Alterations in atrial reactivity in a strain of spontaneously diabetic rats

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- 1 The present study examined the reactivity of atria from control and spontaneously diabetic rats to various adrenoceptor agonists and to adenosine.
- 2 Isoprenaline (1.5 nM–1500 nM) produced concentration-dependent increases in inotropy which were unchanged in diabetic atria. However, the sensitivity to isoprenaline-induced changes in chronotropy was reduced in diabetic preparations.
- 3 In the presence of propranolol (2 μ M), phenylephrine (0.2 μ M–100 μ M) produced concentration-dependent increases in both inotropy and chronotropy; however, atria from diabetic rats exhibited a much greater maximal response. The diabetic state did not alter the sensitivity to phenylephrine.
- 4 Adenosine (0.15 μ M–300 μ M) produced concentration-dependent decreases in both inotropy and chronotropy which were unchanged in diabetic atria.
- 5 Radioligand binding studies revealed that both α_1 - and β -adrenoceptor populations were substantially reduced in atria from diabetic rats. However, there was no change in receptor affinity for either adrenoceptor.
- 6 These results show that diabetes leads to an alteration in atrial reactivity to adrenoceptor stimulation. Future studies examining steps following hormone-receptor coupling are required in order to characterize this defect.

Introduction

Diabetes mellitus is frequently associated with cardiac dysfunction. Clinical and pathological studies along with the epidemiological data from the Framingham study suggest that diabetes mellitus is associated with a specific cardiomyopathy (Kannel *et al.*, 1974; Regan *et al.*, 1977). Animal studies support this concept. Reductions in mechanical performance as well as alterations in myocardial reactivity have been observed in diabetic rats (Fein *et al.*, 1980; Vadlamudi *et al.*, 1982). In particular, animal studies indicate that the responsiveness of the diabetic myocardium to adrenoceptor agonists is changed. However, the degree and nature of this change is still controversial. Foy & Lucas (1976; 1978) observed a decrease in myocardial sensitivity to β -adrenoceptor stimulation under both *in vivo* and *in vitro* conditions. Other studies, however, have failed to detect any difference in either sensitivity or maximum response to β -adrenoceptor stimulation (Ingebretsen *et al.*, 1980; Vadlamudi & McNeill, 1984). Studies examining α -adrenoceptor stimulation are also conflicting. Heyliger *et al.* (1982) demonstrated that methoxamine-stimulated increases in contractile force were significantly attenuated in diabetic preparations. In direct contrast, more recent investiga-

tions have revealed an enhanced inotropic and chronotropic response to α -adrenoceptor stimulation in hearts from diabetic animals (Jackson *et al.*, 1986; Canga & Sterin-Borda, 1986). All of the above animal studies have relied on chemical substances as diabetogenic agents. These substances purportedly selectively destroy beta cells of the pancreas and eventually lead to an insulin-deficient state. Undoubtedly differences in experimental protocol, such as the diabetogenic agent used (alloxan or streptozotocin) or the doses of the agent used, contributed to some of the observed discrepancies.

In an attempt to clarify work in this area we examined the reactivity of atria from a strain of spontaneously diabetic rats to both β - and α -adrenoceptor stimulation. In addition, atrial reactivity to adenosine, an important cardiac regulator, was studied.

Methods

Animal model

Experiments were conducted on a strain of spontaneously diabetic Bio-Breeding (BB) rats. The BB rats

were obtained from a colony maintained at the C.H. Best Institute, University of Toronto. They were initially derived by crossing Long Evans rats (RT1^u haplotype) with seventh generation BB rats from the University of Massachusetts, Worcester. Subsequently, the colony was maintained by random matings between a diabetic male and a non-diabetic female. The overall incidence of overt diabetes is consistently over 80%. As these rats are strictly insulin-dependent they received daily subcutaneous injections of protamine zinc insulin (1.0 u per 100 g body weight) until the day before their sacrifice. At the time the rats were killed (approximately 18 h after the last insulin injection) they were weighed and venous blood collected. Plasma glucose concentration was measured by the glucose-oxidase method (Sigma). Animals used in this study were diabetic for approximately 10 weeks. Age-matched littermates which did not develop diabetes served as controls.

Isolated atrial preparations

Animals were anaesthetized with ether and injected with heparin (1.5 mg kg⁻¹, i.v.). The heart was quickly excised and perfused via the aorta for 15 min at 37°C with freshly oxygenated (95% O₂: 5% CO₂) modified Krebs-Henseleit bicarbonate buffer solution of the following composition (mM): NaCl 117.56, NaHCO₃ 25.00, KCl 5.36, NaH₂PO₄ 1.17, CaCl₂ 1.25, MgSO₄ 1.16, dextrose 11.10, glycerol 9.90 and sodium lactate 5.30 (pH 7.4). After visible blood had been removed from the tissue, left and right atria were excised and suspended vertically in a chamber containing 25 ml of the above solution. Atria were attached on one end to a stimulating electrode and the other end was attached by silk thread to a Grass FTO3C force transducer. Right atria were allowed to beat spontaneously while left atria were electrically stimulated (Grass Stimulator SD9) at a frequency of 1 Hz with rectangular wave pulses of 4 ms duration at a voltage approximately 20% above the threshold. The tissues were allowed to equilibrate for 60 min under a resting tension of 1 g. Based on preliminary resting tension/developed tension curves, this level of resting tension produced approximately 90% maximal force development in atria from both control and diabetic animals. The tension developed was recorded on a Grass Model 7 oscillograph. The force signal arising from the right atria was used to trigger a cardi tachometer (Grass 7P4) from which contraction rate could be recorded.

After a 60 min equilibration period, concentration-response curves were obtained for isoprenaline, phenylephrine, and adenosine. Inotropic (left atria) and chronotropic (right atria) responses to increasing agonist concentrations were obtained by a cumulative increase in the total concentration of the drug in

the organ chamber. Each atria was allowed to remain in contact with the drug for 3 min before each successive concentration of the agonist was added. In order to prevent any β -adrenoceptor mediated effects, the concentration-response curves for phenylephrine were obtained in the presence of a β -adrenoceptor antagonist (Benfey, 1982). Propranolol (2 μ M) was added to the bathing solution and the atria were allowed to equilibrate for 30 min before phenylephrine was added. When the maximum inotropic or chronotropic effect to an agonist was attained, the atria were washed twice and allowed to equilibrate for 30 min before another concentration-response curve was obtained. No more than three concentration-response curves were performed in each atria in order to maintain maximum tissue responsiveness.

At the end of each experiment the tissues were blotted dry, weighed, frozen with liquid nitrogen and stored at -70°C.

Radioligand binding assays

Binding studies were performed on crude atrial membrane fractions. Frozen atrial tissue from 5 animals was thawed and added to 10 ml of ice-cold 50 mM Tris-HCl buffer containing 250 mM sucrose and the following protease inhibitors: 5 mM EDTA, 0.1 mM benzamidine, 1 mM iodoacetamide, 1 μ M pepstatin A, and 1 mM phenylmethylsulphonyl fluoride (pH 7.4). The homogenate was initially prepared by ten passes of a motor-driven glass-Teflon homogenizer. Further homogenization was effected with a Brinkman Polytron by three 5 s bursts at half-maximal setting. The resulting homogenate was passed through 4 layers of surgical gauze under vacuum and centrifuged at 105,000 *g* for 90 min at 4°C using an International B-60 Ultracentrifuge. The resulting membrane pellet was resuspended and washed twice in 8 ml of buffer containing 50 mM Tris-HCl, 1 mM EDTA, 5 mM KCl, 1.5 mM CaCl₂, 4 mM MgCl₂ and 120 mM NaCl (pH 7.4). Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard while sialic acid content of atrial membranes was determined by the enzymatic method of Araki & Yamada (1983).

Radioligand binding experiments were performed as outlined by Williams & Lefkowitz, 1976. Membrane fractions (200 μ l, 100–150 μ g protein) were incubated with varying concentrations of [³H]-dihydroalprenolol (0.5–20 nM) or [³H]-prazosin (0.02–5 nM) in a final volume of 600 μ l. All assays were performed in duplicate. Binding was initiated by placing the tubes in a shaking water bath at 32°C for 30 min. These conditions permitted the complete equilibration of the radioligand with its receptor.

Figure 1 Cumulative concentration-response curves showing the inotropic (a) and chronotropic (b) responses to isoprenaline in atria from control (■) and diabetic (▲) animals. Means ($n = 5-8$) are shown with vertical bars indicating s.e.mean.

The reaction was terminated by the rapid filtration through a Titertek Cell harvester (Skatron Inc.) using glass fibre 'receptor binding' filter mats (Skatron). Filters were washed with 5 ml of 50 mM Tris-HCl buffer (pH 7.4), placed in plastic mini-scintillation vials along with 4 ml of scintillant and monitored for tritium 16 h later in a Packard 4660 liquid scintillation spectrometer at approximately 50% efficiency. Specific binding was defined as that radioligand displaced by either 10 μ M ($\pm$)-propranolol or 10 μ M phentolamine for [3 H]-dihydroalprenolol or [3 H]-prazosin, respectively. Radioligand binding from saturation isotherms was analyzed by the non-linear least squares, curve fitting programme LIGAND (Munson & Rodbard, 1980).

Drugs

The following drugs were used in the present study: (–)-phenylephrine hydrochloride (Sigma), ($\pm$)-isoprenaline (Sigma), adenosine (Sigma), ($\pm$)-propranolol hydrochloride (Sigma), phentolamine hydrochloride (Ciba-Geigy), and protamine zinc insulin (Eli Lilly and Company). [3 H]-dihydroalprenolol (95 Ci mmol $^{-1}$) and [3 H]-prazosin (82 Ci mmol $^{-1}$) were obtained from New England Nuclear. All other chemicals were of analytical grade. Drugs were prepared in Krebs-Henseleit buffer containing ascorbic acid (0.1 μ M). All concentrations are expressed as final molar concentrations of the base in the organ bath.

Statistical analysis

The inotropic and chronotropic responses were expressed as % change from baseline. The EC $_{50}$ values were calculated by computer fitting concentration-response curves to a logistic equation:

$$E = 100 - M \times A^p / (A^p + K^p)$$

Where E is normalized response, M is the maximum response to each drug, A is drug concentration, K is the EC $_{50}$ value of each drug and p is the slope parameter (Parker & Waud, 1971). The EC $_{50}$ values are presented as pD $_2$ values where pD $_2 = -\log$ EC $_{50}$. Experimental values are given as mean $\pm$ s.e.mean. Statistical significance was tested by unpaired Student's *t* test and considered significant if $P < 0.05$.

Results

Animal model

Approximately 10 weeks after the onset of diabetes, body weights were significantly less in diabetic rats relative to age-matched controls ($P < 0.01$). Body weights were 386 ± 8 g and 307 ± 15 g in control and diabetic rats, respectively. Both left and right atrial weight also tended to be lower in diabetic animals but this was not statistically significant ($P > 0.05$). Left atrial weights were 14.8 ± 1.1 mg and 13.1 ± 0.9 mg; while right atrial weights were 12.9 ± 1.4 mg and 11.8 ± 1.2 mg in control and diabetic rats, respectively. Finally, diabetic animals exhibited significantly elevated ($P < 0.01$) plasma glucose levels (22.3 ± 1.3 mM) in comparison to control rats (8.0 ± 0.6 mM).

Inotropic and chronotropic studies

Mechanical properties of atria isolated from diabetic animals were similar to the atria obtained from age-

Table 1 pD_2 values and maximum response of various agonists in atria from control and diabetic rats

	Control	pD_2 Diabetic	Maximum response (%) Control	Maximum response (%) Diabetic
Inotropy (left atria)				
Isoprenaline	7.70 ± 0.12	7.59 ± 0.11	174.8 ± 29.8	162.7 ± 14.3
Phenylephrine	5.60 ± 0.06	5.70 ± 0.06	117.2 ± 15.3	$153.0 \pm 11.3^*$
Adenosine	4.19 ± 0.09	4.37 ± 0.11	84.5 ± 2.6	85.7 ± 2.3
Chronotropy (right atria)				
Isoprenaline	7.82 ± 0.09	$7.55 \pm 0.11^*$	81.3 ± 5.1	74.3 ± 8.6
Phenylephrine	5.49 ± 0.13	5.61 ± 0.15	18.3 ± 1.9	$41.8 \pm 4.6^*$
Adenosine	4.42 ± 0.05	4.35 ± 0.15	80.5 ± 15.8	86.6 ± 8.3

Values are means $\pm$ s.e.mean of 4–7 animals.

* Statistically different from control ($P < 0.05$).

matched control rats. Both basal developed tension and spontaneous contraction rate did not differ significantly ($P > 0.05$) between control and diabetic animals. Basal developed tension of left atria was 40.4 ± 5.1 mg mg^{-1} tissue for control animals while it was 38.8 ± 4.0 mg mg^{-1} tissue for diabetic animals. The spontaneous contraction rates of right atria were 214 ± 10 beats min^{-1} and 198 ± 14 beats min^{-1} for control and diabetic preparations, respectively.

Cumulative additions of isoprenaline (1.5 nM–1500 nM) produced concentration-dependent increases in developed tension in tissues from both control and diabetic animals (Figure 1a). There was no significant difference in either the sensitivity (Table 1) or in the maximum inotropic responses between the two groups of atria. Along with producing changes in inotropy, isoprenaline also produced a concentration-dependent increase in the spontaneous rate of contraction (Figure 1b). Although the maximum chronotropic response was unchanged, diabetic atria exhibited a decreased sensitivity to the isoprenaline-induced chronotropy (Table 1).

In the presence of β -adrenoceptor antagonism (propranolol; $2 \mu M$), phenylephrine ($0.2 \mu M$ – $100 \mu M$) also produced cumulative increases in both developed tension and contraction rate. However, atria from diabetic animals exhibited a significantly greater maximum response to both the inotropic and chronotropic effects of phenylephrine than corresponding atria from control rats (Figure 2a,b). There was no shift in the concentration-response curves to either the inotropic or chronotropic effects in atria from diabetic rats as reflected by the pD_2 values (Table 1).

In contrast to α - and β -adrenoceptor agonists, cumulative additions of adenosine ($0.15 \mu M$ – $300 \mu M$) produced concentration-dependent decreases in both developed tension and spontaneous contraction rate. The maximum responses to both the inotropic and

Figure 2 Cumulative concentration-response curves showing the inotropic (a) and chronotropic (b) responses to phenylephrine in the presence of propranolol ($2 \mu M$) in atria from control (■) and diabetic (▲) animals. Means ($n = 4$) are shown with vertical bars indicating s.e.mean.

chronotropic effects of adenosine were similar in diabetic and control preparations (Table 1). Furthermore, the diabetic state did not significantly alter the pD_2 value for adenosine-induced changes in contraction rate and developed tension (Table 1).

Receptor binding study

As adrenoceptor stimulation resulted in altered responses in atria from diabetic animals further experiments were undertaken to characterize the adrenoceptors. Previous studies have shown that [3H]-dihydroalprenolol and [3H]-prazosin bind specifically and with high affinity to β - and α_1 -adrenoceptors, respectively (U'Prichard *et al.*, 1978; Sastre *et al.*, 1984). In our study, specific binding for [3H]-dihydroalprenolol ranged from 40–60% while specific binding for [3H]-prazosin was between 70–90%.

Results from saturation experiments with [3H]-dihydroalprenolol binding to atrial membranes of control and diabetic membranes are shown in Table 2. Membranes from diabetic rats demonstrated a significant decrease in the total number (B_{max}) of β -adrenoceptors. The density of β -adrenoceptors in membranes from diabetic animals was reduced by 64% and 45% in left and right atria, respectively, compared to corresponding control values. Equilibrium K_d values, on the other hand, were similar in both groups. Saturation isotherms for [3H]-prazosin also yielded a reduction in receptor density that was comparable to the decline seen in β -adrenoceptors.

The α_1 -adrenoceptor population, decreased by 66% and 56% in left and right atria, respectively, in tissues from diabetic animals (Table 2). The K_d for [3H]-prazosin was similar in both groups of membranes.

Finally, diabetes did not alter the yield of membrane protein per mg of initial atrial mass produced by our homogenization procedure nor did it alter the sialic acid content of the membranes relative to the protein concentrations (Table 2).

Discussion

Myocardial reactivity in experimental diabetes has been studied, but no consensus, especially concerning adrenoceptor-mediated stimulation has been reached. In the present study, spontaneously diabetic BB rats were used. These rats have now become an important animal model of type I insulin-dependent diabetes mellitus (Marliss *et al.*, 1982). Although there are numerous investigations relating to various metabolic and biochemical abnormalities in these animals, studies examining cardiovascular function are just beginning (Agrawal & McNeill, 1987; Durante *et al.*, 1988).

This study examined the ability of atria obtained from BB rats to respond to various drugs. It was observed that only the response to adrenoceptor agonists was changed in diabetic animals. Both the inotropic and chronotropic effects of adenosine

Table 2 Membrane properties of atria obtained from control and diabetic rats

	Control	Diabetic
Membrane protein yield ($\mu\text{g mg}^{-1}$ atrial wet weight)	54.0 $\pm$ 2.6	52.6 $\pm$ 2.0
Sialic acid content (nmol mg^{-1} protein)	47.0 $\pm$ 3.1	44.9 $\pm$ 1.9
Left atria		
[3H]-dihydroalprenolol binding		
B_{max} (fmol mg^{-1} protein)	6.68 $\pm$ 1.06	2.41 $\pm$ 0.83*
K_d (nM)	4.52 $\pm$ 1.51	4.84 $\pm$ 1.41
[3H]-prazosin binding		
B_{max} (fmol mg^{-1} protein)	12.08 $\pm$ 2.05	4.16 $\pm$ 1.47*
K_d (nM)	0.38 $\pm$ 0.06	0.30 $\pm$ 0.09
Right atria		
[3H]-dihydroalprenolol binding		
B_{max} (fmol mg^{-1} protein)	8.29 $\pm$ 0.76	4.55 $\pm$ 1.71*
K_d (nM)	4.73 $\pm$ 1.62	4.60 $\pm$ 1.33
[3H]-prazosin binding		
B_{max} (fmol mg^{-1} protein)	22.03 $\pm$ 2.85	9.66 $\pm$ 2.96*
K_d (nM)	0.46 $\pm$ 0.06	0.49 $\pm$ 0.15

Values are means $\pm$ s.e.mean of 4 membrane preparations. Each membrane preparation utilized 5 atria.

* Statistically different from control ($P < 0.05$).

remained unaltered in diabetic preparations. Compared to atria from control animals atria from diabetic rats demonstrated a hyperresponsiveness to both the inotropic and chronotropic effects of phenylephrine while exhibiting no change in sensitivity to α -adrenoceptor stimulation. On the other hand, atria obtained from diabetic rats demonstrated a subsensitivity to the chronotropic effects of the β -adrenoceptor agonist isoprenaline. In contrast, the inotropic effects of isoprenaline were unchanged by the diabetic state.

Recent work seems to support our finding of an augmented myocardial response following α -adrenoceptor stimulation in diabetic animals. Downing *et al.* (1983), utilizing diabetic lambs, observed that changes in contractility as reflected by dP/dt_{max} were twice as great in diabetics as in controls. More relevant to our work, Canga & Sterin-Borda (1986) were able to find an enhanced efficacy in tension development of left atria from streptozotocin-treated rats to α_1 -receptor stimulation. In addition, a study by Jackson *et al.* (1986) showed that right atria from streptozotocin-treated rats produced a significantly greater increase in contraction rate in response to phenylephrine.

A variety of alterations in heart rate have been described in human diabetics. These include a resting tachycardia, reduced heart rate response to postural change, and in some studies a blunted heart rate response to exercise (Rubler & Arvan, 1976; Hilsted *et al.*, 1980; Sundkvist *et al.*, 1980). These abnormalities may result from a change in output of the autonomic nervous system and/or from an altered responsiveness of the sinus node to autonomic stimuli. The decrease in pD_2 value for isoprenaline-induced chronotropy in diabetic atria suggests that there may be a diminished sinus node sensitivity to catecholamines in diabetes. The fact that the inotropic response to isoprenaline was unchanged in diabetic preparations further suggests that diabetes may lead to a very selective change in cardiac β -adrenoceptor function.

Alteration of the hormonal status induced by pathological or pharmacological manipulations is known to cause diverse effects on various receptor-mediated events. These effects are often attributed to changes in the number or function of receptors. In the current study diabetes resulted in a significant decrease in both β - and α_1 -receptors with no significant effect on the equilibrium dissociation constants of the receptors. This confirms previous work which demonstrated a decline in adrenoceptor density in left ventricular tissue from streptozotocin-treated rats (Savarese & Berkowitz, 1979; Williams *et al.*, 1983). Our study extends this observation to atrial tissue of spontaneously diabetic rats. In addition, our results also indicate that changes in receptor density

were not the result of differential membrane purification since the yields of membrane protein and the content of the sarcolemmal membrane marker sialic acid were similar in both control and diabetic groups.

It has been suggested that changes in adrenoceptors may be responsible for altered cardiac responses to β - and α -agonists in diabetic tissue (Heyliger *et al.*, 1982). Data obtained in our study do not support this notion. Despite the fact that β -adrenoceptor density was reduced by 64% in left atria from diabetic rats there was no significant change in maximum β -adrenoceptor-mediated inotropic response or in sensitivity for isoprenaline-mediated contractility. In addition, a comparable loss of cardiac α_1 -adrenoceptors in atria from diabetic animals was not associated with a corresponding decrease in inotropic or chronotropic responses but rather with an enhanced responsiveness to phenylephrine. Clearly, changes in adrenoceptor density in diabetes are not responsible for the altered myocardial response to adrenoceptor agonists. It is possible that steps following drug-receptor coupling may be altered in diabetes.

The importance of endogenous adenosine in biological functions is being increasingly recognized. In the heart, adenosine is a potent extracellular messenger. Aside from causing coronary vascular smooth muscle relaxation (Schnarr & Sparks, 1972), adenosine inhibits noradrenaline release from sympathetic nerve endings (Verhaeghe & Vanhoutte, 1977), reduces atrioventricular conduction velocity (Belardinelli *et al.*, 1983) and has negative inotropic (Endoh & Yamashita, 1980) and chronotropic effects (Belardinelli *et al.*, 1983). Recent studies have reported diminished coronary vascular responses to exogenous adenosine in diabetic animals (Downing *et al.*, 1982; Durante *et al.*, 1989). As such, we wanted to examine whether other cardiac effects of adenosine were also changed in diabetic animals. Our atrial experiments showed that the cumulative addition of adenosine produced negative inotropic and chronotropic effects which were unaltered in diabetic preparations. These results suggest that diabetes leads to a selective alteration in adenosine action in cardiac tissue.

In conclusion, this study demonstrated that isolated atria from spontaneously diabetic rats exhibited an altered response to adrenoceptor agonists. Atria from diabetic animals displayed a marked hyperresponsiveness to both the inotropic and chronotropic effects of selective α_1 -adrenoceptor activation by phenylephrine. In addition, the sensitivity to the chronotropic effect of the β -adrenoceptor agonist isoprenaline is reduced in diabetic preparations. Radioligand binding studies revealed that both α_1 - and β -adrenoceptor populations were substantially

reduced in atria from diabetic rats. However, there was no change in receptor affinity for either adrenoceptor. Future studies examining post receptor events may provide further insight as to the nature of the defect in these diabetic animals.

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