the endothelin receptor subtypes in the rat adrenal gland. *Cell and Tissue Research* **288** 345–352.
- Bitar MS, Koulu M, Rapoport SI & Linnola M 1987 Adrenal catecholamine metabolism and myocardial adrenergic receptors in streptozotocin diabetes. *Biochemical Pharmacology* **36** 1011–1016.
- Breslow MJ 1992 Regulation of adrenal medullary and cortical blood flow. *American Journal of Physiology* **262** H1317–H1330.
- Cameron OG, Kronfol Z, Greden JF & Carroll BJ 1984 Hypothalamic–pituitary–adrenocortical activity in patients with diabetes mellitus. *Archives of General Psychiatry* **41** 1090–1095.
- Cardillo C, Nambi SS, Kilcoyne CM, Choucair WK, Katz A, Quon MJ & Panza JA 1999 Insulin stimulates both endothelin and nitric oxide activity in the human forearm. *Circulation* **100** 820–825.
- Clozel M, Breu V, Burri K, Cassal JM, Fischli W, Gray GA, Hirth G, Löffler BM, Müller M, Neidhart W & Ramuz H 1993 Pathophysiological role of endothelin revealed by the first orally active endothelin receptor antagonist. *Nature* **365** 759–761.
- Cohen RA 1993 Dysfunction of vascular endothelium in diabetes mellitus. *Circulation* **87** Supplement V67–V76.
- Cozza EN, Chiou S & Gomez-Sanchez CE 1992 Endothelin-1 potentiation of angiotensin II stimulation of aldosterone production. *American Journal of Physiology* **262** R85–R89.
- De Nicola AF, Fridman O, Del Castillo EJ & Foglia VG 1976 The influence of streptozotocin diabetes on adrenal function in male rats. *Hormone and Metabolic Research* **8** 388–392.
- Frank HJ, Levin ER, Hu RM & Pedram A 1993 Insulin stimulates endothelin binding and action on cultured vascular smooth muscle cells. *Endocrinology* **133** 1092–1097.
- Fukumoto Y, Yoshida M, Dokita S, Kamai T, Weiss RM & Latifpour J 1993 The reversal effect of insulin on diabetes-induced alterations in beta adrenergic and muscarinic receptors in rat prostate. *Journal of Urology* **149** 1602–1606.
- Fushimi H, Inoue T, Kishino B, Nishikawa M, Tochino Y, Funakawa S, Yamatodani A & Wada H 1984 Abnormalities in plasma catecholamine response and tissue catecholamine accumulation in streptozotocin diabetic rats: a possible role for diabetic autonomic neuropathy. *Life Sciences* **35** 1077–1081.
- Gellai M, Fletcher T, Pullen M & Nambi P 1996 Evidence for the existence of endothelin-B receptor subtypes and their physiological roles in the rat. *American Journal of Physiology* **271** R254–R261.
- Goto K 1999 History of endothelin. In *Pulmonary Actions of the Endothelins*, pp 1–20. Eds RG Goldie & DWP Hay. Basel: Birkhäuser Verlag.
- Gregersen S, Thomsen JL & Hermansen K 2000 Endothelin-1 (ET-1)-potentiated insulin secretion: involvement of protein kinase C and the ET(A) receptor subtype. *Metabolism: Clinical and Experimental* **49** 264–269.
- Herkenham M & Pert CB 1982 Light microscopic localization of brain opiate receptors: a general autoradiographic method which preserves tissue quality. *Journal of Neuroscience* **2** 1129–1149.
- Hinojosa-Laborde C & Lange DL 1999 Endothelin regulation of adrenal function. *Clinical and Experimental Pharmacology and Physiology* **26** 995–999.
- Hu RM, Levin ER, Pedram A & Frank HJ 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 ETA receptor. *Life Sciences* **50** 247–255.
- Ikeda K, Wada Y, Foster Jr HE, Wang Z, Weiss RM & Latifpour J 2000 Experimental diabetes-induced regression of the rat prostate is associated with an increased expression of transforming growth factor-beta. *Journal of Urology* **164** 180–185.
- Imai T, Hirata Y, Eguchi S, Kanno K, Ohta K, Emori T, Sakamoto A, Yanagisawa M, Masaki T & Marumo F 1992 Concomitant expression of receptor subtype and isopeptide of endothelin by human adrenal gland. *Biochemical and Biophysical Research Communications* **182** 1115–1121.
- Kemp PA, Gardiner SM, March JE, Rubin PC & Bennett T 1999 Assessment of the effects of endothelin-1 and magnesium sulphate on regional blood flows in conscious rats, by the coloured microsphere reference technique. *British Journal of Pharmacology* **126** 621–626.
- Korth P, Bohle RM, Corvol P & Pinet F 1999 Cellular distribution of endothelin-converting enzyme-1 in human tissues. *Journal of Histochemistry and Cytochemistry* **47** 447–461.
- Lange D, Haywood JR & Hinojosa-Laborde C 1999 ET-A receptor antagonism attenuates adrenal medullary catecholamine release in male but not female rats during hypoglycemia. *FASEB Journal* **13** A1025.
- Latifpour J, Fukumoto Y & Weiss RM 1995 Regional differences in the density and subtype specificity of endothelin receptors in rabbit urinary tract. *Naunyn-Schmiedeberg's Archives of Pharmacology* **352** 459–468.
- Lin HY, Kaji EH, Winkel GK, Ives HE & Lodish HF 1991 Cloning and functional expression of a vascular smooth muscle endothelin 1 receptor. *PNAS* **88** 3185–3189.
- Lucas PD 1985 Effects of streptozotocin-induced diabetes and noradrenaline infusion on cardiac output and its regional distribution in pithed rats. *Diabetologia* **28** 108–112.
- Lucas PD & Foy JM 1977 Effects of experimental diabetes and genetic obesity on regional blood flow in the rat. *Diabetes* **26** 786–792.
- Masaki T 1993 Endothelins: homeostatic and compensatory actions in the circulatory and endocrine systems. *Endocrine Reviews* **14** 256–268.
- Masaki T, Vane JR & Vanhoutte PM 1994 International union of pharmacology nomenclature of endothelin receptors. *Pharmacological Reviews* **46** 137–142.
- Mazzocchi G, Rossi GP, Rebuffat P, Malendowicz LK, Markowska A & Nussdorfer GG 1997 Endothelins stimulate deoxyribonucleic acid synthesis and cell proliferation in rat adrenal zona glomerulosa, acting through an endothelin A receptor coupled with protein kinase C- and tyrosine kinase-dependent signaling pathways. *Endocrinology* **138** 2333–2337.
- Mazzocchi G, Malendowicz LK, Musajo FG, Gottardo G, Markowska A & Nussdorfer GG 1998 Role of endothelins in regulation of vascular tone in the *in situ* perfused rat adrenals. *American Journal of Physiology* **274** E1–E5.
- Munson P & Rodbard D 1980 Ligand: a versatile computerized approach for ligand-binding systems. *Analytical Biochemistry* **107** 220–239.
- de Nucci G, Thomas R, D'Orleans-Juste P, Antunes E, Walder C, Warner TD & Vane JR 1988 Pressor effects of circulating endothelin are limited by its removal in the pulmonary circulation and by the release of prostacyclin and endothelium-derived relaxing factor. *PNAS* **85** 9797–9800.
- Nussdorfer GG, Rossi GP & Belloni AS 1997 The role of endothelins in the paracrine control of the secretion and growth of the adrenal cortex. *International Review of Cytology* **171** 267–308.
- Oliver FJ, de la Rubia G, Feener EP, Lee ME, Loeken MR, Shiba T, Quettermous T & King GL 1991 Stimulation of endothelin-1 gene expression by insulin in endothelial cell. *Journal of Biological Chemistry* **266** 23251–23256.

- Öztürk Y, Altan VM & Yildizoglu-Ari N 1996 Effects of experimental diabetes and insulin on smooth muscle functions. *Pharmacological Reviews* **48** 69–112.
- Pecci A, Romero DG, Lantos CP & Cozza EN 1998 Mechanisms of action of endothelin-1 in rat adrenal. *Life Sciences* **63** 1315–1328.
- Pollock DM 1998 Endothelin receptor subtypes and tissue distribution. In *Endothelin: Molecular Biology, Physiology, and Pathology*. Ed. RF Highsmith. Totowa: Humana Press.
- Rodgers BD, Lau AOT & Nicoll CS 1994 Hypophysectomy or adrenalectomy of rats with insulin-dependent diabetes mellitus partially restores their responsiveness to growth hormone. *Proceedings of the Society for Experimental Biology and Medicine* **207** 220–226.
- Rosenthal HE 1967 Graphic methods for the determination and presentation of binding parameters in a complex system. *Analytical Biochemistry* **20** 525–532.
- Roy M, Collier B & Roy A 1990 Hypothalamic–pituitary–adrenal axis dysregulation among diabetic outpatients. *Psychiatry Research* **31** 31–37.
- Rubanyi GM & Polokoff MA 1994 Endothelins: molecular biology, biochemistry, pharmacology, physiology, and pathophysiology. *Pharmacological Reviews* **46** 325–415.
- Saito M, Wada Y, Ikeda K, Wang Z, Foster Jr HE, Smith SD, Nishi K, Weiss RM & Latifpour J 2000a Expression of endothelin receptor subtypes and their messenger RNAs in diabetic rat prostate: effect of insulin treatment. *Molecular and Cellular Biochemistry* **210** 1–12.
- Saito M, Wada Y, Ikeda K, Wang Z, Smith SD, Foster Jr HE, Nishi K, Weiss RM & Latifpour J 2000b Gene expression, localization, and pharmacological characterization of endothelin receptors in diabetic rat bladder dome. *European Journal of Pharmacology* **387** 253–263.
- 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.
- Sakurai T, Yanagisawa M, Inoue A, Ryan US, Kimura S, Mitsui Y, Goto K & Masaki T 1991 cDNA cloning, sequence analysis and tissue distribution of rat preproendothelin-1 mRNA. *Biochemical and Biophysical Research Communications* **175** 44–47.
- Schilling L, Feger GI, Ehrenreich H & Wahl M 1995 Endothelin-1-induced contraction and relaxation of isolated rat basilar artery: effect of the endothelium. *Journal of Cardiovascular Pharmacology* **26** Supplement 3 S197–S199.
- Schwartz MW, Strack AM & Dallman MF 1997 Evidence that elevated plasma corticosterone levels are the cause of reduced hypothalamic corticotrophin-releasing hormone gene expression in diabetes. *Regulatory Peptides* **72** 105–112.
- Scribner KA, Walker CD, Cascio CS & Dallman MF 1991 Chronic streptozotocin diabetes in rats facilitates the acute stress response without altering pituitary or adrenal responsiveness to secretagogues. *Endocrinology* **129** 99–108.
- Scribner KA, Akana SF, Walker C-D & Dallman MF 1993 Streptozotocin-diabetic rats exhibit facilitated adrenocorticotropin responses to acute stress, but normal sensitivity to feedback by corticosteroids. *Endocrinology* **133** 2667–2674.
- Shiba R, Sakurai T, Yamada G, Morimoto H, Saito A, Masaki T & Goto K 1992 Cloning and expression of rat preproendothelin-3 cDNA. *Biochemical and Biophysical Research Communications* **186** 588–594.
- Shimada K, Takahashi M & Tanzawa K 1994 Cloning and functional expression of endothelin-converting enzyme from rat endothelial cells. *Journal of Biological Chemistry* **269** 18275–18278.
- Simms D, Cizdziel PE & Chomczynski P 1993 TRIZOL: a new reagent for optimal single-step isolation of RNA. *Focus* **15** 99–102.
- Steger RW & Rabe MB 1997 The effect of diabetes mellitus on endocrine and reproductive function. *Proceedings of the Society for Experimental Biology and Medicine* **214** 1–11.
- Strack AM, Sebastian RJ, Schwartz MW & Dallman MF 1995 Glucocorticoids and insulin: reciprocal signals for energy balance. *American Journal of Physiology* **268** R142–R149.
- Tsukahara H, Ende H, Magazine HI, Bahou WF & Goligorsky MS 1994 Molecular and functional characterization of the non-isopeptide-selective ETB receptor in endothelial cells. *Journal of Biological Chemistry* **269** 21778–21785.
- Williams DL, Jones KL, Pettibone DJ, Lis EV & Clineschmidt BV 1991 Sarafotoxin S6c: an agonist which distinguishes between endothelin receptor subtypes. *Biochemical and Biophysical Research Communications* **175** 556–561.
- Xu D, Emoto N, Giaid A, Slaughter C, Kaw S, de Wit D & Yanagisawa M 1994 ECE-1: a membrane-bound metalloprotease that catalyzes the proteolytic activation of big endothelin-1. *Cell* **78** 473–485.
- Yanagisawa H, Yanagisawa M, Kapur RP, Richardson JA, Williams SC, Clouthier DE, de Wit D, Emoto N & Hammer RE 1998 Dual genetic pathways of endothelin-mediated intercellular signaling revealed by targeted disruption of endothelin converting enzyme-1 gene. *Development* **125** 825–836.
- Zellers TM, McCormick J & Wu Y 1994 Interaction among ET-1, endothelium-derived nitric oxide, and prostacyclin in pulmonary arteries and veins. *American Journal of Physiology* **267** H139–H147.

Received 11 July 2000

Accepted 7 September 2000