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The In-vitro Effects of Insulin and the Effects of Acute Fasting on Cardiac β-Adrenoceptor Responses in the Short-term Streptozotocin-diabetic Rat

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Abstract—This study demonstrates that in-vitro incubation with insulin results in a reduction of the sensitivity of cardiac tissues from streptozotocin-diabetic rats to isoprenaline. Following incubation with insulin, the sensitivity of left atria and papillary muscles from diabetic animals was not significantly different from those of control animals. Insulin incubation had no effect on the sensitivity of diabetic tissues to forskolin or on ventricular β-adrenoceptor number. Reduction of blood glucose in the absence of insulin by fasting, did not affect the sensitivity of tissues from diabetic animals to isoprenaline. These results suggest that insulin itself can directly reduce β-adrenoceptor sensitivity without altering receptor number.

Diabetes mellitus may be classified as a disorder of metabolism characterized by chronic hyperglycaemia. The raised glucose level in insulin-dependent diabetes mellitus (IDDM or type 1 diabetes) is due to total or partial failure of the cells of the islets of Langerhans to synthesize or secrete insulin. Individuals with type 1 diabetes replace this insulin with injections of the hormone in an attempt to maintain blood glucose levels as close to normal as possible. Good management of this is essential but, despite better dosage schedules, secondary complications arising from the diabetic state continue to present a major problem to the patient. The most important of these is cardiovascular disease which contributes to 80% of all diabetic deaths (Kannel 1978).

Experimentally, these complications may be studied in animals made diabetic by injections of diabetogenic drugs such as streptozotocin. Streptozotocin has been shown to destroy the insulin-secreting cells of the pancreas and produces a state characterized by hypoinsulinaemia and resulting hyperglycaemia (Mansford & Opie 1968). Using such a model we have previously investigated the effect of short-term diabetes on cardiac adrenoceptors and have shown an increase in atrial and ventricular β-adrenoceptor sensitivity in rats diabetic for periods of two to four weeks. This change could be prevented by in-vivo pretreatment of diabetic animals with insulin (Austin & Chess-Williams 1992).

It has previously been shown that insulin in-vitro can antagonize the positive inotropic responses to noradrenaline of non-diabetic cardiac tissues from the lamb (Lee & Downing 1976) and guinea-pig (Bhagat et al 1981). It is therefore possible that in the diabetic rat, insulin reduces the increased cardiac β-adrenoceptor sensitivity by a similar direct effect. In pithed diabetic rats, however, it has been shown that altered pressor responses to catecholamines can be returned to normal in the absence of insulin when blood glucose levels were returned to normal by fasting (Foy & Lucas 1976). This suggests that it is a secondary effect of insulin, i.e. reduction of blood glucose, which is important in the control of adrenoceptor status.

The aim of the present study was to investigate whether in-vitro incubation with insulin or in-vivo reduction of blood glucose in the absence of insulin can reduce the increased cardiac β-adrenoceptor responsiveness previously reported.

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Materials and Methods

Female Wistar rats, 200–250 g, were made diabetic by a single intraperitoneal injection of streptozotocin (50 mg kg^{-1}) dissolved in 0·01 M citrate buffer (pH 4·5). Control rats were age-matched and received citrate buffer alone.

Effect of in-vitro incubation with insulin

Isolated cardiac tissues. Fourteen days after initial injection, animals were killed by a blow to the head and exsanguinated. Left atria and papillary muscles were removed and set up in a Krebs-bicarbonate solution (composition in mM: NaCl 118·4, KCl 4·7, $NaHCO_3$ 25·0, glucose 11·7, $MgSO_4$ 1·2, $KHPO_4$ 1·2 and $CaCl_2$ 1·9) gassed with 5% CO_2 in O_2 at 37°C. Tissues were suspended under 0·8 g tension and paced at 1 Hz by square-wave pulses (5 ms duration, threshold voltage + 50%), delivered via bipolar electrodes from Grass S48 stimulators. Isometric tension was recorded via Lectromed UF1 (57 g sensitivity range) force transducers, on a Devices M19 polygraph.

Following equilibration, tissues were incubated for 1 h with or without insulin (Humulin 5 munits mL^{-1} (Tahiliani & McNeill 1986)). Cumulative concentration-response curves to isoprenaline were constructed on tissues from both control and diabetic animals. The effect of the presence of insulin on responses to forskolin, phenylephrine (both in the presence of 1 μM propranolol) and calcium chloride were examined in a similar manner in tissues from diabetic animals alone. Preliminary experiments have shown that any effects of Humulin insulin are vehicle independent.

All experiments were performed in the presence of metanephrine (10 μM) and desipramine (1 μM) to inhibit extraneuronal uptake and neuronal uptake, respectively.

Radioligand binding. Following removal of atria and papillary muscles, the remaining ventricular tissue was cut into small strips and incubated in aerated Krebs solution in the presence or absence of insulin in a similar manner to isolated tissue experiments. The viability of test strips after 1 h was confirmed by setting up a sample of paced tissues and constructing concentration-response curves to isoprenaline.

Following incubation, the remaining strips were plunged into ice-cold Tris buffer (50 mM, pH 7·4) and a crude membrane fraction was prepared by homogenizing for 15 s using an Ultra-Turrax homogenizer, followed by four times four strokes of a Teflon homogenizer. The homogenate was centrifuged at 1000 *g* for 10 min in a Centaur 2 M.S.E. Chillspin centrifuge. The supernatant was then re-centrifuged at 30 000 *g* for 20 min in an M.S.E.18 centrifuge. The resulting pellet was resuspended in 4·5 mL Tris buffer and used in a receptor binding assay. All procedures were carried out at 4°C.

The crude membrane fraction was assayed for β-adrenoceptor binding sites by use of [^{3}H]dihydroalprenolol. Samples of the membrane suspension were incubated at 37°C for 30 min, with a range of radioligand concentrations (0·5–16·0 nM) in a final volume of 250 μL. Non-specific binding was determined by displacement with 200 μM isoprenaline. Incubations were terminated by addition of 1 mL ice-cold buffer, followed by rapid filtration through Whatman GF/B glass fibre filters. The filters were rapidly washed three times with 5 mL ice-cold buffer. Radioactivity remaining on the filters was measured by standard scintillation-counting techniques. Specific binding as a percentage of the total binding was approximately 65%. Protein content of the membranes was determined by the method of Lowry et al (1951).

Effect of in-vivo alteration of blood glucose

Thirteen days after streptozotocin injection, food was withdrawn and rats were given free access to water only. Twenty-four hours later these fasted rats were killed and isolated cardiac tissues set up as previously described.

Following equilibration, concentration-response curves were obtained to isoprenaline. Experiments were performed in the presence of uptake blockers. Responses were compared with those obtained on tissues from fed fourteen-day control and diabetic animals.

Twenty-four hour fasting has previously been shown to have no significant effects on heart rate, blood free fatty acids or on responses of control animals to isoprenaline (Foy & Lucas 1976).

Statistical analysis

Mean blood glucose levels were calculated with s.e. mean and differences examined by use of Student's *t*-test following analysis of variance.

Isolated tissues. Increases in developed tension were plotted as a percentage of the maximum increase for each drug. Individual EC50 values were determined and geometric mean EC50 values with 95% confidence limits calculated. Student's *t*-test was performed on logarithmic EC50 values. Mean maximum responses (with s.e. mean) were calculated and Student's *t*-test performed on these values to test for differences between control and diabetic animals. In addition, two way analysis of variance was used to examine the effect of diabetes, the effect of insulin, and the interaction between the two.

Binding experiments. Dissociation constants (K_d) and the maximum number of binding sites (B_{max}) for specific binding were calculated by Scatchard analysis of saturation curves performed in duplicate. Mean values (with s.e. mean) were calculated and an unpaired Student's *t*-test employed to examine differences between groups of animals.

Drugs used

[^{3}H]Dihydroalprenolol (100 Ci mmol^{-1}) was obtained from the Radiochemical Centre, Amersham, UK. Humulin S was obtained from Lilly (Basingstoke, UK). All other drugs were obtained commercially as the hydrochloride salts from Sigma (St Louis, MO, USA). All reagents were of Analar grade.

Table 1. Mean tensions (g±s.e.m.) developed by tissues from diabetic and control rats in the presence or absence of insulin.

	n	Left atria	n	Papillary muscle
Control	5	0·24±0·03	4	0·27±0·05
Control+insulin	8	0·15±0·04	6	0·18±0·07
Diabetic	4	0·45±0·18	5	0·87±0·14
Diabetic+insulin	5	0·28±0·10	9	0·41±0·08*

* $P<0·001$ with respect to tissues in the absence of insulin.

Table 2. Geometric mean EC50 values (with 95% confidence limits) (n≥4) and mean maximum increases in developed tension (±s.e.m.) to isoprenaline of left atria and papillary muscles from control and diabetic rats in the presence and absence of insulin.

	Left atria		Papillary muscle	
	EC50 (nM)	Maximum tension (g)	EC50 (nM)	Maximum tension (g)
Control	121·6 (39·6–373·7)	0·3±0·1	147·5 (63·4–343·2)	0·5±0·2
Control+insulin	211·1 (56·4–789·7)	0·4±0·1	523·2 (138·2–1980·6)	0·4±0·2
Diabetic	3·8 (1·3–11·4)***	0·4±0·2	15·8 (7·4–31·0)**	0·4±0·2
Diabetic+insulin	45·5 (14·4–143·6)†††	0·5±0·1	197·4 (156·3–692·3)††	0·6±0·1

** $P<0·01$, *** $P<0·005$ relative to control tissues. †† $P<0·01$, ††† $P<0·005$ compared with insulin absent.

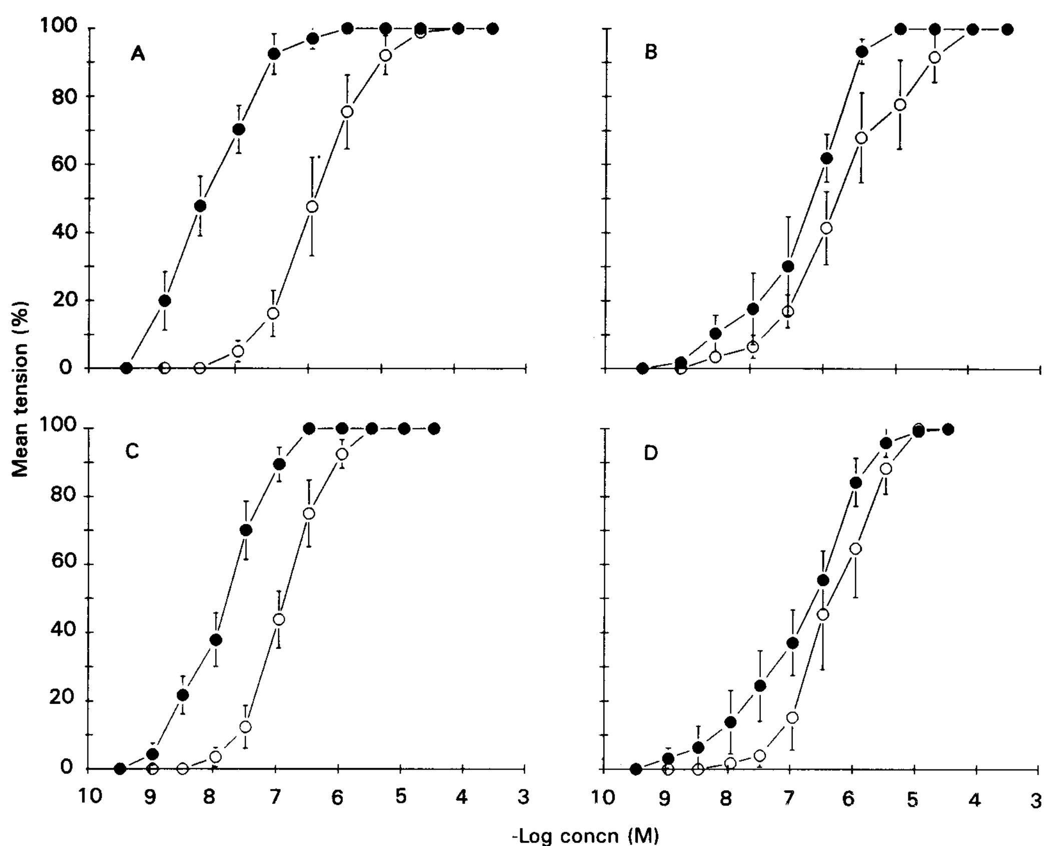

FIG. 1. Concentration-response curves to isoprenaline of left atria (A, C) and papillary muscles (B, D) from control (○) and 14-day streptozotocin-diabetic rats (●) following 1-h incubation in the presence (C, D) and absence (A, B) of insulin.

Results

Effect of insulin incubation

At the time of death, two-week diabetic animals had a mean blood glucose concentration of 20·3 ± 0·6 mM which was significantly elevated ($P < 0·001$) with respect to the mean value of 4·02 ± 0·4 mM for control animals.

Isolated tissues

The resting tensions developed by isolated left atria and papillary muscles from control rats were similar to those for tissues from diabetic animals (Table 1). Incubation with insulin reduced the resting tensions developed by tissues; however, this was only significant for papillary muscles from diabetic animals.

Table 3. Geometric mean EC50 values (with 95% confidence limits) ($n \geq 4$) and maximum increases in developed tensions (±s.e.m.) to phenylephrine, calcium and forskolin of left atria and papillary muscles from diabetic animals in the presence and absence of insulin.

	Insulin absent	Insulin present
Phenylephrine		
Left atria EC50 (μM)	0·76 (0·36–1·62)	1·15 (0·31–4·35)
Maximum tension (% isoprenaline maximum)	43·89 ± 7·70	36·73 ± 14·13
Papillary muscle EC50 (μM)	2·50 (1·17–5·35)	1·28 (0·54–3·00)
Maximum tension (% isoprenaline maximum)	32·75 ± 9·76	20·41 ± 3·88
Calcium		
Left atria EC50 (mM)	7·59 (4·54–12·69)	9·98 (6·60–15·10)
Maximum tension (g)	0·46 ± 0·12	0·45 ± 0·07
Papillary muscle EC50 (mM)	6·63 (4·28–10·26)	11·00 (8·10–14·93)
Maximum tension (g)	0·46 ± 0·10	0·46 ± 0·09
Forskolin		
Left atria EC50 (μM)	0·17 (0·05–0·58)	0·15 (0·02–80·99)
Maximum tension (g)	0·28 ± 0·09	0·35 ± 0·08
Papillary muscle EC50 (μM)	0·34 (0·12–1·00)	10·84 (0·24–2·45)
Maximum tension (g)	0·46 ± 0·12	0·28 ± 0·08

Responses to isoprenaline. Both left atria and papillary muscles from diabetic animals were significantly more sensitive to the positive inotropic effects of isoprenaline than tissues from non-diabetic rats. This was indicated by a reduction of isoprenaline EC50 values in tissues from streptozotocin-treated animals ($P<0.05$ by two-way analysis of variance) without any significant effect being observed on the maximum responses (Table 2).

The presence of insulin in the bathing medium increased isoprenaline EC50 values in both left atria and papillary muscles from control and diabetic rats ($P<0.05$ by analysis of variance). The insulin-induced reduction in sensitivity was greater in tissues from diabetic animals than those from control animals although, when studied by two way analysis of variance, this was not significant ($P<0.08$ for left atria, $P<0.1$ for papillary muscles). In the presence of insulin, however, there was no significant difference between the sensitivity to isoprenaline of tissues from control and streptozotocin-treated animals (Fig. 1).

Maximum developed tensions to isoprenaline were not significantly affected by the presence of insulin (Table 2).

Responses to forskolin, phenylephrine and calcium. The sensitivity and maximum developed tensions to forskolin, phenylephrine and calcium of tissues from diabetic animals were similar in the presence and absence of insulin (Table 3).

[^{3}H]Dihydroalprenolol binding

The density of [^{3}H]dihydroalprenolol binding sites was greater ($P<0.05$) in membranes prepared from diabetic animals (72.1 ± 16.0 fmol (mg protein)$^{-1}$) than in membranes prepared from control animals (41.3 ± 4.7 fmol (mg protein)$^{-1}$) when experiments were performed in the absence of insulin. The K_d values for binding were similar in the two groups (1.5 ± 0.6 and 1.5 ± 0.5 nM in diabetic and non-diabetic experiments, respectively). Ventricular membranes prepared from diabetic animals and incubated with insulin exhibited a B_{max} value of 79.8 ± 9.5 fmol (mg protein)$^{-1}$. Although the difference was not significant this was greater than the B_{max} of 49.3 ± 9.7 fmol (mg protein)$^{-1}$ for control preparations incubated with insulin. Dissociation constants

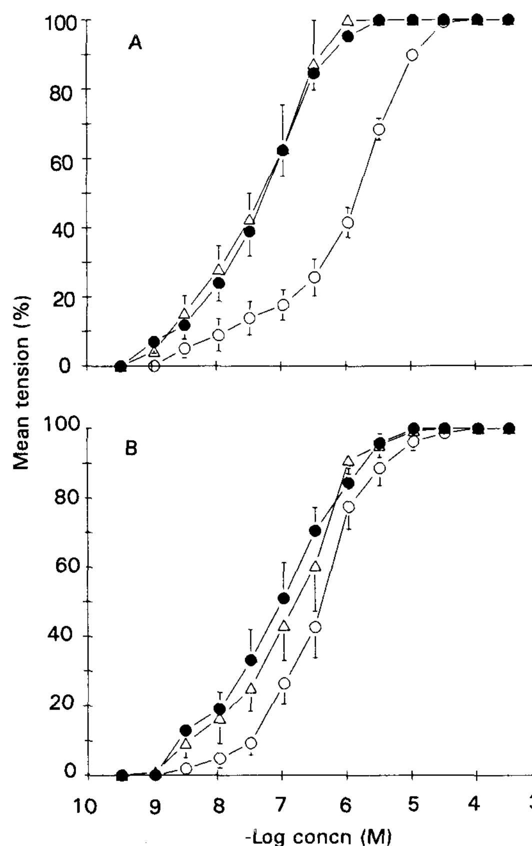

FIG. 2. Concentration-response curves to isoprenaline of left atria (A) and papillary muscles (B) from control (○), 14-day diabetic (●) and fasted 14-day diabetic rats (△).

Table 4. Mean geometric EC50 values (n ≥ 4) and maximum developed tensions (g) to isoprenaline (nM) and calcium (mM) of left atria and papillary muscles from 14-day control, diabetic and fasted diabetic rats.

	Control	Diabetic	Fasted diabetic
Isoprenaline			
Left atria			
EC50	38·39 (21·90–67·29)	5·08 (2·67–9·66)	3·04 (1·30–7·12)
Tension	0·41 ± 0·05	0·40 ± 0·07	0·42 ± 0·08
Papillary muscle			
EC50	57·40 (27·62–119·27)	10·60 (3·45–32·61)	13·14 (4·96–34·82)
Tension	0·59 ± 0·10	0·49 ± 0·05	0·49 ± 0·09
Calcium			
Left atria			
EC50	4·16 (2·86–6·06)	4·86 (2·41–9·77)	4·78 (4·16–5·48)
Tension	0·36 ± 0·10	0·31 ± 0·09	0·40 ± 0·06
Papillary muscle			
EC50	6·12 (1·17–32·01)	3·93 (2·54–6·09)	3·96 (2·91–5·37)
Tension	0·33 ± 0·04	0·46 ± 0·07	0·43 ± 0·12

were similar for control and diabetic preparations being 1·0 ± 0·1 and 1·0 ± 0·3 nM, respectively.

Examination of in-vivo changes in glucose concentrations

Blood glucose and weight changes. Thirteen days after streptozotocin administration, animals used in this study had a mean blood glucose of 17·2 ± 2·3 mM, a value similar to that of the 14-day diabetic animals. Following a 24-h fast, the mean blood glucose had fallen to 5·2 ± 1·0 mM which was similar to control values.

Isolated cardiac tissues

Responses to isoprenaline. Cardiac tissues from 14-day fasted diabetic rats were supersensitive to the positive inotropic response of isoprenaline compared with 14-day controls, as indicated by a shift to the left of the isoprenaline concentration-response curves. This shift was similar to that seen on tissues from non-fasted 14-day diabetic rats (Fig. 2). Maximum developed tensions of both left atria and papillary muscles from all animals were similar (Table 4).

Responses to calcium. Sensitivity and maximum developed tensions to calcium were not altered by fasted diabetic animals (Table 4).

Discussion

The results obtained in the present study confirm the previously reported cardiac β-adrenoceptor supersensitivity observed in streptozotocin-diabetic rats of two and four weeks duration. In-vivo treatment of such diabetic rats with insulin has previously been found to prevent the diabetes-induced increase in β-adrenoceptor sensitivity (Austin & Chess-Williams 1992).

In the present study, in-vitro incubation with insulin was found to reduce the resting tensions developed by tissues from control and diabetic animals. Insulin has previously been shown, however, to increase the force of contraction of isolated cardiac tissues (Lucchesi et al 1972; Lee & Downing 1976; Bhagat et al 1981). The reason for this discrepancy is unknown, although the time of incubation may be important in determining the effects seen (Lucchesi et al 1972).

It was also found that incubation of both left atria and papillary muscles with insulin reduced their sensitivity to isoprenaline relative to the sensitivity of tissues incubated in the absence of the hormone. This was observed in tissues from both control and diabetic animals; however, the reduction in sensitivity by insulin was greater, although not significantly so, in diabetic tissues. In the presence of insulin, EC50 values of both left atria and papillary muscles from diabetic animals were not significantly different from those of similar tissues from control animals.

Insulin has previously been shown to antagonize the positive inotropic response to adrenaline of non-diabetic cardiac tissues from dogs (Hiatt & Katz 1969), and to noradrenaline of tissues from the lamb (Lee & Downing 1976) and guinea-pig (Bhagat et al 1981). The results of the present study support these findings and further demonstrate that insulin can attenuate the inotropic effects of isoprenaline on cardiac tissues from both control and diabetic rats. The effect of insulin is greater on tissues from diabetic animals and, despite this not reaching significance in the present study, it was found to prevent the diabetes-induced β-adrenoceptor supersensitivity.

The results of the present study, demonstrating an effect of in-vitro insulin administration, suggest that the increased diabetic cardiac β-adrenoceptor responsiveness may be prevented by a direct effect of insulin, although it should be noted that the concentration of insulin used in the present study is somewhat higher than that found in-vivo. It has previously been suggested, however, that it may be the influence insulin has on blood glucose which is the important factor in controlling adrenoceptor status (Foy & Lucas 1976). To investigate whether this is important in the increased β-adrenoceptor sensitivity seen in the hearts of our 14-day diabetic rats, elevated blood glucose levels were returned to normal by overnight fasting of streptozotocin-treated animals. These fasted diabetic animals, however, were still supersensitive to the inotropic effects of isoprenaline. The results of the present study, therefore, suggest that the mechanism by which in-vivo administration of insulin prevents or reverses the increased cardiac β-adrenoceptor sensitivity is by some direct effect on the receptor and is not purely a consequence of reduced blood glucose levels.

Previous studies in our laboratory on similar 14-day streptozotocin-diabetic rats have shown that the increased β-adrenoceptor sensitivity was not accompanied by any change in the sensitivity of cardiac tissues to the α-adrenoceptor agonist phenylephrine or to additional calcium. In the present study insulin was found to have no effect on the responses of cardiac tissues from diabetic animals to α-adrenoceptor stimulation or to calcium. The effect of insulin therefore appears to be specific for the β-adrenoceptor and does not appear to be due to any general change in responsiveness of the tissue as indicated by the unaltered responses to phenylephrine and calcium.

Experiments were subsequently carried out in an attempt to obtain more information about the mechanism by which insulin reduces or reverses the diabetic cardiac β-adrenoceptor supersensitivity. Previous studies have shown that the increased sensitivity is accompanied by an increase in the number of cardiac β-adrenoceptors as indicated by an increase in the number of [^{3}H]dihydroalprenolate-binding sites to ventricular membranes (Austin & Chess-Williams 1992). This was also seen in the present study. The elevated adrenoceptor number was observed in diabetic tissues incubated in both the presence and absence of insulin. The mechanism by which insulin, when added to the organ bath, reduces the sensitivity of cardiac tissues to isoprenaline does not, therefore, appear to involve a decrease in the density of β-adrenoceptors. This is in agreement with studies on human mononuclear leucocytes where 35-min in-vitro exposure to insulin was found to reduce β-adrenoceptor responsiveness whilst not affecting receptor density (Sager 1990).

It has been established that insulin combines with a specific glycoprotein receptor (Czech 1985). The receptor is regulated by the concentration of insulin at the cell surface and states of hypoinsulinaemia, such as diabetes mellitus, have been shown to be associated with an increased receptor density (Hepp 1977). This may explain the enhanced effects of insulin on the β-adrenoceptor sensitivity of tissues from diabetic animals relative to those of controls. The second

messenger system for the insulin receptor is poorly understood. The hormone, however, is known to affect a variety of systems and, although cyclic (c) AMP is not thought to be its second messenger, insulin has been reported to suppress increased levels of cAMP response to catecholamines in adipocytes (Manganiello et al 1971). We have previously shown that the increased cardiac β-adrenoceptor sensitivity is accompanied by a change in the cAMP-generating second messenger system of the β-adrenoceptor. This was demonstrated by an increase in the sensitivity of tissues from diabetic animals to forskolin, an agent which elevates intracellular cAMP levels by direct activation of adenylate cyclase (Seamon & Daly 1981; Daly 1984). It seemed possible, therefore, that insulin may reverse the diabetic β-adrenoceptor supersensitivity by inhibiting this elevated second messenger system. In the present study, however, insulin was found to have no effect on the positive inotropic responses of tissues from diabetic animals to forskolin. Insulin may, therefore, be having its effect at some part in the second messenger-generating cascade before adenylate cyclase, presumably on the mechanism coupling receptor occupation to stimulation of adenylate cyclase.

The present study, therefore, demonstrates that insulin in-vitro can reduce the elevated cardiac β-adrenoceptor sensitivity observed in 14-day streptozotocin-diabetic rats. In-vivo insulin administration has previously been shown to reverse this supersensitivity and, as reducing blood glucose in the absence of insulin has no effect on responses, this is probably due to a similar direct effect of insulin on the β-adrenoceptor.

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