

Streptozotocin-induced diabetes reduces the density of [¹²⁵I]-endothelin-binding sites in rat cardiac membranes

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The effect of acute, streptozotocin-induced diabetes on the affinity (K_D), density (B_{max}) and selectivity of specific, high affinity binding sites for [¹²⁵I]-endothelin ([¹²⁵I]-ET) in rat cardiac membrane fragments was determined. Three days after a single i.v. bolus dose of streptozotocin (60 mg kg⁻¹), the density of [¹²⁵I]-ET binding sites was reduced ($P < 0.01$) without changes in affinity or selectivity.

Introduction Endothelin (ET), a vasoconstrictor polypeptide secreted by vascular endothelial cells (Yanagisawa *et al.*, 1988), has positive inotropic (Ishikawa *et al.*, 1988a; Hu *et al.*, 1988) and chronotropic (Ishikawa *et al.*, 1988b) effects on the heart, increases the amplitude and duration of the cardiac action potential (Ishikawa *et al.*, 1988a), stimulates phospholipase C (Resink *et al.*, 1988) and phosphatidylinositol metabolism (Sugiura *et al.*, 1989) and causes bronchoconstriction (Uchida *et al.*, 1988).

High affinity ET (as [¹²⁵I]-ET) binding sites have been identified in the heart (Ambar *et al.*, 1989; Gu *et al.*, 1989a,b), where the bound [¹²⁵I]-ET is displaceable by cold ET and sarafotoxin S6b (Gu *et al.*, 1989b; Ambar *et al.*, 1989) but not by Ca²⁺- or α -adrenoceptor antagonists (Gu *et al.*, 1989a). Pretreatment with ET causes 'down-regulation' of [¹²⁵I]-ET binding sites (Hirata *et al.*, 1988). Little else is known about the factors which modify their density, affinity or selectivity. Our results show that streptozotocin-induced acute diabetes, a model often used to study the cardiac effects of diabetes (Tani & Neely, 1988), alters the density of cardiac [¹²⁵I]-ET binding sites.

Methods Hearts from adult (200–250 g) Sprague Dawley rats which had been fasted overnight were used for these experiments. Half the rats were injected via the tail vein with a bolus dose of streptozotocin (60 mg kg⁻¹) dissolved in sodium-citrate buffer (pH 4.5), to produce non-ketoacidotic diabetes (Mansford & Opie, 1968). The others received an equal volume of citrate buffer i.v. All rats received rat chow and water *ad libitum*.

On the third day the rats were anaesthetized with a diethylether-O₂ mixture and heparinized. Blood was taken for non-fasting plasma glucose determinations, measured by a glucose oxidase technique (Beckman Astra autoanalyser, U.S.A.).

Cardiac membranes were harvested (Gu *et al.*, 1989a), in a homogenizing medium containing 20 mM NaHCO₃ and 0.1 mM phenylmethylsulphonylfluoride (PMSF) pH 7.4. Protein was assayed by the Lowry method (Lowry *et al.*, 1951) with bovine serum albumin as standard. [¹²⁵I]-ET binding was monitored as previously described (Gu *et al.*, 1989a) with a final protein concentration of 0.16–0.24 mg protein ml⁻¹ in 0.25 ml. Non-specific binding was defined in the presence of 2×10^{-7} M ET. The reaction mixture contained 50 mM Tris and 0.1 mM PMSF, pH 7.4, with 2×10^{-11} M– 1×10^{-9} M [¹²⁵I]-ET. Incubation was at 37°C for 60 min. Bound and free [¹²⁵I]-ET were separated by rapid vacuum filtration across GF/C Whatman filters after dilution with 3.5 ml of ice-cold 10 mM Tris buffer containing 6.6% polyethyleneglycol 6000 (PEG), pH 7.4. After two additional washes with Tris-PEG buffer the radioactivity of the filters was counted in a LKB multiwell γ counter (80% efficiency).

Binding selectivity was established with 10^{-13} – 10^{-8} M ET, 10^{-12} – 10^{-7} M (+)-PN200-110 (isopropyl-4 (2,1,3-benzoxadiazol-4- γ -1)-1,4 dihydro-2,6-dimethyl-5-methoxycarbonyl-pyridine-3-carboxylate), (10^{-13} – 10^{-8} M) sarafotoxin S6b, and [¹²⁵I]-ET (10^{-10} – 2×10^{-10} M).

Data analysis was as previously described (Gu *et al.*, 1988). K_D is the concentration of ligand required to occupy 50% of binding sites, B_{max} the density of binding sites, K_i the inhibition constant of the competing ligand, and IC_{50} the concentration of ligand which displaces 50% of the specifically bound ligand ([¹²⁵I]-ET). Results are presented as mean \pm s.e.mean of 6 experiments, unless otherwise stated. Tests of significance were calculated by Student's *t* test, with $P = 0.05$ as the limit of significance.

Porcine ET, (Protein Research Foundation, Osaka, Japan) was iodinated (Gu *et al.*, 1989a) to a specific activity of approximately 1600 Ci mmol⁻¹. Sarafotoxin S6b, (+)-PN200-110 and streptozotocin came from Peninsula Research Laboratories, Cali-

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fornia, U.S.A., Sandoz Ltd, Basle, Switzerland, and Boehringer Mannheim, Mannheim, Germany, respectively.

Results Plasma from control and streptozotocin-treated diabetic rats contained 10.22 ± 0.35 and 28.93 ± 1.07 mmol glucose l^{-1} respectively. The membrane yields were similar for both groups (9.01 ± 0.32 , and 9.12 ± 0.42 mg g^{-1} for control and diabetic hearts respectively). [^{125}I]-ET binding reached asymptote within 60 min, and was linear over a protein range of 0.16 – 0.24 mg ml^{-1} . Binding to reaction tubes and filters was negligible.

Specific [^{125}I]-ET binding for non-diabetic rat membranes was to a single population of sites (Hill coefficient, 0.999 ± 0.004), with a K_D of 0.098 ± 0.007 nM, and a B_{max} of 100.6 ± 5.3 fmol mg^{-1} protein (Figure 1a). [^{125}I]-ET binding for diabetic rat membranes was also to a single population of sites (Hill coefficient, 0.999 ± 0.003), with a K_D (0.090 ± 0.05 nM) not significantly different from the controls. Non specific binding was unchanged. However, (Figure 1a) the B_{max} was reduced ($P < 0.01$) to 70.9 ± 1.4 fmol mg^{-1} protein. Despite this reduction in B_{max} , the selectivity was maintained, with cold ET and sarafotoxin S6b, but not (+)-PN200-110, displacing bound [^{125}I]-ET (Figure 1b and c). The K_i and IC_{50} values for ET and sarafotoxin S6b displacement of [^{125}I]-ET for control membranes were:— K_i , 0.04 nM for ET and 0.12 nM for sarafotoxin S6b; $IC_{50} = 0.08$ nM for ET and 0.23 nM for sarafotoxin S6b. For diabetic rat membranes the K_i was 0.04 nM for ET and 0.17 nM for sarafotoxin S6b, with IC_{50} values of 0.08 nM for ET and 0.33 nM for sarafotoxin S6b.

Adding streptozotocin to isolated membranes, to provide a concentration equivalent to the maximum plasma levels achievable after the bolus injection, altered neither the B_{max} nor the affinity of the [^{125}I]-ET binding.

Discussion These results confirm the ability of [^{125}I]-ET to bind to a single population of sites in rat cardiac membranes (Gu *et al.*, 1989a), and extend them by showing that the density of these sites is reduced in membranes harvested from streptozotocin-treated rats, without changes in affinity or selectivity.

This effect of acute diabetes on the density of cardiac high affinity [^{125}I]-ET binding sites will have to be considered in future studies in which hearts from acutely diabetic rats are used as experimental models. For example, Tani & Neely (1988) used hearts from acutely diabetic rats to study the

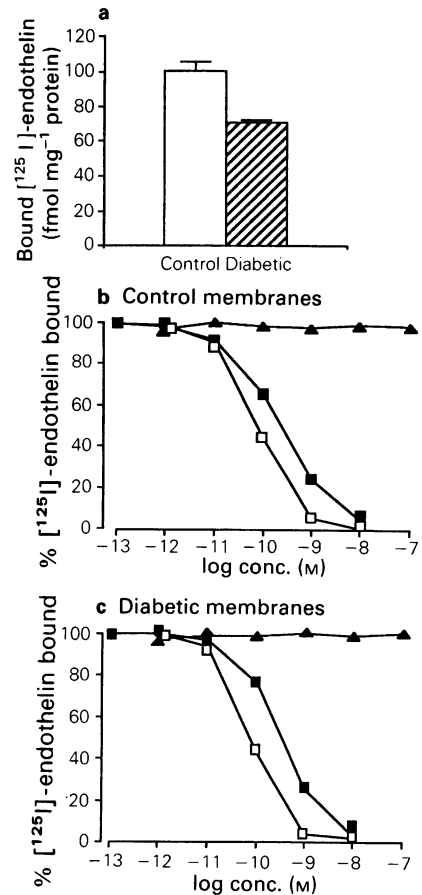


Figure 1 Effect of streptozotocin-induced acute diabetes on [^{125}I]-endothelin ([^{125}I]-ET) binding to cardiac membranes. (a) [^{125}I]-endothelin bound to cardiac membranes from control (open column) and diabetic (hatched column) rats. Each column is mean of 6 experiments (with s.e.mean shown by vertical bars), the estimates for which were performed in duplicate. * = $P < 0.01$. Control (b) + diabetic (c) displacement curves for the effect of cold endothelin (10^{-12} – 10^{-8} M) (□), sarafotoxin S6b (10^{-12} – 10^{-8} M) (■) and (+)-PN200-110 (10^{-13} – 10^{-8} M) (▲) on specifically bound [^{125}I]-endothelin from cardiac membranes. Similar results were obtained in 3 other experiments.

effect of diabetes on ischaemia- and reperfusion-induced injury. They described a protective effect of diabetes. The reduction in [^{125}I]-ET binding site density described here could contribute to this protection, since ET promotes Ca^{2+} influx (Ishikawa *et al.*, 1988a) and increases energy utilization by way of its positive inotropic and chronotropic activity (Hu *et al.*, 1988). These effects, together with the stimulant effect of ET on phosphoinositol metabolism (Sugiura *et al.*, 1989) and Na^+/H^+ exchange (Wann

et al., 1989) could implicate ET in the loss of Ca^{2+} homeostasis observed during ischaemia and reperfusion.

Our results do not indicate whether the diabetes-induced reduction in [^{125}I]-ET binding site density

is due to receptor 'down regulation'. However, they rule out a direct effect of the streptozotocin.

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