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.

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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. 1997). 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 neuroendocrine 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 adrenocorticotropic 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 (D116). 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 D116) after STZ-administration. The adrenal glands were immediately dissected and frozen in liquid nitrogen for subsequent RNA and membrane–particle 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 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 were cut with a microtome cryostat (2800 Frigocut N; Leica Inc., Malver, PA, USA) at −22 °C and mounted on aminoalkysilane-coated microscope slides. Autoradiographic localization of 125I-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 125I-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 KH2PO4, 1 mM MgCl2, 1 mM CaCl2, 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 125I-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 125I-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-2H; 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), 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 × 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, Shibata 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 MgCl2, 200 µM 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.

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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. 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 \(^{125}\text{I}-\text{ET-1} \) 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 \(^{125}\text{I}-\text{ET-1} \) binding to the adrenal glands are shown in Table 2. The density of binding sites for \(^{125}\text{I}-\text{ET-1} \) was significantly higher in the diabetic adrenal gland than in the age-matched controls (Fig. 1; Table 2). \( K_d \) values for \(^{125}\text{I}-\text{ET-1} \) 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 \(^{125}\text{I}-\text{ET-1} \) 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 1 Specific primers for RT-PCR

<table>
<thead>
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<th>Product</th>
<th>Sequence</th>
<th>Size (bp)</th>
<th>References</th>
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<tr>
<td>ECE-1</td>
<td>Sense 5'-CGTAGCGGATAGTGCTTAGGAC-3'</td>
<td>529</td>
<td>Shimada et al. 1994</td>
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<td></td>
<td>Antisense 5'-GTCCACCCAAATGACTACAG-3'</td>
<td>500</td>
<td>Sakurai et al. 1991</td>
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<tr>
<td>ET-1</td>
<td>Sense 5'-GCTCCTCTCCCTACTAGG-3'</td>
<td>384</td>
<td>Shiba et al. 1992</td>
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<tr>
<td></td>
<td>Antisense 5'-GGGCTCCTGCAAGCAGG-3'</td>
<td>780</td>
<td>Lin et al. 1991</td>
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<tr>
<td>ET-3</td>
<td>Sense 5'-GAGGTCATGAGGCTTTTG-3'</td>
<td>919</td>
<td>Sakurai et al. 1990</td>
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</table>
Table 2 Maximal binding capacities and dissociation constants of ET receptors in rat adrenal gland

<table>
<thead>
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<th></th>
<th>Control</th>
<th>Diabetic</th>
<th>Diabetic–Insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>8 weeks</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$B_{\text{max}}$</td>
<td>fmol/mg protein</td>
<td>84·3 ± 5·6</td>
<td>129·8 ± 5·8$^a$</td>
</tr>
<tr>
<td></td>
<td>fmol/g tissue</td>
<td>1893 ± 125</td>
<td>2912 ± 131$^a$</td>
</tr>
<tr>
<td>$K_d$ (pM)</td>
<td>95·5 ± 11·2</td>
<td>102·0 ± 7·2</td>
<td>–</td>
</tr>
<tr>
<td><strong>16 weeks</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$B_{\text{max}}$</td>
<td>fmol/mg protein</td>
<td>90·0 ± 7·9</td>
<td>136·3 ± 4·7$^a$</td>
</tr>
<tr>
<td></td>
<td>fmol/g tissue</td>
<td>2020 ± 178</td>
<td>3005 ± 524$^a$</td>
</tr>
<tr>
<td>$K_d$ (pM)</td>
<td>101·8 ± 10·4</td>
<td>104·0 ± 9·5</td>
<td>113·3 ± 13·0</td>
</tr>
</tbody>
</table>

*B$_{\text{max}}$ 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.

*Significantly 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.
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 \(B_{\text{max}}\) 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 \(B_{\text{max}}\) 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).
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

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Diabetic</th>
<th>Diabetic–Insulin</th>
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<tr>
<td>8 weeks</td>
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<tr>
<td>BQ 123</td>
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<tr>
<td>$K_{dH}$ (nM)</td>
<td>1·8 ± 0·3</td>
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<td>$K_{L}$ (µM)</td>
<td>0·61 ± 0·11</td>
<td>0·66 ± 0·17</td>
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</tr>
<tr>
<td>%R$_{H/UT}$</td>
<td>37·0 ± 1·1</td>
<td>18·5 ± 0·3*</td>
<td>–</td>
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<tr>
<td>STXc</td>
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<tr>
<td>$K_{dH}$ (nM)</td>
<td>8·7 ± 1·0</td>
<td>8·2 ± 1·1</td>
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<td>$K_{L}$ (µM)</td>
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<td>%R$_{H/UT}$</td>
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<td>BQ 123</td>
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<td>$K_{L}$ (µM)</td>
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<tr>
<td>%R$_{H/UT}$</td>
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<td>22·0 ± 0·8*</td>
<td>32·9 ± 2·2</td>
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<td>STXc</td>
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<td>%R$_{H/UT}$</td>
<td>46·7 ± 2·0</td>
<td>58·3 ± 1·7*</td>
<td>45·4 ± 3·2</td>
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$K_{dH}$ and $K_{L}$ are estimated dissociation constants for high- and low-affinity binding sites respectively.
%R$_{H/UT}$ 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. *Significantly 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 regulators 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...
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 vasocontractile and vasorelaxant responses to ET isopeptides are mediated primarily by ETA and ETB receptors respectively (Masaki et al. 1994). Vasodilating resulting from ET-1-induced activation of ETB receptors is modulated by endothelium-derived relaxing substances, such as nitric oxide and prostaglandin I₂, 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 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 autogradiographic 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.
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 diabetic rats is consistent with this hypothesis.

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 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 < 0.05); **, Significantly different from C16 and DI16 (P < 0.05).

Figure 7 Autoradiograms of $^{125}$I-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 $^{125}$I-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 $^{125}$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. 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.

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.

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