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ROLE OF THROMBOXANES IN ALTERATIONS OF THE DIABETIC β-ADRENERGIC SYSTEM

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Abstract—The inotropic effects of isoproterenol (ISO), as well as the β-adrenoceptors population, were measured in cardiac tissues from normal and short-term (3 days) diabetic rats. ISO increased the tension of both normal and diabetic ventricles, but the efficacy (E_{max}) of the concentration–response curve was greater on ventricles from diabetic rats than in those from the normal control. This phenomenon was accompanied by a decrease in the number of β-adrenoceptor sites (B_{max}) during diabetes. Insulin-treated diabetic hearts partially reversed the phenomenon. Propanolol blocked, in a competitive manner, the positive inotropic action of ISO in both types of ventricles. Inhibition of the synthesis and receptors of thromboxane (TX) reduced the hyperreactivity to ISO and increased the number of β-adrenoceptors during diabetes, producing B_{max} values almost similar to those of the normal heart. Additionally, the diabetic heart generated and released a greater amount of TXB_2 than the normal heart, even in the presence or absence of ISO. The stimulatory effect of ISO upon TXB_2 release was altered by the specific β-adrenergic blockade and by verapamil. In addition, the drugs able to induce a sustained increase of endogenous cAMP also inhibited the release of TXB_2 by diabetic ventricles. Exogenous TXB_2 exerted the same type of hyperreactivity in diabetic ventricles. This phenomenon was accompanied by an inhibition of $Na^+ + K^+$-ATPase activity. These results suggest that β-adrenergic inotropic stimulation is secondary to receptor-mediated hydrolysis of arachidonic acid with subsequent release of thromboxanes, which, in turn, may be responsible for both the superreactivity and the decrease in the number of β-adrenoceptors during diabetes. The abnormal reactivity to β-agonists also could be associated with alterations of the diabetic cardiac $Na^+ + K^+$-ATPase activity induced by TXB_2 whose production is increased during diabetes.

Autonomic neuropathy has been associated with the occurrence of early cardiac disturbances in diabetes.

Important alterations in β-adrenoceptor-stimulated events have been demonstrated during diabetes. Foy and Lucas [1, 2] observed a decrease in myocardial sensitivity to isoproterenol (ISO) under both *in vivo* and *in vitro* conditions. Other studies were undertaken to analyze the origins of β-adrenergic-altered response in diabetic conditions. The decrease in the number of β-adrenoceptors [3–11] has been associated with alterations in the cAMP protein kinase system and with the alteration of β-adrenergic stimulation of adenylate cyclase activity [12]. The time of onset of these alterations is still a matter of controversy.

Studies performed after a long period of diabetes (1–2 months) revealed the development of subsensitivity to the inotropic effect of ISO with a concomitant decrease in β-adrenoceptor density [3, 8–10]. However, studies performed after a short period of diabetes (< 2 weeks) are conflicting [6, 13, 14], and little is known about myocardial reactivity to β-adrenergic agonists after 3 days of streptozotocin treatment. There is evidence, however, that one or more metabolites of arachidonic acid (AA) may be implicated in the pathophysiological alterations observed in diabetes [15, 16]. Thus, AA is the biological precursor of diverse physiologically active products that regulate cardiac function.

AA is a fatty acid bound to membrane phospholipids released by the stimulation of specific cell-surface receptors and the subsequent activation of phospholipase A_2 [17]. The transformation of AA into cyclic endoperoxides by the cyclooxygenase system is a pivotal step in the synthesis of prostaglandins and thromboxanes [18]. The synthesis of thromboxanes (TXs) has been reported to increase significantly in diabetes [19, 20].

Recently, we demonstrated that the metabolism of AA is shifted toward TXB_2 formation during diabetes; whereas in normal heart it is directed to prostaglandin I_2 (PGI_2) production [21]. We also suggested that the hypersensitivity to the α-agonist observed in atria from acute diabetic rats may be mediated by a mechanism that couples activation of cardiac α-adrenoceptors, triggering the generation of oxidative products of endogenous AA; they in turn, enhance the positive inotropic effect of the α-agonist [22].

In this paper, the existence of supersensitivity to β-adrenergic agonists in ventricular myocardium from acutely-diabetic rats accompanied by a reduction in the number of β-adrenergic receptor binding sites will be demonstrated. The mechanism appears to involve the generation of TXs that are released upon specific β-agonist receptor recognition. Both the binding assay and the mechanical effect of the β-agonist were modulated by the thromboxane recep-

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Table 1. 5'-Nucleotidase activity

Conditions	P_i (μmol/mg protein/10 min)	
	Homogenate	Microsomes
Normal	0.16 ± 0.05	1.62 ± 0.06
Diabetic	0.15 ± 0.04	1.57 ± 0.05

Values are the means ± SE of five experiments in each group.

tor blockade, inducing alterations in the membrane β-adrenergic receptor. The hypersensitivity to β-agonists may be associated with alterations of the cardiac enzymatic activity induced by TXB_2, whose production is increased during diabetes.

MATERIALS AND METHODS

Animals

Experimental short-term diabetes was induced, as reported previously [21, 22], in Wistar male rats (200–250 g) by a single i.p. dose of streptozotocin (STZ: 85 mg/kg body weight, Sigma Chemical Co., St Louis, MO). STZ was dissolved in citrate buffer (pH 4.8) prior to the injection. A colorimetric enzymatic method (glycemia enzymatic) for "true glucose" determination in blood was used. Animals with plasma glucose above 300 mg/dl and with glycosuria were considered diabetic. The rats were killed 72 hr after STZ injection.

Insulin treated. Diabetic rats were treated with protamina-zinc insulin (Eli Lilly, Indianapolis, U.S.A.), 4.6 units per animal s.c., 36 hr prior to being killed. The insulin dose was adjusted to take into account the values of blood glucose and glucosuria.

Isolated ventricular preparation to measure contractile activity

The animals were decapitated with a guillotine. The entire heart was quickly removed and placed in Petri dishes filled with a modified Krebs–Ringer–bicarbonate (KRB) solution of the following composition (mM): Na^+, 145; K^+, 6.02; Ca^{2+}, 1.22; Mg^{2+}, 1.33; Cl^- 126; HCO_3^-, 25.3; SO_4^{2-}, 1.33; PO_4^{2-}, 1.20; and glucose, 5.5. Right ventricle strips were cut, attached to a glass holder, and immersed in a tissue chamber filled with 20 ml of KRB solution, gassed with 95% oxygen in 5% CO_2 and kept at 30° and pH 7.4 throughout the experiments. One end of the preparation was anchored to the glass holder, and other was connected to a force transducer (Statham UC-3 Gold Cell) coupled to an ink writing oscillograph (SAN EI 180). A constant resting tension of 1 g was applied to the strips by means of a micrometric device. Right ventricle strips were electrically excited with field stimulation using square wave pulses of 1-sec duration and slightly suprathreshold intensity (10%) at a frequency of 1 Hz delivered from a conventional stimulator.

The mechanical activity was analyzed in terms of the maximum rate of isometric force (dF/dt) developed above the externally applied resting tension. Tissues were allowed to equilibrate for 60 min. Control values (100%) refer to dF/dt before drug delivery. The absolute values of dF/dt at the end of the equilibrium period (60 min) were 16.5 ± 1.6 g/sec for normal and 15.7 ± 1.2 g/sec for diabetic ventricles.

Cumulative concentration–response curves for ISO and TXB_2 were made according to the method described by Van Rossum [23]. Single concentrations were administered in a volume of 0.01 to 0.025 ml of KRB solution. The total volume added to the bath never exceeded 0.1 ml. The time interval between concentrations was that required by each one to produce a maximal effect for TXB_2 (10 min) and for ISO (2 min).

Preparation of purified membranes

Diabetic and normal cardiac membranes used for identification of β-adrenoceptors and enzymatic assays were prepared essentially as described by Limas and Limas [24] and Borda *et al.* [25]. Briefly, ventricle tissue from eight rats was mixed in 4 vol. of cold buffer containing 0.25 M sucrose, 5 mM Tris–HCl (pH 7.4), 1 mM $MgCl_2$ and was homogenized with a Polytron PT-20 at a setting of 3–5 and 10 for 15 sec, three times. The homogenates were centrifuged at 1000 *g* for 10 min. The supernatant fraction was centrifuged at 12,000 *g* for 10 min and then at 30,000 *g* for 120 min. The pellet was resuspended in 2–5 ml of 50 mM Tris–HCl (pH 7.4) and 10 mM $MgCl_2$. The yield of membranes was 3.35 ± 0.19 and 3.38 ± 0.27 mg protein/g tissue in normal and diabetic preparations respectively. Table 1 shows the relative activity of 5'-nucleotidase, indicating the degree of purity of the microsomal fraction [26]; enzyme activities were similar in normal and diabetic conditions.

Binding assay

For (−)-[^{3}H]dihydroalprenolol [(−)-[^{3}H]DHA] binding of 100 μl of membrane suspension and different concentrations of (−)-[^{3}H]DHA (New England Nuclear, Boston, MA, sp. act. 95 Ci/mmol) were incubated with shaking for 15 min at 37° in a total volume of 150 μl of 30 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$. At the end of the incubation period a 150-μl sample was placed into 4 ml buffer and immediately filtered through GF/c glass fiber filters. The filters were washed with 8 ml of buffer, dried, placed in 10 ml of Triton–Toluene Based Scintillation Fluid, and counted. Non-specific binding, which did not exceed 22% of the specific binding, was determined by filtering the membranes incubated in the presence of 10^{-5} M (−)-propranolol. Results are expressed as femtomoles of (−)-[^{3}H]DHA specifically bound per milligram of protein.

Enzymatic assay

The K^+-activation pattern of *p*-nitrophenylphosphatase (pNPPase) activity was assayed by a modification of the method described by Liu and Onji [27]. Briefly, the reaction was carried out in duplicate in test tubes containing a total volume of 2 ml of a reaction solution of the following composition: 5 mM $MgCl_2$, 0.35 mM ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA), 5 mM *p*-nitro-

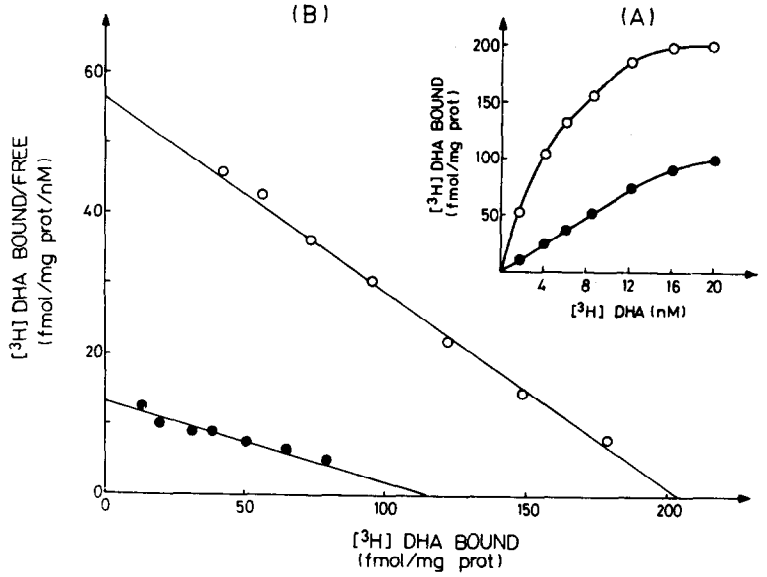

Fig. 1. (A) Specific binding of (−)-[^{3}H]DHA to rat cardiac membranes from normal (○) and diabetic (●) rats. Specific binding is defined as total binding minus non-specific binding as measured in the presence and absence of 10^{-5} M (−)-propranolol expressed as fmol/mg protein. (B) Scatchard plot of saturation binding data from (A). The ratio (B/F) of bound (−)-[^{3}H]DHA (fmol/mg protein) to free (−)-[^{3}H]DHA (nM) is plotted as a function of the (−)-[^{3}H]DHA bound per mg protein. The intercept with the abscissa is the number of binding sites (B_{max}) and the negative reciprocal of the slope is the dissociation constant (K_d). Values are the means of eight experiments in each group.

phenylphosphate (pNPP) and 20 mM Tris–HCl buffer (pH 7.4) (from the Sigma Chemical Co.). The protein concentration of the membrane suspension prepared as described above was adjusted to 0.1 mg/ml. Prior studies have shown that Mg^{2+} and pNPP concentrations above 4 mM yield maximal pNPPase activity [27]. Concentrations of KCl in the reaction medium were varied from 0 in blanks representing only Mg^{2+}-dependent pNPPase to 8.0 mM. After a 10-min preincubation period without pNPP, the reaction was initiated by the addition of pNPP and allowed to proceed for 6 min at 37°. Previous studies have established that the reaction rate is linear for up to 10 min [27]. Termination of the reaction was affected by the addition of 0.2 ml of 50% trichloroacetic acid (TCA) solution, followed by a 10-min centrifugation at low speed to sediment precipitated proteins. One milliliter of the cleared solution was pipetted into 2 ml of 0.5 M Tris–HCl to permit color development of the reaction product, *p*-nitrophenol (pNP). Optical density was measured at 400 nm and related to the standard curve of pNP absorbance. The K^+-activated pNPPase activity is defined as the result of each K^+ concentration minus the basal Mg^{2+}-dependent activity for which no K^+ is added [28].

Thromboxane B_2 assay

Rat ventricles (normal or diabetic) were excised immediately after the animals were killed, and left, with their spontaneous activity, in 1 ml of KRB with 5% CO_2 in oxygen at 37° for 30 min with or without blockers and then for an additional 5, 15 and 30 min alone or with 10^{-8} M ISO or for 30 min with 10^{-6} M db-cAMP. In all cases, total incubation time was the same under similar experimental conditions. The TXB_2 released by the tissues was measured in aliquots of incubation medium with a radioimmunoassay procedure using a TXB_2-[^{125}I]RIA Kit (New England Nuclear). Values are expressed as nanograms as of TXB_2 per gram tissue wet weight.

Table 2. Binding of (−)-[^{3}H]DHA to cardiac membranes from control and diabetic rats: effects of thromboxane-receptor blockers

	B_{max} (fmol/mg protein)	K_d (nm)
Normal	206 ± 2	3.66 ± 0.36
Normal + AH-23848	203 ± 10	3.52 ± 0.57
Diabetic	118 ± 11*	8.89 ± 0.61*
Diabetic + AH-23848	181 ± 5†	3.76 ± 0.42†
Diabetic + insulin	185 ± 12†	4.25 ± 0.52†

Ventricles were incubated in KRB with 5% CO_2 in O_2 at 30° during 1 hr with or without AH-23848. Then membranes were obtained. Aliquots of 100 μl were incubated for 20 min with different concentrations of (−)-[^{3}H]DHA. Values are the means ± SE of eight experiments in each group.

* Significantly different from normal values, $P < 0.001$.

† Significantly different from diabetic values, $P < 0.001$.

Drugs

Freshly prepared solutions of the following drugs were used: ISO, TXB_2, theophylline (1×10^{-4} M), dibutyryl cAMP (1×10^{-6} M), (−)-propranolol (5×10^{-9} M), and imidazole (5×10^{-4} M), (Sigma Chemical Co.); verapamil (1×10^{-5} M) (Knoll,

Ludwigshafen, West Germany); L-8027 (1×10^{-5} M) (Labaz Lab., U.S.A.) and AH-23848 (5×10^{-6} M) (Glaxo Ltd., U.S.A.). Ventricle strips were incubated for 30 min with β-adrenoceptor antagonist and with inhibitors of thromboxane receptors or synthesis before adding increasing amounts of ISO or TXB_2. The effects of receptor blocker and synthesis inhibitor drugs were not reversible.

Statistics

Students *t*-test for unpaired values was used to determine the levels of significance. Differences between means were considered significant if P was equal to or less than 0.05. Statistics of the binding assays were determined using a simple computer program from Scatchard plot analysis (Sagripanti *et al.*) [29].

RESULTS

Binding assay

(−)-[^{3}H]DNA binding to cardiac membrane was studied under normal and diabetic conditions. As shown in Fig. 1A, (−)-[^{3}H]DHA specifically bound to normal membrane preparations more than to diabetic membrane at seven concentrations of the radioligand employed.

Cumulative Scatchard analysis of data demonstrated a statistically significant decrease in the maximum number of binding sites (B_{max}) with a decrease of the dissociation constant (K_d) in the cardiac membranes of diabetic rats (Fig. 1B and Table 2).

Insulin-treated diabetic hearts partially reversed the phenomenon (Table 2).

Contractile assay

To determine if β-adrenergic receptor alteration observed during short-term diabetes may have influenced the mechanical response to β-agonist, the effect of ISO upon dF/dt of normal and diabetic ventricles was examined. Figure 2 shows the effect of increasing concentrations of ISO on the contractility of ventricles from normal and diabetic rats. It can be seen that ISO induced a concentration-dependent increase in dF/dt in both groups of ventricles. The maximal force of contraction (E_{max}) was greater on ventricles from diabetic rats than on those from normal control, whereas the potencies (K_d) were similar (Table 3). Insulin treatment reduced the E_{max} (57 ± 5 g/sec), while the K_d (0.36 ± 0.03 mM) was unaffected. This type of hyperreactivity was also observed with TXB_2. In Fig. 2 it can be seen that TXB_2 induced a concentration-dependent increase in dF/dt, that was greater on ventricles from diabetic (E_{max}: 33 ± 2 g/sec; K_d: 84 ± 3 nM) than from normal rats (E_{max}: 21 ± 1 g/sec; K_d: 96 ± 4 nM). The inotropic action of TXB_2 was blunted by AH-23848, a thromboxane receptor blocker (data not shown). It is important to note that the contractile effect of TXB_2 started at 1–2 min, reaching maximum at 8–10 min, and persisted until it was eliminated by washing.

To assess the role of β-adrenoceptors in the contractile effect of ISO, ventricles were incubated with β-adrenoceptor antagonist such as (−)-propranolol. Results shown in Table 3 indicate that, in the presence of this agent, the positive inotropic action of ISO was antagonized in a competitive manner both in normal ventricles and in those from diabetic rats. The dF/dt developed after the addition of the β-antagonist, both in normal and diabetic ventricles, had a similar magnitude.

To determine the nature of the mechanism which triggers the hyperreactivity to ISO in diabetic ventricles, the actions of inhibitors of the synthesis and receptors of thromboxanes were explored. It can be seen in Fig. 3 and Table 3 that L-8027 and imidazole reduced the inotropic positive effect of ISO in diabetic ventricles reaching values similar to those observed in normal ventricles. Table 3 also shows that a subthreshold TXB_2 concentration partially reversed the action of L-8027. The inhibitors of thromboxane synthesis did not modify the action of ISO in normal ventricles (Table 3). To elucidate if the thromboxane receptor activation could be involved in this effect, ventricles were incubated with AH-23848 for 30 min. As can be seen in Fig. 3 and Table 3, in the presence of AH-23848 the stimulant effect of ISO was reduced significantly in diabetic ventricles, reaching values similar to those observed under normal conditions. AH-23848 was unable to modify the concentration–response curve of ISO in normal ventricles (Table 3). None of the drugs modified *per se* the dF/dt of normal and diabetic ventricles.

The ability of AH-23848 to modify (−)-[^{3}H]DHA binding in both normal and diabetic membranes was also tested. In the presence of AH-23848, the B_{max} and the affinity constant increased to cardiac membranes from diabetic rats, almost reaching values similar to those observed in normal conditions (Table 2). AH-23848 had no effect on (−)-[^{3}H]DHA binding in normal ventricles.

TXB_2 assay

To verify if thromboxane is involved in the effect of ISO on diabetic ventricles, TXB_2 release was measured in normal and diabetic tissues, in the presence and absence of ISO. Table 4 shows that both normal and diabetic ventricles were able to generate TXB_2, but the amount of TXB_2 was significantly higher in the supernatant fractions from diabetic ventricles than in those from normal ones. Inhibitors of TXA_2 synthesis prevented the release of TXB_2 by normal and diabetic ventricles (data not shown). It should be noted that the amount of basal TXB_2 accumulation in the diabetic preparation appears not to alter the functionality of heart, because the absolute values of dF/dt at the end of the equilibration period were similar in control and diabetic preparations. In the presence of ISO, the release of TXB_2 from the diabetic heart increased, whereas the β-agonist was unable to modify the basal values of TXB_2 in normal tissue.

Values of TXB_2 released by ISO were similar at 5, 15 and 30 min of β-adrenergic stimulation (data not shown).

The stimulatory effect of ISO upon TXB_2 release was altered by specific β-adrenergic blockade. Verapamil also inhibited the release of TXB_2 induced by ISO on diabetic ventricles and decreased the basal release in both normal and diabetic ventricles (Table 4).

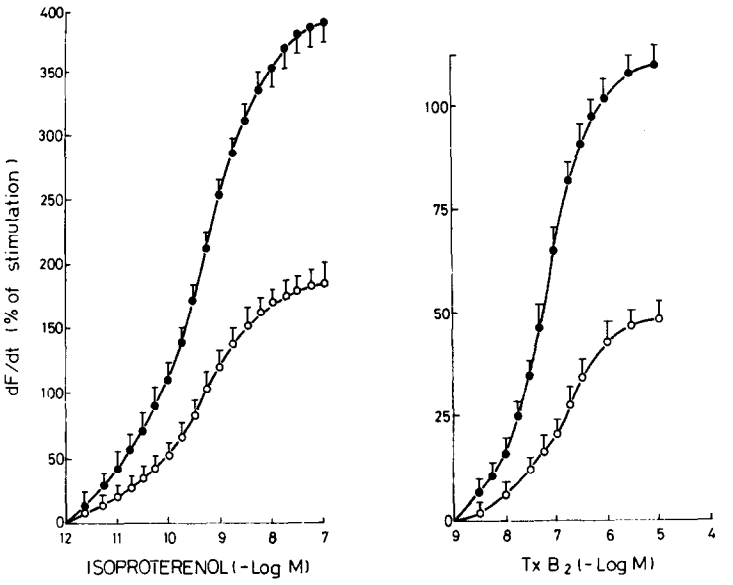

Fig. 2. Cumulative concentration–response curves of isoproterenol and thromboxane B_2 (TXB_2) in ventricles isolated from normal (○) and diabetic (●) rats. dF/dt is expressed as percent change versus initial controls. Values are the means ± SE of eight experiments in each group.

Table 3. Influence of β and thromboxane (TX) receptor blockers and inhibitors of TX synthesis on the positive inotropic effect of ISO in normal and diabetic ventricles

	Normal			Diabetic		
Drugs	E_{max} (g/sec)	K_d (M)	N	E_{max} (g/sec)	K_d (M)	N
ISO	44 ± 3	0.37 ± 0.01 × 10^{-9}	8	73 ± 4	0.38 ± 0.02 × 10^{-9}	8
ISO + propranolol	41 ± 2	0.57 ± 0.02 × 10^{-8}*	7	71 ± 3	0.59 ± 0.03 × 10^{-8}*	7
ISO + imidazole	43 ± 3	0.39 ± 0.02 × 10^{-9}	6	46 ± 4*	0.37 ± 0.02 × 10^{-9}	6
ISO + L-8027	42 ± 1	0.36 ± 0.01 × 10^{-9}	6	44 ± 2*	0.36 ± 0.02 × 10^{-9}	6
ISO + L-8037 + TXB_2	42 ± 3	0.38 ± 0.03 × 10^{-9}	5	62 ± 4	0.35 ± 0.02 × 10^{-9}	5
ISO + AH-23848	45 ± 3	0.37 ± 0.02 × 10^{-9}	6	39 ± 3*	0.37 ± 0.01 × 10^{-9}	6

Ventricles were exposed to various concentrations of ISO in the absence or presence, for 30 min of thromboxane (TX) receptor blockers or enzymatic inhibitors. TXB_2 (10^{-9} M) was added 2 min before ISO. Values are means ± SE. The antagonistic drugs were used at the following concentrations: propranolol, 5 × 10^{-9} M; imidazole, 5 × 10^{-4} M; L-8027, 1 × 10^{-5} M; and AH-23848, 5 × 10^{-6} M. All of these values were obtained by fitting the data of Figs. 2 and 3 to the Michaelis–Menten equation, taking linear relationships and fitting the equivalent to Lineweaver–Burk plots.

* Significantly different from ISO alone, $P < 0.001$.

In addition, TXB_2 was assayed in supernatant fractions from normal and diabetic ventricles that had been incubated with substances able to induce a sustained increase of endogenous cAMP, such as db-cAMP and theophylline. It can be seen in Table 4 that either db-cAMP or theophylline plus ISO decreased the release of TXB_2 in diabetic conditions, whereas in normal conditions they did not modify it (Table 4).

Enzymatic assay

To investigate the mechanism by which the inotropic effect of TXB_2 is potentiated during diabetes, $Na^+ + K^+$-ATPase activity was measured in the presence of TXB_2 under normal and diabetic conditions. The $Na^+ + K^+$-ATPase activity was assessed by the measurement of K^+-activated pNPPase. The pNPPase was chosen because a previous report showed no change in the pattern of $Na^+ + K^+$-ATPase activity but a depression in K^+-activated pNPPase activity in sarcolemmal from diabetic rat heart [30].

It can be seen in Table 5 that pNPPase activity was lower in diabetic than in normal ventricles. TXB_2 inhibited the enzymatic activity in both normal and diabetic ventricles. However, the inhibition of thromboxane synthetase or thromboxane receptor blocker only modified the basal activity of the enzyme in diabetic conditions.

DISCUSSION

The present results demonstrate an increased ability of acute diabetic rat ventricular muscle to respond to ISO. This phenomenon, which appeared to be secondary to receptor-mediated hydrolysis of arachi-

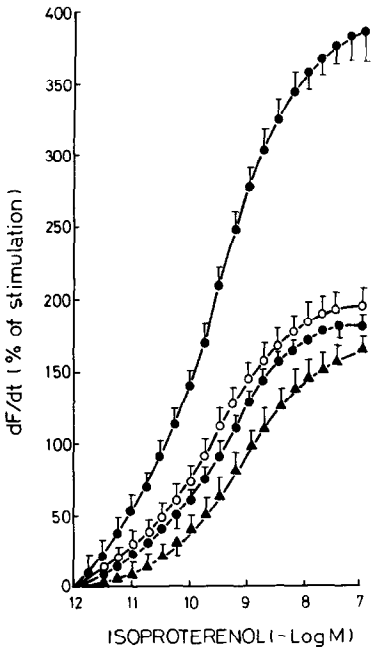

Fig. 3. Contractile effect of isoproterenol (●——●) in diabetic ventricles and the influence of 1×10^{-5} M L-8027 (●---●), 5×10^{-4} M imidazole (○——○) and 5×10^{-6} M AH-23848 (▲——▲). Values are the means ± SE of six experiments in each group.

The sensitivity of diabetic tissue for the agonist (K_d), as derived from the concentration–effect relationship, appeared not to be altered. This observation contrasts with data from receptor binding studies obtained in this paper, in which a decrease in the affinity (increased K_d) and a decrease in β-receptor density (E_{max}) during acute diabetic conditions were observed. Although it is hazardous to correlate antagonist binding data to functional agonist activity, it is probable that, in the contractile effects of ISO, other inotropic factors, such as the movement of calcium, could be involved. An increase in the maximal velocity and the affinity in the sarcolemmal Ca^{2+}-dependent ATPase activity associated with an increase in the affinity to the inotropic action of low extracellular calcium concentration has been reported in ventricles from acute diabetic rats [31]. Another factor to take into account is the influence of inotropic action of thromboxanes in the contractile effect of ISO. Here we show that the contractile response to ISO in diabetic ventricles was reduced significantly by imidazole and L-8027, two thromboxane synthetase inhibitors [32, 33]. Furthermore, a subthreshold TXB_2 concentration reversed this inhibitory effect. The fact that ISO was able to trigger the release of TXB_2 by the diabetic heart suggests that thromboxanes could be mediating the hyperreactivity to ISO observed during diabetic conditions. AH-23848, a thromboxane receptor blocker, significantly inhibited the hyperreactivity of the diabetic heart to the β-agonist. This is consistent with the hypothesis that TXB_2 can act synergistically with ISO to induce the inotropic positive effect through its own specific receptors. AH-23848 in diabetic preparations, however, increased the affinity constant and the number of β-adrenoceptors, almost reaching values similar to those observed in normal conditions. These data may indicate that thromboxane induces down-regulation, attenuating the abnormal reactivity to β-agonists during diabetes.

Table 4. Release of TXB_2 by normal and diabetic ventricles: effects of β-adrenergic drugs and calcium blocker agents

Drugs	TXB_2 (ng/g tissue wet weight) Normal	Diabetic
Basal	22.5 ± 2.8	45.0 ± 3.6
ISO	20.9 ± 2.2	65.8 ± 4.2†
Propranolol (10^{-7} M) + ISO	20.7 ± 2.4	41.3 ± 3.2‡
Verapamil (10^{-5} M) + ISO	27.0 ± 2.6	30.6 ± 2.7‡
Theophylline (10^{-4} M) + ISO	26.3 ± 2.7	26.1 ± 2.2‡
Dibutyryl cAMP	27.5 ± 2.9	28.5 ± 2.2†
Verapamil (10^{-5} M)	15.2 ± 1.3*	19.2 ± 2.0†

Values are means ± SE of six experiments in each group performed in duplicate. Ventricles were incubated in 1 ml of KRB with 5% CO_2 in O_2 at 37° for 30 min with or without blockers and then for 30 min more alone or with ISO (5×10^{-7} M) or dibutyryl cAMP (1×10^{-6} M). In all cases total incubation time was 1 hr.

* Significantly different from basal normal, $P < 0.01$.

† Significantly different from basal diabetic, $P < 0.01$.

‡ Significantly different from ISO, $P < 0.01$.

donic acid (AA), was accompanied by a decrease in the density of β-adrenoceptor sites.

The pharmacological analysis of both β-agonist and antagonist tends to support the theory that β-adrenoceptors are the most important mediators of the positive inotropic response to ISO in both diabetic and normal ventricles. We found, however, that ventricles from diabetic rats, at a time when the isolated working heart had a basal dF/dt similar to the normal control, were hyperresponsive (increased E_{max}) to selective β-adrenoceptor stimulation by ISO.

A dissociation between the mechanical effect of

Table 5. *p*-Nitrophenylphosphatase (pNPPase) activity in normal and diabetic ventricles: effect of TXB_2 and inhibitors of TX receptors

Drugs	*p*-Nitrophenylphosphatase (nmol/hr/mg protein) Normal	Diabetic
Basal	3.25 ± 0.13	1.58 ± 0.08*
TXB_2 (10^{-9} M)	2.02 ± 0.08*	0.90 ± 0.09*
TXB_2 (10^{-6} M)	0.85 ± 0.03*	0.39 ± 0.01*
TXB_2 (10^{-9} M) + AH-23848	3.15 ± 0.11	2.70 ± 0.12*
Imidazole (10^{-3} M)	3.10 ± 0.12	2.98 ± 0.12*
L-8027 (10^{-5} M)	3.40 ± 0.14	2.57 ± 0.15*
AH-23848 (5×10^{-6} M)	3.39 ± 0.13	2.61 ± 0.13*

Values are means ± SE of eight experiments performed in duplicate in each group. Ventricles were incubated in KRB with 5% CO_2 in O_2 at 30° during 1 hr with or without AH-23848, imidazole or L-8027. Then membranes were obtained. Aliquots of 80–120 μg protein were incubated with or without TXB_2 (10^{-6} M and 10^{-9} M) for 30 min before starting the reaction by the addition of pNPP (see Methods).

* Significantly different at $p < 0.001$; basal diabetic vs basal normal; TXB_2 normal and diabetic vs basal; heart treated with thromboxane enzymatic and receptor inhibitors vs basal diabetic.

the agonists and the number of receptor sites has been found for short-term diabetic rats. Carrier *et al.* [34] reported a post-junctional muscarinic supersensitivity to the negative chronotropic effects of cholinergic agonists accompanied by a decrease in the atrial muscarinic receptors during diabetes. Moreover, hyperresponsiveness to the contractile effect of α-agonists was also found in isolated left atria from chronic diabetic rats [35] and in atria from acute diabetic rats accompanied by a decrease in the number of α-adrenoceptors [36]. On the contrary, during chronic diabetic conditions a reduction in inotropic response of ventricular muscle to methoxamine and ISO, coinciding with a decrease in α- and β-adrenoceptor density of the heart, without changes in the affinity of the receptors was described [3, 7, 37].

The increase in the intrinsic activity of the β-agonist observed in this paper during acute diabetes may have been due to an alteration in the utilization of calcium by the myocardium, after receptor occupancy-mediated hydrolysis of AA with subsequent release of thromboxanes. This has been described for some of the contractile effects of the α_1-adrenergic agonist, PGE_2, TXA_2; PGI_2 and exogenous AA during stimulation of diabetic heart and vessels [21, 22, 38–40].

In this paper, we also demonstrated that TXB_2 triggered the same type of hyperreactivity that was induced by ISO on diabetic ventricles. All of these data, taken together with the results of others [35, 41], are in agreement with the notion that cardiac calcium content is higher during diabetes and that this intracellular calcium overload may subserve the hyperreactivity of the heart of acute diabetic rats to various inotropic interventions.

The fact that verapamil prevented the release of TXB_2 induced by ISO, reaching values similar to those obtained in normal conditions, suggests that free-cytosolic calcium plays an important role in the increased release of TXB_2 during diabetes. On the other hand, the interventions able to induce sustained increases of endogenous cAMP decrease the release of TXB_2. These data suggest that cAMP-dependent protein kinase and the influx of calcium act in opposite ways: calcium enhances the release of TXB_2, whereas cAMP inhibits it.

Another factor in the synergistic action of TXB_2 on the inotropic effect of ISO appears to be the inhibitory action that TXB_2 induced on pNPPase activity. Similarly, an inhibitory action of ISO and cAMP on $Na^+ + K^+$-ATPase activity has been described [26]. The inhibition of $Na^+ + K^+$-ATPase activity is expected to result in a positive inotropic action on the myocardium [42]. We observed that TXB_2 inhibited the pNPPase activity in both normal and diabetic ventricles. Moreover, basal values of the enzyme are low during diabetes, suggesting that in this condition the inhibition of the enzyme was probably related to an accumulation of TXB_2. The fact that the inhibition of thromboxane synthetase affected the behavior of the enzyme in the diabetic heart, switching it from normal activity, confirms this notion. In addition, the specific thromboxane receptor blocker abrogated the inhibitory effect of TXB_2 upon the pNPPase activity, suggesting that thromboxane inhibited the enzyme through its own receptor.

In this paper we postulate that the overload of thromboxane may modulate the β-adrenergic reactivity of the diabetic heart, potentiating the inotropic positive response via direct interaction with thromboxane receptors and exerting a negative feedback control to β-adrenoceptors. The increase in the positive inotropic effects of TXB_2 and ISO is probably related to the inhibition of $Na^+ + K^+$-ATPase activity observed during diabetes.

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