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β_3-Adrenoceptor-mediated responses in diabetic rat heart

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Abstract. β_3-adrenoceptors mediate negative inotropic effect in contrast to classical β_1- and β_2-adrenoceptors. Cardiac β_3-adrenoceptors are upregulated in experimental diabetes. Thus, cardiodepressant effect mediated by β3-adrenoceptors has been proposed to contribute to the impaired cardiac function in this pathology. In our study, we investigated the influence of streptozotocin-diabetes on cardiac contractility to β_3-adrenoceptors stimulation by using Langendorff-perfused rat hearts. BRL 37344, a selective β_3-adrenoceptor agonist, induced dose-dependent decreases in left ventricular developed pressure (LVDP) in hearts from control rats. BRL 37344 also dose-dependently decreased +d*P*/d*t* and –d*P*/d*t* values. Effects of BRL 37344 were abolished by SR 59230, but not altered by nadolol pre-treatment. On the other hand, these effects of BRL 37344 were all significantly increased in hearts from diabetic rats. We also observed that diabetes significantly increased the mRNA levels encoding cardiac β_3-adrenoceptors. In addition, $G_{i\alpha 2}$ mRNA expressions were found to be increased in the cardiac tissue of diabetic rats as well. The effect of BRL 37344 on cardiac contractility was normalized upon treatment of diabetic rats with insulin. These data demonstrate an increased effect of β_3-adrenoceptor stimulation on hemodynamic function of the heart in accordance with an increased mRNA levels encoding cardiac β_3-adrenoceptors in 8-week diabetic rats.

Key words: β_3-adrenoceptors — Diabetes — LVDP — Negative inotropy — Negative lusitropy

Introduction

Diabetic patients are at increased risk for cardiovascular complications such as atherosclerosis, acute myocardial infarction and congestive heart failure compared to non-diabetics (Kannel and McGee 1979). Diabetes also can affect cardiac structure and function independently of vascular or valvular pathology, a condition called diabetic cardiomyopathy (Sharma and McNeill 2005; Boudina and Dale Abel 2007). Thus, cardiovascular diseases are the leading cause of diabetes-related morbidity and mortality. In experimental models of diabetes, decreased responsiveness of cardiac preparations to both inotropic and chronotropic effects of β-adrenoceptor (β-AR) agonist stimulation is among the easily seen functional abnormalities of the heart (Vadlamudi and McNeill 1984; Yu and McNeill 1991; Karasu and Altan 1993; Ozuari et al. 1993). The mechanisms underlying depressed cardiac responses are poorly understood. However, they may be associated with the decrease in the density of cardiac β-ARs (Latifpour and McNeill 1984; Sundaresan et al. 1984; Gando et al. 1997). In fact, there has been reported a 28% reduction in the number of β-ARs, accompanied by a 24% decrease in the heart rate of streptozotocin-induced diabetic rats, when compared with controls (Savarese and Berkowitz 1979). Similarly, we have demonstrated that cardiac β_1-AR expression was decreased by 55% in 14-week-diabetic rats (Dincer et al. 1998). In another set of experiments, we have also shown that maximum chronotropic response of the right atria to β_1-AR stimulation was decreased by almost 30% in 14-week-diabetic rats (Dincer et al. 2001). These results suggest that a decrease in cardiac β-AR expression may contribute to the decreased functional responses in diabetic hearts.

On the other hand, the presence of functional β_3-AR in the human heart has been demonstrated (Gauthier et al. 1996). β_3-AR differs from β_1- and β_2-ARs by its molecular structure and pharmacological profile. Interestingly, in contrast to positive inotropic effects of β_1- and β_2-ARs, β_3-ARs mediate negative inotropic effects. Therefore, the

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cardiodepressant effect mediated by β_3-ARs may also play a role in cardiac dysfunction. In fact, cardiac β_3-AR expressions were demonstrated to be significantly increased in certain pathological states such as diabetes and heart failure (Dincer et al. 1996, 2001; Gauthier et al. 1996; Moniotte et al. 2001). However, no data, to our knowledge, are available on the influence of diabetes on selective β_3-AR-mediated functional responses in the heart. Therefore, in the present study we investigated the effect of 8-week diabetes on cardiac contractility in response to β_3-AR stimulation in rats.

Materials and Methods

Animals

Male Sprague Dawley rats weighing 200–250 g were obtained from Bilkent University Genetics and Biotechnology Research Center (Ankara, Turkey). The rats were housed individually on a 12 h light/dark cycle at constant room temperature, and given standard laboratory chow (Purina Rat Chow; Optima AS, Turkey) and tap water ad libitum in the Ankara University Faculty of Pharmacy Animal Care Unit. All experiments were approved by the Ankara University Animal Care and Use Committee.

Induction and verification of experimental diabetes

Diabetes was induced with 45 mg/kg streptozotocin (STZ, Sigma-Aldrich) dissolved in citrate buffer (pH 4.5), administered as a single intravenous tail-vein injection. Three days after STZ injection, blood glucose was measured and those with blood glucose levels ≥ 300 mg/dl were accepted as diabetic.

Insulin treatment protocol

After five weeks of STZ injection, diabetic rats were randomly divided into treated and untreated groups. Treated rats were given daily subcutaneous neutral protamine Hagedorn (NPH) insulin injections (Humulin® N, Eli Lilly, USA) for three weeks, after five weeks of untreated diabetes. Insulin doses were given once *per* day, and individually adjusted based on each animal's blood glucose level in order to maintain the euglycemic state (8–15 U/kg/day). Blood glucose levels were monitored daily using Accu-check® (Roche Diagnostics) strips.

Measurement of in-vivo basal hemodynamics

Hemodynamic parameters were measured under ketamine/xylazine anaesthesia. A Millar catheter (SPR-249, Millar Institute, Houston, TX, USA) was inserted into the left ventricle (LV) *via* the left carotid artery to obtain LV systolic and diastolic pressure. The heart rate, systolic and diastolic blood pressure of each rat were also measured with the catheter in the aorta. The maximal rate of LV pressure increase (+dP/dt) and decrease (–dP/dt) and LV end diastolic pressure (LVEDP) were calculated from LV derivatives with the use of AcqKnowledge software (MP30 Data Acquisition System, Biopac System Inc, Santa Barbara, USA).

Myocardial functional analysis

Langendorff preparation. β_3-AR-mediated effects on cardiac function were studied in the isolated heart preparations with the Langendorff technique. Rats were anaesthetized with an intraperitoneal injection of ketamin (60 mg/kg) and xylazine (10 mg/kg) combination. After rapid excision, the hearts were perfused retrogradely with a modified Krebs-Henseleit solution of the following composition (in mmol/l): 120 NaCl, 4.8 KCl, 1.25 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, and 11 glucose (37°C, pH 7.4). The perfusate was equilibrated continuously with a standard 95%O_2/5% CO_2 gas mixture and maintained at 37°C. Coronary perfusion flow rate was adjusted to 10 ml/min. Heart rate was maintained at 300 b.p.m by right ventricular pacing performed using two electrodes connected to a stimulator (Grass Instrument. Inc., Quincy, MA, USA). A compliant latex balloon, constructed as described previously (Sutherland et al. 2003), attached to a pressure transducer by polyethylene tubing was inserted into the LV to measure LV pressure and the maximal rate of LV pressure development (+dP/dt) and the maximal rate of relaxation (–dP/dt). The left ventricular end diastolic pressure was set at 10 mmHg by adjusting the balloon volume.

BRL 37344 ((±)-(R*,R*)-[4-[2-[[2-(3-chlorophenyl)-2-hydroxyethyl]amino]propyl]phenoxy]acetic acid sodium hydrate; Sigma Aldrich, USA), which is a selective β_3-AR agonist with low potency at β_1- and β_2-AR (Gauthier et al. 2007), was used to evaluate the functional effects of β_3-AR activation in the isolated heart. In the first group, the hearts were perfused with solution containing incremental concentrations of BRL 37344 (10^{-10}–10^{-6} mol/l). In the second group, the hearts were perfused with solution containing incremental concentrations of BRL 37344 (10^{-10}–10^{-6} mol/l) in the presence of nadolol (10^{-5} mol/l; Sigma Aldrich, USA), which is a potent β_1- and β_2- antagonist. In the third group, the hearts were perfused with solution containing incremental concentrations of BRL 37344 (10^{-10}–10^{-6} mol/l) in the presence of SR 59230A (10^{-7} mol/l; 3-(2-ethylphenoxy)-1-[[(1S)-1,2,3,4-tetrahydronaphth-1-yl]amino]-(2S)-2-propanol oxalate salt; Sigma Aldrich, USA), which is a selective β_3-AR antagonist

(Kitamura et al. 2000). LV function was measured when LV pressure had reached a stable level.

Isolation and quantitation of total RNA

LV of the hearts removed from killed rats were quickly frozen in liquid nitrogen and were stored at –80°C. Tissue samples were homogenized in TRIzol® Reagent (Sigma). After homogenization, samples were centrifugated at 10000 rpm for 10 min. After incubation of the homogenized samples for 5 min at room temperature, 0.2 ml of chloroform was added *per* 1 ml of TRIzol® Reagent. The samples were mixed vigorously and then centrifuged at 10000 rpm for 15 min at 4°C. Centrifugation separated the biphasic mixtures into the lower red, phenol-chloroform phase and the upper colorless, aqueous phase. The RNA was precipitated from the aqueous phase by mixing with 0.5 ml of isopropanol (for each initial milliliter of TRIZOL Reagent). The samples were centrifugated at 10000 rpm for 20 min. The supernate was removed and the RNA pellet was washed once with 75% ethanol. The pellet was air dried and dissolved in diethyl pyrocarbonate (DEPC)-treated water. The RNA was quantitated by determination of optical denstiy (OD) values of each sample spectrophotometrically using Nanodrop (NanoDrop Technologies, Wilmington, Del., USA). Samples with an OD value lower than 1.7 were eliminated.

Reverse transcription-polymerase chain reaction (RTPCR)

RT-PCR experiments were performed using ImProm-II Reverse Transcription System (Promega). At first, mRNAs were obtained from total RNAs using oligo dT primer after a reaction in the thermocycler which was held at 70°C for 5 min. Thereafter, mRNAs were used for the synthesis of cDNA strains by reverse transcriptase. ImProm II™ Reverse Transcriptase Reaction Buffer (Promega, USA), deoxynucleotide triphosphate (dNTP), $MgCl_2$, RNasin and reverse transcriptase were added; water was then added to a final volume of 20 µl. The tubes were again placed into the thermocycler and heated for 60 min at 42°C for reverse transcriptase reaction, followed by 15 min at 70°C for denaturation. First strand cDNA samples were then cooled to 4°C and stored at –80°C until use. The single strand cDNAs were consequently amplified by PCR as a way of determining the amount of transcripts present in each sample. cDNA, 5X Go Taq®Green Reaction Buffer (Promega, USA), dNTP, $MgCl_2$, Taq DNA polymerase, sense and antisense primers (Table 1) were added to PCR tubes. Diethylpyrocarbonate-treated water was then added to bring it to a final volume of 50 µl. The samples were then mixed and placed in the thermocyler, and denaturated for 3 min at 94°C. Thereafter, segments were amplified using the sequence 1.5 min denaturation (94°C) followed by 1 min annealing (58–60°C) and 2 min extension (72°C), this sequence was repeated for a total of 35 cycles. β-actin was amplified in each set of PCR reactions and served as internal references during quantitation to correct for operator and/or experimental variations. At the end of reactions, each PCR product were mixed with blue/orange loading dye. The samples were then loaded onto a agarose gel containing ethidium bromide and electrophoresed. The resulting gels were visualized using an UV transilluminator (Viber Loumat TFX 20M UV, France) and photographed using UV gel camera (Kodak EDAS 290, USA).

Statistical analysis

Two different sets of animals were used to perform basal haemodynamic studies *in vivo* and the Langendorff studies *in vitro*. Values of *in vivo* studies and mRNA expressions are presented as mean ±S.E.M. One-way ANOVA with Bonferroni correction was used for statistical analysis of these results. Data from the Langendorff studies were expressed as the mean of percentages ±S.E.M. Two-way ANOVA was used to compare the different concentration-response curves and unpaired *t*-test with Bonferroni correction was performed to make pairwise comparisons for each concentration. In all cases, *p* values less than 0.05 were considered to be statistically significant. GraphPad Prism 5 for Windows (GraphPad Software Inc, USA) was used for all statistical analyses.

Table 1. The sequences and product sizes of primers

mRNA	Forward (3'-5')	Reverse (3'-5')	Product size	Accession number
β_3-AR	CGCTTAGCTACGACGAAC	AGTGGGACTCCTCGTAATG	444	NM-013108
eNOS	ATATCTTCAGCCCCAAACGCA	ACCACTTCCATTCTTCGTAGCG	553	U53142
$G_{i\alpha 2}$	CATCTTCTGTGTCGCCTTGA	CTCAGAAGAGGCCACAGTCC	404	99597-AT

β_3-AR, β_3-adrenoceptor; eNOS, endothelial nitric oxide synthase.

Results

General features of control and diabetic rats

8 weeks after STZ injection, blood glucose levels were significantly increased in diabetic rats (444 ± 22.9 mg/dl) in comparison with control (102.4 ± 3.1 mg/dl). In insulin-treated diabetic group, blood glucose levels returned to control values (111.2 ± 24.0 mg/dl). At the end of the experimental protocol, mean body weights of diabetic rats were lower (291 ± 16.1 g) than those of control rats (416 ± 13.2 g). Insulin treatment corrected the body weight loss in diabetic group (354 ± 17.9 g).

Hemodynamic functions

The data in Table 2 describe *in vivo* basal cardiac hemodynamic parameters of control and diabetic rats at the end of 8 weeks. These data show that cardiac dysfunction was induced in diabetic rats and insulin treatment ameliorated this impaired hemodynamic parameters as we expected. LVP and LVDP values were decreased, while LVEDP was found to be increased in diabetic rats compared to those of control rats. In insulin treated rats, however, these values were not significantly different from control rats.

The first derivatives of the maximal rate of LV pressure development (+d*P*/d*t*) and of the maximal rate of relaxation (–d*P*/d*t*) were also decreased in diabetic rats while these values were restored to control values with insulin treatment (Table 2). Moreover, heart rate values were decreased in diabetic rats. Insulin treatment of diabetic rats corrected these changes in heart rate, as well.

On the other hand, blood pressure values were not significantly influenced by diabetes although there was a slight decrease in diabetic rats compared to control rats (Table 2).

Effects of BRL 37344 on mechanical responses

BRL 37344, a preferential β_3-AR agonist, induced a dose-dependent negative inotropic effect at concentrations ranging from 10^{-10} to 10^{-7} mol/l in the hearts of control rats. However, at higher concentration (10^{-6} mol/l), a positive inotropic effect was seen instead of negative inotropic effect. The same tendency was also observed on both +d*P*/d*t* and –d*P*/d*t* values at 10^{-6} mol/l (Figure 1). The negative inotropic effect of BRL 37344 was not modified by pre-treatment with nadolol (10^{-5} mol/l), a β_1/β_2-AR antagonist (Figure 2), but abolished by SR 59230 (10^{-7} mol/l), a β_3-AR antagonist (Figure 3). This negative inotropic effect of BRL 37344, on the other hand, was significantly increased in isolated hearts of diabetic rats. Similarly, BRL 37344 also induced dose-dependent decreases in +d*P*/d*t* and –d*P*/d*t* values at the concentrations that cause negative inotropic effect. The effects of BRL 37344 on these parameters were also found to be increased in diabetic rats. In insulin-treated diabetic rats, all these effects of BRL 37344 on these parameters were not different from control rats.

Quantitation of β3-AR, eNOS and Giα2 transcripts

Diabetes significantly increased cardiac β_3-AR mRNA expression levels. Insulin treatment slightly attenuated this increase observed in diabetic rats. However, β_3-AR mRNA levels were still higher than those of the control rats (control, 100 ± 17%; diabetic, 167.9 ± 3.9%; insulin-treated diabetic, 152.3 ± 6.1%) (Figure 4A). In addition, eNOS mRNA levels were found to be increased in cardiac tissue of diabetic rats. However, this change was not found to be significantly different from those of the control rats (control, 100 ± 13%; diabetic, 134.7 ± 39.0%; insulin-treated

Table 2. *In vivo* basal cardiac hemodynamic parameters of control ($n = 5$), diabetic ($n = 5$) and insulin-treated diabetic ($n = 4$) rats at the end of 8 weeks.

	Control	Diabetic	Insulin-treated diabetic
LVP (mmHg)	115.1 ± 5.6	97.1 ± 3.1*	110.4 ± 2.9#
LVEDP (mmHg)	7.5 ± 0.5	14.5 ± 1.1***	8.0 ± 0.1##
LVDP (mmHg)	107.6 ± 6.0	82.6 ± 2.1**	106.0 ± 3.9##
+*dP/dt* mmHg/sec)	3955.4 ± 312.5	3207.8 ± 73.1*	3785.5 ± 197.5#
–*dP/dt* (mmHg/sec)	3467.6 ± 205.4	2736.0 ± 127.8*	3483.3 ± 206.7#
Heart rate (bpm)	329.4 ± 22.9	241.4 ± 21.2*	290.8 ± 11.5
SBP (mmHg)	115.4 ± 5.6	105.4 ± 5.3	114.5 ± 6.6
DBP (mmHg)	79.4 ± 3.3	71.0 ± 7.5	79.5 ± 4.9

Values are the mean ±S.E.M. One-way ANOVA with Bonferroni test was used for comparisons within groups (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, controls *vs.* diabetics; # $p < 0.05$, ## $p < 0.01$, diabetics *vs.* insulin-treated diabetics). LVP, left ventricular pressure; LVEDP, left ventricular end-diastolic pressure; LVDP, left ventricular developed pressure; +d*P*/d*t,* maximal rate of LV pressure increase; –d*P*/d*t*, maximal rate of LV pressure decrease; SBP, systolic blood pressure; DPB, diastolic blood pressure.

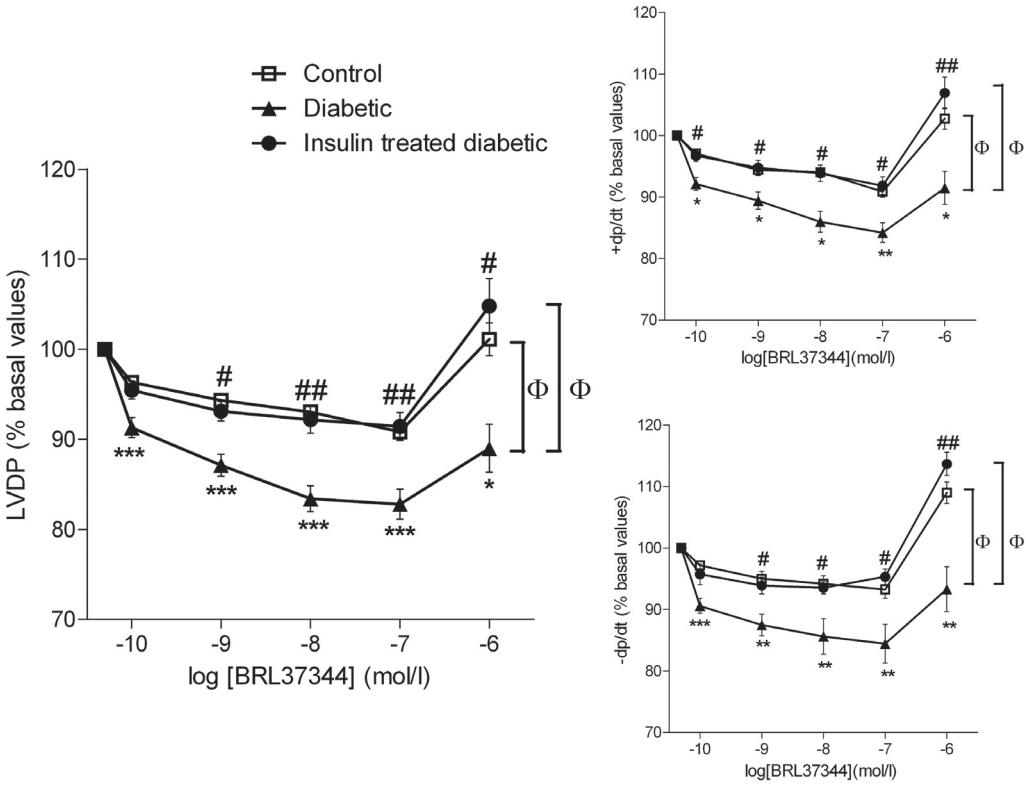

Figure 1. Concentration-response curve for BRL 37344 on left ventricular developed pressure (LVDP), the first derivatives of the maximal rate of left ventricular pressure development (+d*P*/d*t*), and of the maximal rate of relaxation (–d*P*/d*t*). Values are the mean ±S.E.M. ($n = 10$). The response is expressed as the percentage of LVDP, +d*P*/d*t* and –d*P*/d*t* measured at baseline. Unpaired *t*-test with the Bonferroni correction was used for pairwise comparisons within groups (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, controls *vs.* diabetics; # $p < 0.05$, ## $p < 0.01$, diabetics *vs.* insulin-treated diabetics). Comparison of the different concentration-response curves was performed by two-way ANOVA (Φ, $p < 0.05$).

diabetic, 128.4 ± 37.0%) (Figure 4B). Moreover, $G_{i\alpha 2}$ mRNA expressions were significantly increased in diabetic rats, and insulin treatment had no influence on this increase (control, 100 ± 3%; diabetic, 126.8 ± 4.9%; insulin-treated diabetic, 125.2 ± 4.7%) (Figure 4C).

Discussion

Cardiac dysfunction induced by 8-week STZ-induced diabetes was verified by the results of *in vivo* basal hemodynamics obtained by Millar catheterisation. LVP and LVDP, were decreased while LVEDP was increased in diabetic rats. In addition, the rate of contraction and relaxation, +d*P*/d*t* and –d*P*/d*t*, were both significantly decreased as a result of 8 week diabetes. Basal heart rate of diabetic animals was less than that observed in nondiabetic control rats, as well. Our findings are in accordance with those of previous studies (Heyliger et al. 1985; Ramanadham et al. 1989; Dai et al. 1994; Dincer et al. 2001). Insulin treatment of diabetic rats, on the other hand, restored these alterations to control values.

A primary finding of the present study is that cardiac function measured as LVDP, +d*P*/d*t* and –d*P*/d*t* to selective β_3-AR stimulation is significantly altered in 8-week STZ-induced diabetes. BRL 37344, a selective β_3-AR agonist, induced negative inotropic effect in the isolated hearts of control rats and this effect of BRL 37344 is significantly increased in diabetic rats. The negative inotropic effect of BRL 37344, obtained by assessment of LVDP values was observed at concentrations ranging from 10^{-10}–10^{-7} mol/l. Barbier et al. also demonstrated that BRL 37344 induced dose-dependent negative inotropic effects in rats at the same concentrations. However, the reduction in LVDP, particularly at 10^{-7} mol/l, was significantly greater in their study compared to that of ours. Even though we have no accurate explanation for this discrepancy, it might be attributed to the differences in Langendorff technique (constant flow *vs.* constant pressure) and the strain of the rats (Sprague Dawley *vs.* Wistar) used in both studies. On the other hand, at higher concentration (10^{-6} mol/l), BRL 37344 induced positive inotropic effect. In previous studies, selective β_3-AR stimulation was also demonstrated to lead to positive inotropic effect at higher concentrations (Takayama

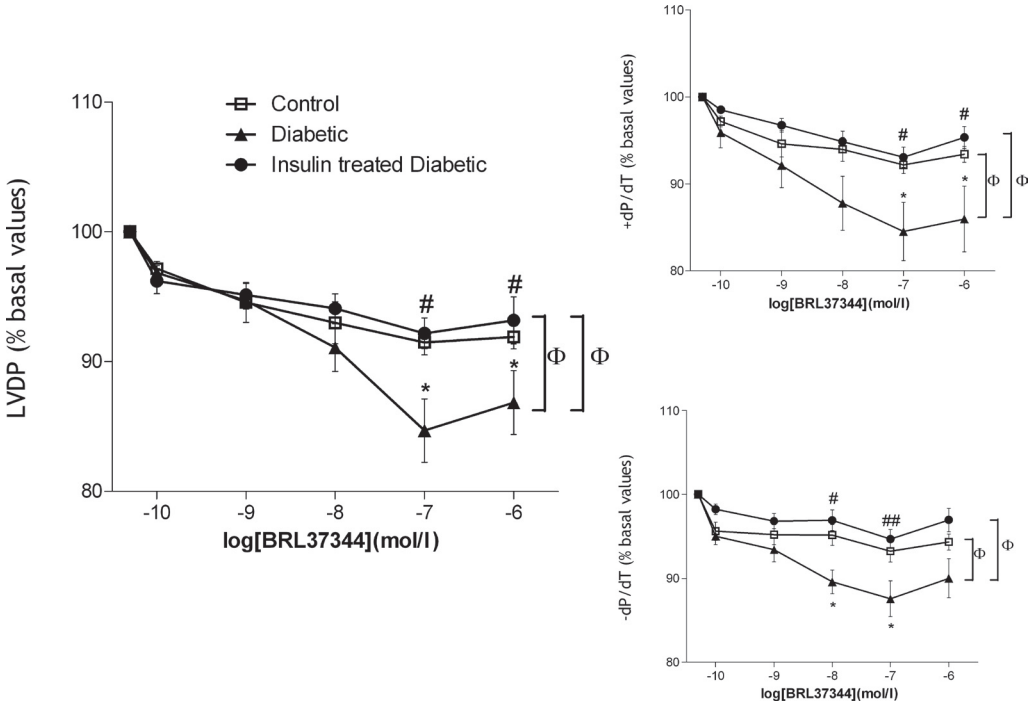

Figure 2. Concentration-response curve for BRL 37344 in the presence of nadolol (10^{-5} mol/l) on left ventricular developed pressure (LVDP), the first derivatives of the maximal rate of left ventricular pressure development (+dP/dt), and of the maximal rate of relaxation (–dP/dt) Values are the mean ±S.E.M. (n = 8). The response is expressed as the percentage of LVDP, +dP/dt and –dP/dt measured at baseline. Unpaired t test with the Bonferroni correction was used for pairwise comparisons within groups (* $p < 0.05$, controls vs. diabetics; # $p < 0.05$, ## $p < 0.01$, diabetics vs insulin treated diabetics). Comparison of the different concentration-response curves was performed by two-way ANOVA (Φ, $p < 0.05$).

et al. 1993; Wheeldon et al. 1993; Berlan et al. 1994; Barbier et al. 2007). The positive inotropic effect of β_3-AR agonists at higher concentrations has been suggested to result from affecting β_1-/β_2-ARs instead of β_3-ARs (Kitamura et al. 2000). Therefore, the effects of BRL 37344 were also studied in the presence of nadolol, a β_1-/β_2-AR antagonist, to abolish potential β_1-/β_2-AR-mediated effects. On these conditions, inhibitory effect of BRL 37344 on cardiac contractility was not modified by pre-treatment with nadolol but abolished by SR 59230A, a β_3-AR antagonist, suggesting that β_3-ARs are responsible for negative inotropic effect. However, significant inhibition of positive inotropic effect of BRL 37344 at the concentration of 10^{-6} M in the presence of nadolol, supported that this effect is indeed mediated by β_1-/β_2-ARs. In contrast to our expectations, this positive inotropic effect was abolished in the presence of SR 59230A, as well. This finding is quite difficult to interpret, as we would expect the positive inotropic effect of BRL 37344 to be unchanged by SR 59230A pretreatment. However, it should be noted that SR 59230A was demonstrated to interact with other subtypes of β-ARs (Hutchinson et al. 2001; Hoffman et al. 2004). This partly explains why SR 59230A pretreatment abolished the positive inotropic effect of BRL 37344 at the higher concentration. On the other hand, our finding that negative inotropic effect of BRL 37344 is enhanced in diabetic heart was not surprising as cardiac β_3-AR mRNA expression levels were also found to be increased in this study. As is known, an increase in mRNA levels is not necessarily translated into an increase in protein levels. However, we have previously shown a 100% increase in β_3-ARs protein levels of 14-week diabetic rat hearts when compared with those of controls (Dincer et al. 2001). From our previous finding on β_3-ARs protein levels, coupled with data from the present study, it might be speculated that augmentation of negative inotropic effect mediated by cardiac β_3-ARs is most likely associated with the increased expression of this subtype in diabetes. Although our results that treatment of diabetic rats reverses the increase in negative inotropic effect of BRL 37344 but not β_3-AR mRNA levels do not seem to support this possibility, it should be pointed out that the effect of insulin could be more prominent at the level of protein turnover. Indeed, we have previously shown that insulin treatment significantly attenuated the increase in the expression of β_3-ARs whereas it only partially decreased the increase in β_3-AR mRNA levels in diabetic rats (Dincer et al. 2001). As a matter of fact, in the present study, we observed a significant increase in mRNA levels encoding $G_{i\alpha 2}$ from diabetic rat hearts. In human ven-

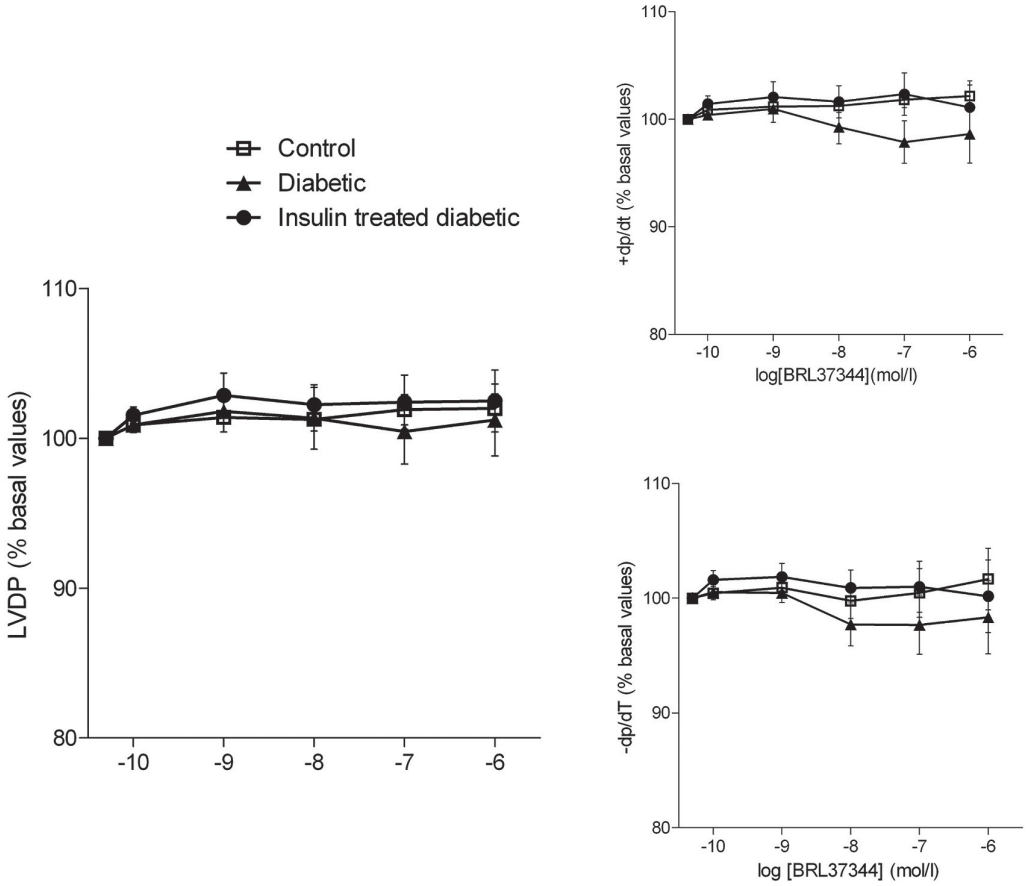

Figure 3. Concentration-response curve for BRL 37344 in the presence of SR 59230A (10^{-7} mol/l) on left ventricular developed pressure (LVDP), the first derivatives of the maximal rate of left ventricular pressure development (+dP/dt), and of the maximal rate of relaxation (–dP/dt) Values are the mean ±S.E.M. (n = 9). The response is expressed as the percentage of LVDP, +dP/dt and –dP/dt measured at baseline. Unpaired t test with the Bonferroni correction was used for pairwise comparisons within groups. Comparison of the different concentration-response curves was performed by two-way ANOVA.

tricular muscle, β_3-ARs were shown to couple to $G_{i/o}$ proteins, which are upregulated in heart failure and diabetes (Gauthier et al. 1996; Rozec et al. 2003; Gauthier et al. 2011). Stimulation of G_i proteins activates NO pathway, probably implicating endothelial eNOS (Ursino et al. 2009). Accordingly, the effects of β_3-AR agonists on contractility were shown to be associated with parallel increases in the production of NO and intracellular cGMP in human ventricule, which were also inhibited by NOS inhibitors (Gauthier et al. 1998). Therefore, it has been proposed that the negative inotropic effect induced by β_3-AR stimulation at least partly results from the activation of $G_{i/o}$ with downstream signalling through NO (Audigane et al. 2009; Gauthier et al. 1998). Even though we observed an increase in mRNA levels encoding eNOS in diabetic rat hearts, it was not found to be significant. However, it should be kept in mind that diabetes might affect the phosphorylation status of eNOS without altering total eNOS expression. Hence, it could be interesting to determine whether any alteration occurs in the phosphorylation status of eNOS and NO generation due to diabetes. Although we were not able to define underlying mechanism(s) of increased β_3-AR mediated effect in diabetic rats, our results are important, because experimental evidence indicates that alterations in the function of cardiac β_3-AR subtypes might be involved in the development of diabetes-induced cardiac dysfunction. As to why diabetes enhances the selective β_3-AR responsiveness in the heart, on the other hand, is unknown. In human heart, the regulation of cardiac contractility and/or heart rate is provided *via* the stimulation of β_1/ β_2-ARs. Therefore, short-term stimulation of these receptors is considered as positive for heart function (Balligand 2009). In heart failure, sympathetic nervous system is activated to compensate the decrease in myocardial contractility and is still beneficial to maintain cardiac performance at the earlier stage. However, chronic overactivation becomes detri-

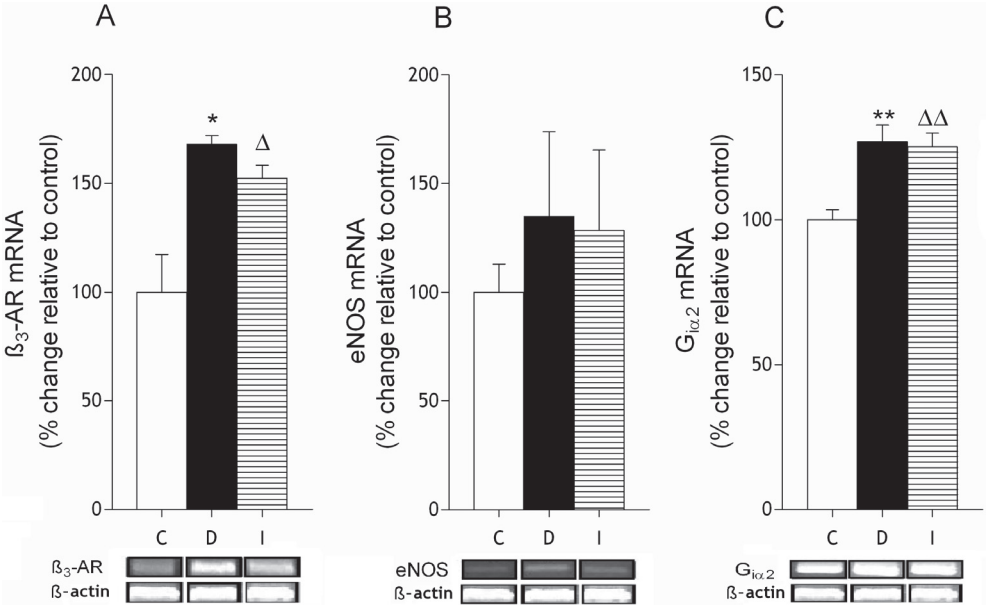

Figure 4. A. Cardiac β_3-adrenoceptor mRNA expression obtained from control (n = 5), diabetic (n = 4) and insulin-treated diabetic rat (n = 4) hearts. **B.** Cardiac eNOS mRNA expression obtained from control (n = 4), diabetic (n = 5) and insulin-treated diabetic rat (n = 4) hearts. **C.** Cardiac $G_{i\alpha 2}$ mRNA expression obtained from control (n = 4), diabetic (n = 5) and insulin-treated diabetic rat (n = 4) hearts. Expression levels were represented as the ratio of signal intensity for β_3-adrenoceptor mRNA relative to β-actin mRNA. All samples were derived at the same time and processed in parallel. One-way ANOVA with Bonferroni test was used for comparisons within groups (** $p < 0.01$, controls *vs.* diabetics; $^{\Delta}$ $p < 0.05$, controls *vs.* insulin-treated diabetics).

mental by leading adverse remodeling of the myocardium through toxic effects on cardiomyocytes (Engelhardt et al. 1999). Chronic stimulation of β_1-ARs has been reported to lead to hypertrophy, interstitial fibrosis and heart failure in β_1-ARs transgenic mice (Engelhardt et al. 2001). As a result, β-adrenergic signal transduction is reduced secondary to downregulation of β_1-ARs (Brodde 1991) and desensitization in β_2-ARs (Ungerer et al. 1993) to suppress the pressure on the heart caused by increased sympathetic drive. This endogenous antiadrenergic strategy could be considered as a self-protective mechanism and it constitutes the rationale for the use of β-adrenergic blockers in the treatment of chronic heart failure (Bristow 2000). β_3-ARs, on the other hand, are stimulated at higher catecholamine concentrations, suggesting that this subtype could be activated in situations where sympathetic tone is high (Gauthier et al. 2011). Several studies have shown a significant upregulation of β_3-ARs in both experimental (Cheng et al. 2001) and clinical heart failure (Moniotte et al. 2001). In contrast to β_1-ARs, no histological evidence of myocyte hypertrophy or fibrogenesis was observed in mice overexpressing β_3-ARs, suggesting that activation of this subtype was not associated with cardiac damage during heart failure (Tavernier et al. 2003). Moreover, lack of β_3-AR signalling has recently been shown to worsen cardiac pressure-overload remodeling, implying a cardioprotective role for this subtype for modulating adverse remodeling in the failing heart (Moens et al. 2009). Therefore, it has been proposed that the negative inotropic effect induced by the β_3-AR stimulation in healthy heart might have a protective role during intense adrenergic stimulation such as stress and heavy physical effort (Rozec and Gauthier 2006). In a similar way, at earlier stages of heart failure, β_3-ARs activation, may provide protection against catecholamine-induced tissue remodeling without significant effects on left ventricle contractility (Gauthier et al. 2011). Thus, it has been proposed that β_3-AR pathway might act as an "endogenous β_1-AR blocker" in circumstances of adrenergic overdrive (Dessy and Balligand 2010). At chronic stages, however, the role of β_3-ARs is uncertain. In advanced heart failure, the consequence of β_3-ARs activation might be even worse with a persistent negative inotropic effect further enhancing myocardial depression (Gauthier et al. 2007). Previous observations from our laboratory have estimated that the ratio of β_1-AR:β_2-AR:β_3-AR in control rat hearts to be approximately 62:30:8, respectively. In diabetic rats, this ratio changed to 40:36:23 (Dincer et al. 1998). As a result, diabetes has many similarities to heart failure in terms of the alterations in the expression of cardiac β-AR subtypes. However, it is not clear at present whether the increase in the expression and/or responsiveness of β_3-ARs in diabetes occurs in response to excessive catecholamine stimulation of the heart, because there are studies

demonstrating either an increase, a decrease or no change in noradrenaline concentration in the diabetic heart (Vadlamudi and McNeill 1984; Gando et al. 1993; Wisniewska and Wisniewski 1996; Schmid et al. 1999).

Our findings on the effects of BRL 37344 on +d*P*/d*t* and –d*P*/d*t* values support this conclusion. We have observed that BRL 37344 produced a negative lusitropic effect in control rats as previously described in several studies (Kitamura et al. 2000; Barbier et al. 2007). We have also found that this negative lusitropic effect of BRL 37344 was significantly increased in diabetic rats. In a recent study, it has been reported that the negative lusitropic effect of BRL 37344 involves the activation of NO-cGMP-protein kinase G pathway (Angelone et al. 2008). In addition, in the same study, BRL 37344 has been shown to counteract the positive lusitropic effect induced by isoproterenol suggesting that lusitropic control mediated by β_3-ARs might oppose the effects of excessive β_1/β_2-ARs stimulation, thereby allows preserving normal cardiac function. However, at present, whether the increased negative lusitropic effect of β_3-AR stimulation is still beneficial in long-term diabetes is uncertain. Nevertheless, overexpression of β_3-ARs in cardiac disease might have important implications as abnormal β-AR signal transduction appears to be one of the major causes of systolic diastolic dysfunction in heart failure and diabetes. It has been demonstrated that β_3-AR stimulation activates cardiac Na^+/K^+-ATPase, which may counteract Na^+overload (Golfman et al. 1998). As increased intracellular myocyte Na^+ levels represent a key adverse pathophysiological feature of heart failure, β_3-AR upregulation may be a useful compensatory mechanism that facilitates β_3-AR-mediated stimulation of the Na^+/K^+-ATPase, which is the main export route for Na^+. Consistent with this, it has been proposed that β_3-AR activation is beneficial in severe heart failure (Rasmussen et al. 2009). On the other hand, sarcolemmal Na^+/K^+-ATPase activity was found to be depressed in diabetic rats as well (Golfman et al. 1998). By analogy, it might be postulated that the activation of Na^+/K^+-ATPase *via* upregulated β_3-ARs is also beneficial in diabetic heart.

In conclusion, the present study shows that 8-week diabetes increased both the effects of selective β_3-AR stimulation on cardiac hemodynamics (LVDP, +d*P*/d*t*, –d*P*/d*t*) and mRNA levels encoding β_3-ARs in rats. However, the consequences of increased β_3-AR stimulation on cardiac function in diabetes can not be determined based on these findings. At present, there is little information in the literature concerning the spatial nature of β_3-AR signalling. As a matter of fact, it has been proposed that the effects of β_3-ARs on cardiac contractility might be subsidiary (Michel et al. 2011). In this regard, the possibility that β_3-ARs might have a different role in the heart can not be excluded. Thus, the possible, yet, undetermined effect(s) of cardiac β_3-ARs should be elucidated for a healthy prediction.

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