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Characterization of Endothelin Receptors in Streptozotocin-Induced Diabetic Rat Vas Deferens

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ABSTRACT. As there is increasing evidence that diabetes induces changes in the plasma levels of endothelins (ETs) and in the properties of ET receptors in peripheral tissues, and as there are reports indicating the presence of significant amounts of ET receptors in mammalian vasa deferentia, we studied possible alterations in ET receptor characteristics in the vasa deferentia of the following groups of rats: 8 weeks diabetic (D_8), 8 weeks age-matched control (C_8), 16 weeks diabetic (D_{16}), 16 weeks diabetic-insulin-treated (started 8 weeks after the onset of diabetes) (DI_{16}), and 16 weeks age-matched control (C_{16}). Diabetes was induced by the i.v. injection of 65 mg/kg streptozotocin (STZ). Diabetic rats had hyperglycemia, hypoinsulinemia, glucosuria, polydipsia, and polyuria and had smaller vasa deferentia than control and diabetic-insulin-treated animals. Receptor binding experiments with [^{125}I]ET-1 demonstrated that the densities of ET receptors in vasa deferentia from D_8, C_8, D_{16}, DI_{16}, and C_{16} animals were 377 ± 11, 255 ± 24, 315 ± 18, 210 ± 12, and 214 ± 7 fmol/mg of protein, respectively. [^{125}I]ET-1 binding to the ET receptors was inhibited by ET-1 (non-selective), BQ 610 (ET_A selective), ET-3 (ET_C selective), and IRL 1620 (ET_B selective) with the following rank order of K_i values: ET-1 < BQ 610 < ET-3 << IRL 1620. The pharmacological profile of the ET receptors was similar in all groups and was consistent with the predominance of the ET_A receptor subtype in the rat vasa deferentia. Our data indicate that experimental diabetes up-regulates the density of ET receptors in the rat vasa deferentia and that the receptor up-regulation is reversed by insulin treatment. BIOCHEM PHARMACOL 52;10:1593–1598, 1996. *Copyright © 1996 Elsevier Science Inc.*

KEY WORDS. endothelin receptors; diabetes; vas deferens

ET-1,† described as a 21 amino acid peptide, was isolated from the medium of cultured porcine aortic endothelial cells [1]. The ETs are classified into three isoforms, i.e. ET-1, ET-2, and ET-3, and have been reported to produce a wide variety of biological actions that are thought to be mediated by ET receptors [2]. ET receptors are distributed widely in a number of tissues and cell types [3, 4]. Distinct potencies of ETs in inducing a variety of pharmacologic responses and differences in the rank order of binding affinities of ET receptors for ET isopeptides in a number of tissues suggest the existence of multiple ET receptor subtypes [3–6]. The ET_A receptor subtype shows a selectivity of ET-1 = ET-2 >> ET-3, whereas the ET_B receptor subtype is non-selective for ET-1, ET-2, and ET-3 [3, 4]. More recently, the ET_C receptor subtype to which ET-3 shows a higher affinity than ET-1, has been identified in bovine endothelial cells [7] and on *Xenopus* dermal melanophores [8]. There are, however, no agonists or antagonists that can clearly distinguish the ET_C receptor subtype from the other ET receptor subtypes. ET_A, ET_B, and ET_C receptor subtypes have been confirmed from cloning and sequencing studies of cDNAs [8–10].

ETs induce prolonged contractile responses in isolated vas deferens from rat [11–15], guinea pig [16, 17], and mouse [18]. These data suggest that ETs and ET receptors may be important in the function of mammalian vasa deferentia. Urogenital dysfunction, i.e. impotence, infertility, and retrograde ejaculation, is a common clinical dysfunction of diabetes mellitus [19]. It has been reported that vasa deferentia from chemically induced diabetic rats are supersensitive to contractile agents such as adrenergic and/or cholinergic agonists [20–23] and that the increased contractile responses correlate with the increase in the expression of relevant receptor systems in this tissue [22, 24, 25].

The present study was undertaken to investigate the effect of STZ-induced diabetes on biochemical and pharmacological characteristics of endothelin receptors in rat vasa deferentia.

MATERIALS AND METHODS

Animals

Experimental diabetes was induced as previously reported [26]. Male Sprague-Dawley rats (55 to 56-day-old, weighing

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† *Abbreviations:* ET-1, endothelin-1; D_8, 8 weeks diabetic; C_8, 8 weeks age-matched control; D_{16}, 16 weeks diabetic; DI_{16}, 16 weeks diabetic-insulin-treated; C_{16}, 16 weeks age-matched control; STZ, streptozotocin; PMSF, phenylmethylsulfonyl fluoride; B_{max}, maximum number of binding sites; K_D, equilibrium dissociation constant; K_i, inhibition constant; and n_H, Hill coefficient.

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250–300 g) were divided into five groups: (1) D_8, (2) C_8, (3) D_{16}, (4) DI_{16} and (5) C_{16}. Diabetes was induced in rats in the D_8, D_{16}, and DI_{16} groups with a single injection of 65 mg/kg STZ dissolved in 0.1 M citrate buffer (pH 4.5) via the lateral tail vein. C_8 and C_{16} groups were injected with the same volume of citrate buffer. Eight weeks after the induction of diabetes, DI_{16} rats started to receive 5–8 U of protamine zinc insulin subcutaneously daily for another 8 weeks. Eight or sixteen weeks after the initial injection of STZ or vehicle, rats were killed by decapitation, blood samples were collected for measurement of serum glucose, serum insulin, and serum testosterone, and vasa deferentia were rapidly dissected, trimmed, frozen in liquid nitrogen, and stored at −80° until assayed.

Glucose concentration was measured by the hexokinase method (Glucose H. K., Sigma Chemical Co., St. Louis, MO). Serum testosterone and serum insulin levels were determined with radioimmunoassay kits (Diagnostic Systems Laboratories Inc., Webster, TX).

Preparation of Membrane Particulates

Frozen vasa deferentia were thawed, cut into small pieces, and homogenized in 40–50 vol. 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 PMSF and 10 μg/mL each of aprotinine, leupeptine, pepstatin A, and soybean trypsin inhibitor using a Brinkmann Polytron at 70% of the maximum power with four 15-sec bursts separated by 30-sec cooling intervals. The homogenate was centrifuged at 49,000 *g* for 15 min at 4°. The pellet was rehomogenized, filtered through a 250 μm metallic mesh, and recentrifuged under the same conditions as above. The final pellet was suspended in 20 vol. of the same buffer, and samples were taken for determination of the protein concentration according to Lowry *et al.* [27], using BSA as standard.

Binding Experiments

Binding assays were performed on frozen tissue samples as previously described [28].

In saturation experiments, the membrane suspensions from all groups 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-methione, 0.25% BSA, and 0.14% bacitracin) to 2000 vol. to yield 15–30 μg/mL of protein concentration. Aliquots of vasa deferentia membrane preparations were incubated, in triplicates, for 120 min with increasing concentrations of [^{125}I]ET-1 (4–120 pM) in a total of 0.25 mL at 23° in the presence or absence of 100 nM unlabeled ET-1. At the end of the incubation period, the reaction mixtures were filtered rapidly under a vacuum through Whatman GF/B glass fiber filters using a Brandel Cell Harvester (model M-24R, Brandel Instruments, Gaithersburg, MD). The GF/B glass fiber filters had been treated previously with a 1% BSA solution to reduce the non-specific binding to the filter papers. Each filter disc was washed intensively with 80 mL of ice-cold 50 mM Tris–HCl buffer (pH 8.0). The radioactivity trapped on filter discs was counted by a gamma counter at an efficiency of 70–75%. Specific bindings were calculated by subtracting non-specific binding, obtained from binding activity measured in the presence of 100 nM unlabeled ET-1, from the total binding.

In inhibition binding studies, membrane suspensions were prepared as described above. Aliquots of membrane particulates from C_{16}, D_{16}, and DI_{16} containing 15–30 μg/mL of protein were incubated, in duplicates, with a fixed concentration of [^{125}I]ET-1 (approximately 25 pM) in the presence or absence of increasing concentrations of the following unlabeled peptides: ET-1 (non-selective), ET-3 (ET_C selective), BQ 610 (ET_A selective), and IRL 1620 (ET_B selective) [29–31] in a total volume of 0.25 mL at 23° for 120 min. The remainder of the procedure was the same as that of the saturation studies.

Analysis of Binding Assay

Saturation data were analyzed according to Rosenthal [32] using computer-assisted linear regression of bound/free versus bound in order to calculate the maximum number of binding sites, B_{max} values, and the equilibrium dissociation constant, K_D values. The inhibition data were calculated with a non-linear least-squares parametric curve-fitting program in order to calculate the inhibition constants (K_i values), and Hill coefficient (n_H). The interactive curve fitting for inhibition data provided IC_{50} values (the concentrations of unlabeled drug that inhibit 50% of specific binding) for each non-labeled ET receptor agonist or antagonist. K_i values (the inhibition constants) were obtained from $K_i = IC_{50} / (1 + F/K_D)$, where F and K_D are the free concentration of [^{125}I]ET-1 and its equilibrium dissociation constant [33]. The n_H values were calculated according to Bennett and Yamamura [34]. K_i values were calculated as geometric means, whereas B_{max} and n_H values were calculated as arithmetic means. 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.

Drugs and Chemicals

[^{125}I]ET-1 (2200 Ci/mmol) was purchased from New England Nuclear (Boston, MA). ET-1, ET-3, BQ 610, and IRL 1620 were obtained from Peninsula Laboratory Inc. (Belmont, CA). PMSF, aprotinine, leupeptine, pepstatin A, *N*-acetyl-DL-methionine, BSA, soybean trypsin inhibitor, and bacitracin were obtained from the Sigma Chemical Co. (St. Louis, MO).

RESULTS

General Features

Induction of diabetes was confirmed 2 days after STZ injection by noting that urinary glucose levels were greater

than 1000 mg/dL. The general features of experimental groups are shown in Table 1. At the time the rats were killed, body weights of the D_8 and D_{16} groups were significantly lower than those of C_8 and C_{16} (45 and 52% of control animals, respectively), and weights of vasa deferentia in the D_8 and D_{16} groups were significantly smaller than those in the age-matched control groups (66 and 79% of their control groups, respectively). However, the ratio of vas deferens weight to final body weight was increased in D_8 and D_{16} rats compared with their age-matched controls. Furthermore, D_8 and D_{16} had significantly greater serum glucose levels and lower serum testosterone and serum insulin levels than C_8 and C_{16}. Body weight of DI_{16} was significantly larger than that of D_{16}, and the weight of vasa deferentia of DI_{16} was slightly although not significantly greater than that of D_{16}, indicating that the insulin treatment was effective in alleviating abnormalities in the general features of diabetic animals.

Receptor Binding Data

Our preliminary binding experiments showed that freezing had no significant effect on binding properties of ET-1 in rat vas deferens (data not shown). In saturation experiments with [^{125}I]ET-1, the presence of a single class of specific, saturable, high-affinity binding sites were identified in rat vasa deferentia from the diabetic, diabetic-insulin-treated, and control groups (Fig. 1, Table 2). The B_{max} values, i.e. the density of ET receptors, in D_8 vasa deferentia, calculated on the basis of femtomoles per milligram of protein, were significantly higher than those of the C_8. The receptor density in D_8, however, was moderately, but not significantly, greater than that of C_8 when the B_{max} values were normalized in terms of gram wet weight of tissue, which was probably due to the greater variability in the tissue wet weight measurements and/or to a loss of tissue in the homogenization process. The density of endothelin receptors in D_{16} vasa deferentia was significantly higher than that in the C_{16} group when calculated either on the basis of femtomoles per milligram of protein or per gram wet weight of tissue. The B_{max} values of ET receptors in vasa deferentia of the DI_{16} group were similar to those in the C_{16} group whether normalized to protein content or wet tissue weight. No significant differences were found in the K_D values for [^{125}I]ET-1 binding sites between any of the groups studied. These data indicate that STZ-induced diabetes increases the densities of ET receptors in rat vasa deferentia and that insulin treatment reverses this ET receptor up-regulation.

To examine possible diabetes- and/or insulin treatment-induced alterations in the pharmacological properties of endothelin receptors, inhibition experiments were performed in which [^{125}I]ET-1 binding to vasa deferentia was inhibited competitively with selective endothelin agonists or an antagonist: ET-1 (non-selective), ET-3 (ET_C selective), BQ 610 (ET_A selective), IRL 1620 (ET_B selective) [29–31] (Figs. 2 and 3 and Table 3). Inhibition of [^{125}I]ET-1 binding to vasa deferentia by ET-1, ET-3, BQ 610, and IRL 1620 generated steep competition curves with n_H values

TABLE 1. General features of experimental animals

	Control	Diabetic	Diabetic-Insulin
8 Weeks			
Body wt, g			
Initial	283 ± 3	275 ± 2	
Final	581 ± 24	263 ± 12*	
Vas deferens wt, mg	131 ± 9	87 ± 4*	
Serum glucose, mM	8.6 ± 0.3	30.7 ± 1.5*	
Serum insulin, μU/mL	19.6 ± 2.6	2.2 ± 0.5*	
Serum testosterone, ng/mL	2.6 ± 0.4	0.8 ± 0.4*	
16 Weeks			
Body wt, g			
Initial	287 ± 5	283 ± 4	290 ± 5
Final	645 ± 24	336 ± 19†	482 ± 26‡
Vas deferens, wt, mg	130 ± 11	103 ± 8*	113 ± 6
Serum glucose, mM	8.3 ± 0.3	29.0 ± 0.8*	26.3 ± 2.3*
Serum insulin, μU/mL	20.7 ± 2.0	4.4 ± 0.9†	45.5 ± 18.0
Serum testosterone, ng/mL	3.0 ± 0.5	1.4 ± 0.3†	3.7 ± 0.4

Diabetes was induced in diabetic and insulin-treated diabetic groups with a single i.v. injection of 65 mg/kg STZ. 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. Eight or sixteen weeks after the initial injection of STZ or vehicle, rats were killed by decapitation. Data are means ± SEM of 12–15 determinations in each group.

* Significantly different ($P \leq 0.05$) from control group.

† Significantly different ($P \leq 0.05$) from control and diabetic-insulin-treated groups.

‡ Significantly different ($P \leq 0.05$) from control and diabetic groups.

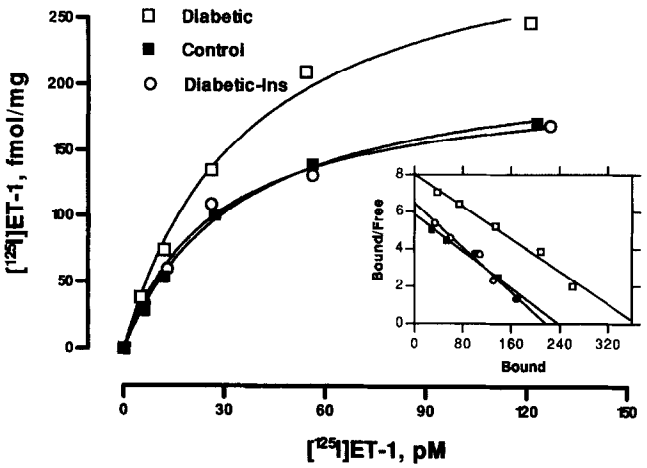

FIG. 1. Saturation of [^{125}I]ET-1 binding in rat vas deferens. Aliquots of vas deferens membrane particulates from 16-week experimental groups were incubated with increasing concentrations of [^{125}I]ET-1 for 2 hr at 23°. Specific binding was defined in the presence and absence of 100 nM ET-1. In the inset, the same data are plotted according to Rosenthal [32]. Each curve represents the mean of a single experiment performed in triplicate.

TABLE 2. Saturation of [^{125}I]ET-1 binding in rat vas deferens

	Control	Diabetic	Diabetic-Insulin
8 Weeks			
B_{max}			
fmol/mg protein	255 ± 24	377 ± 11*	
fmol/g tissue	9,330 ± 1,220	12,200 ± 720	
K_D, pM	40 ± 3	39 ± 1	
16 Weeks			
B_{max}			
fmol/mg protein	214 ± 7	315 ± 18†	210 ± 12
fmol/g tissue	9,080 ± 956	14,800 ± 792†	9,200 ± 905
K_D, pM	36 ± 2	38 ± 3	31 ± 2

Aliquots of membrane particulates were incubated with increasing concentrations of [^{125}I]ET-1 for 2 hr at 23°. Specific binding was defined in the presence and absence of 100 nM ET-1. Data were analyzed by the linear regression of bound vs bound/free according to Rosenthal [32]. B_{max}, maximum number of binding sites; K_D, equilibrium dissociation constant. Data are means ± SEM of 5–6 experiments performed in triplicate.

* Significantly different ($P \leq 0.05$) from control group.

† Significantly different ($P \leq 0.05$) from control and diabetic-insulin treated groups.

close to unity, suggesting an interaction with a single class of binding sites. These curves were better fit to a one-site model than to a two-site model. The rank order of potency of the compounds in inhibiting [^{125}I]ET-1 binding in rat vasa deferentia in all groups was ET-1 > BQ 610 > ET-3 >> IRL 1620. The inhibition data suggest the predominance of the ET_A receptor subtype in rat vasa deferentia. Furthermore, these data indicate that the induction of diabetes and its treatment with insulin do not affect the pharmacological properties and subtype specificities of ET receptors in rat vasa deferentia.

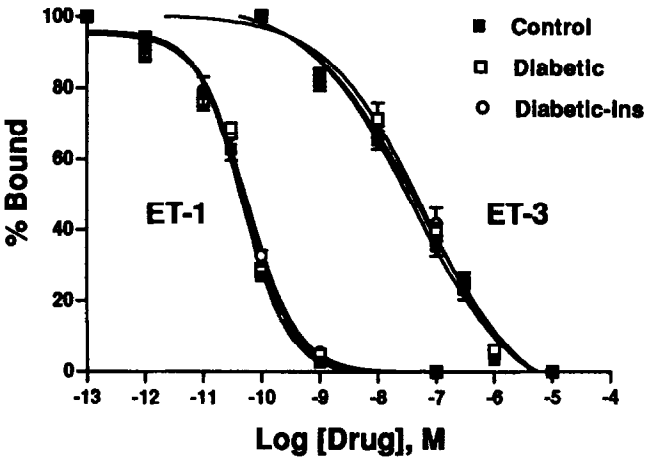

FIG. 2. Inhibition of [^{125}I]ET-1 binding in vas deferens by ET-1 and ET-3. Aliquots of vas deferens membrane particulates from 16-week experimental groups were incubated with increasing concentrations of unlabeled compound and a fixed concentration of [^{125}I]ET-1 for 2 hr at 23°. Each curve represents the mean of 3–5 separate experiments performed in duplicate. The curves are the computer fits of data points according to a one-site binding model.

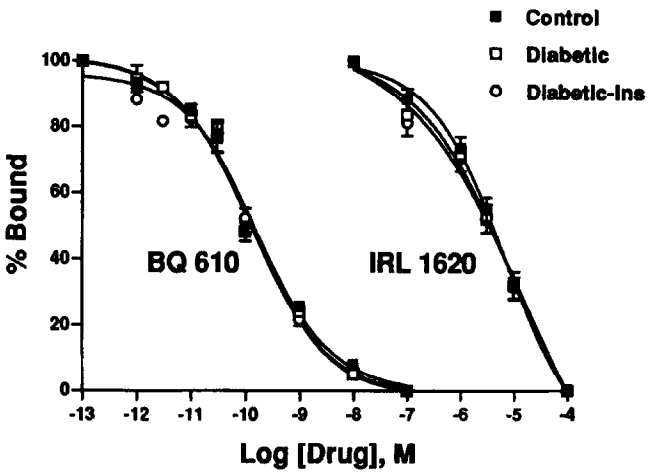

FIG. 3. Inhibition of [^{125}I]ET-1 binding in vas deferens by BQ 610 and IRL 1620. Aliquots of vas deferens membrane particulates from 16-week experimental groups were incubated with increasing concentrations of an unlabeled compound and a fixed concentration of [^{125}I]ET-1 for 2 hr at 23°. Each curve represents the mean of 3–5 separate experiments performed in duplicate. The curves are the computer fits of data points according to a one-site binding model.

DISCUSSION

The present study provides the first report of experimental diabetes-induced alterations in endothelin receptors in rat vasa deferentia. Our data demonstrated that experimental diabetes increases the expression of ET receptors in the rat vas deferens and that the receptor up-regulation is reversed by insulin treatment. Furthermore, these data indicated that the induction of diabetes and insulin treatment does not alter the predominance of the ET_A receptor subtype in rat vasa deferentia.

Diabetes-induced dysfunctions such as impotence, infertility, and retrograde ejaculation are attributed to autonomic neuropathy and/or to disorders in hormonal homeo-

TABLE 3. Inhibition of [^{125}I]ET-1 binding to rat vas deferens by various compounds

	Control	Diabetic	Diabetic-Insulin
ET-1			
K_i, pM	20 ± 4	24 ± 3	24 ± 4
n_H	0.93 ± 0.07	0.89 ± 0.08	0.85 ± 0.01
ET-3			
K_i, nM	12 ± 2	18 ± 5	15 ± 4
n_H	0.82 ± 0.03	0.76 ± 0.04	0.83 ± 0.07
BQ 610			
K_i, nM	0.97 ± 0.11	0.93 ± 0.25	0.67 ± 0.20
n_H	0.80 ± 0.05	0.78 ± 0.02	0.82 ± 0.03
IRL 1620			
K_i, nM	2464 ± 108	2748 ± 1134	2414 ± 880
n_H	0.82 ± 0.02	0.80 ± 0.05	0.84 ± 0.01

Aliquots of membrane particulates were incubated with increasing concentrations of ET-1, ET-3, BQ 610, or IRL 1620 and a fixed concentration of [^{125}I]ET-1 for 2 hr at 23°. K_i, inhibition constant; n_H, Hill coefficient. Values are means ± SEM of 3–5 separate experiments.

stasis [19, 35]. STZ-induced diabetes is reported to decrease the weights of sex accessory organs such as the vas deferens and prostate and to decrease serum testosterone levels [19, 24, 35]. The general features of 8-week and 16-week diabetic rats were similar to those reported previously from our laboratories and from those of others in that STZ-induced diabetes causes a variety of changes including a decrease in body and vas deferens weights [22–24]. However, it is to be noted that in spite of high levels of serum glucose, DI_{16} rats showed normalization of vas deferens weights and testosterone levels with insulin treatment. Thus, it appears that the normalization of vas deferens weights and testosterone levels by insulin treatment of STZ-induced diabetic rats is not related to serum glucose levels. The normalizing effects of insulin replacement on vas deferens weight, and possibly on its receptor composition, are probably related to the normalization of testosterone in DI_{16} as replacement of testosterone levels in diabetic and castrated rats has been shown to normalize vas deferens weights [23].

Vasa deferentia from STZ-induced diabetic rats have been shown to be supersensitive to contractile agents such as norepinephrine [20, 21], acetylcholine [21], and carbachol [22, 23]. Moreover, data from our laboratory and those of others have demonstrated that experimental diabetes causes increases in the densities of muscarinic cholinergic receptors [22, 24] and α_1-adrenergic receptors [25] in the rat vasa deferentia, and that the receptor changes can be prevented by the administration of insulin. These biochemical and functional studies suggest that alterations of autonomic receptors, i.e. muscarinic cholinergic and α_1-adrenergic, may play a role in diabetes-induced sexual dysfunction. Diabetes-induced changes in the biochemical and functional properties of autonomic receptors in reproductive organs also have been observed in castrated animals [22, 23]. As correcting testosterone deficiency in these diabetic animals, either directly by testosterone administration or indirectly by insulin replacement, can normalize receptor-related changes, it has been postulated that the autonomic receptor changes in the diabetic rats may be due to low levels of serum testosterone [24].

Both 8 and 16 weeks of experimental diabetes caused a similar up-regulation in the density of ET receptors, indicating that a further increase in the duration of diabetes beyond 8 weeks did not increase the magnitude of the ET receptor up-regulation. It is to be noted that insulin treatment begun 8 weeks after the induction of diabetes could reverse the diabetes-induced up-regulation of ET receptors in the rat vas deferens.

The subtype specificity, the proportion of ET receptor subtypes, and the physiological function of ET receptors in mammalian vas deferens are yet to be determined. As ET-3 was shown to be more potent than ET-1 in the enhancement of field stimulation-induced contractions in the rat vasa deferentia, Telemaque and D'Orléans-Juste [14] suggested that ET_B receptors mediate the contractile response in this tissue. This is contrary to results reported by Maggi *et al.* [13] who showed an opposite rank order of potency for ET-1 and ET-3 in mediating contractions in the same preparation. In a more recent study, Warner *et al.* [15], using a series of selective agonists and antagonists, showed that ET-1 potentiates field stimulation-induced contractile responses in the prostatic part of the rat vasa deferentia and suggested that the response is consistent with the involvement of postjunctional ET_A receptor subtypes in this process. Furthermore, as it was shown that ET-1 is significantly more potent than ET-3 and sarafotoxin S6c in inducing contractile responses in vas deferens, and as the response is resistant to the inhibitory effect of 1 μM BQ 123, a potent ET_A antagonist, the presence of an atypical ET receptor subtype, possibly ET_C, in the rat vas deferens was postulated [12]. The possibility of an atypical endothelin receptor subtype in mouse vasa deferentia also has been reported by Maas *et al.* [18]. Sudjarwo *et al.* [36] suggested that ET_A receptor subtypes be subclassified into two subclasses based on the sensitivity to the ET_A receptor antagonist BQ 123, i.e. ET_{A1} which is BQ 123-sensitive and ET_{A2} which is BQ 123-insensitive.

In the present study, using a variety of ET receptor subtype selective and non-selective compounds, we examined possible alterations in endothelin receptor subtype specificities in vasa deferentia of 16 week diabetic, 16 week diabetic-insulin-treated (insulin treatment began 8 weeks after the induction of diabetes), and age-matched control rats. The rank order of potency of these selective compounds for inhibiting [^{125}I]ET-1 binding in membrane particulates from rat vasa deferentia (ET-1 > BQ 610 > ET-3 >> IRL 1620) indicates that ET_A is the predominant receptor subtype in all tissues examined and that pharmacological properties and receptor subtype specificities of endothelin receptors in rat vasa deferentia are not affected by the induction of diabetes or by insulin treatment. However, it should be noted that although our inhibition data indicate that the majority of ET receptors in these tissues are of the ET_A subtype, the presence of a minority population, possibly less than 10% of the total, of the ET_B receptor subtype may not be ruled out. This is an important question as it is still unknown what ET receptor subtype mediates the contractile response in vas deferens.

In summary, experimental diabetes caused an up-regulation of ET receptors in rat vas deferentia, and insulin treatment started 8 weeks after the induction of diabetes reversed these alterations. The predominance of the ET_A receptor subtype in the rat vasa deferentia was not affected by either the induction of diabetes or by insulin treatment of the diabetes.

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