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# The role of insulin–thyroid hormone interaction on β-adrenoceptor-mediated cardiac responses

Ebru Arioglu-Inan [a,*], Isil Ozakca [a], Gizem Kayki-Mutlu [a], Aylin Sepici-Dincel [b], Vecdi Melih Altan [a]

[a] Ankara University, Faculty of Pharmacy, Department of Pharmacology, 06100 Tandogan, Ankara, Turkey
[b] Gazi University, Faculty of Medicine, Department of Biochemistry, 06500 Teknikokullar, Ankara, Turkey

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ABSTRACT

*β*-adrenoceptor-mediated responses are known to be attenuated in diabetic rat hearts, related to decreased receptor sensitivity and density. These impaired responses were improved with insulin in diabetic rats, but not in thyroidectomized diabetic rats. We aimed to investigate the possible interaction between insulin and thyroid hormones to restore diabetes-induced alterations on *β*-adrenoceptor-mediated responses. Male Sprague-Dawley rats were divided into seven groups: control (C), diabetic (D), insulin-treated diabetic (DI), thyroidectomized diabetic (TxD), insulin-treated thyroidectomized diabetic (TxDI), insulin+low dose 3,3′,5-triiodo-L-thyronine ($T_3$) treated ($TxDIT_{2.5}$) or insulin+high dose $T_3$ ($TxDIT_5$) treated thyroidectomized diabetic rats. Diabetes was induced with 38 mg/kg streptozotocin. Cardiac function was assessed through pressure-volume analysis and papillary muscle experiments. QPCR and western blot experiments were performed to evaluate cardiac gene expressions. Hemodynamic parameters were impaired in diabetes, and were mostly corrected in DI and $TxDIT_5$ groups. Isoprenaline- and BRL37344-induced contractile responses were also decreased in diabetes. Isoprenaline responses were improved significantly in DI and $TxDIT_5$ groups, whereas BRL 37344-mediated responses were increased slightly. Reduced $\beta_1$-adrenoceptor and SERCA 2A mRNA levels in diabetes were corrected in DI and $TxDIT_5$ groups. Decreased SERCA 2A and increased $\beta_3$-adrenoceptor protein levels in diabetes were improved in DI and $TxDIT_5$ groups. No significant changes were found in phospholamban or endothelial nitricoxide synthase protein levels. These results show that the beneficial effects of insulin on *β*-adrenoceptor-mediated responses in diabetic rats are dependent upon adequate concentrations of thyroid hormones.



## 1. Introduction

Cardiovascular complications are the most important factors of diabetic morbidity and mortality (Fowler, 2008). Ventricular dysfunction and hyperthrophy, coronary artery disease and hypertension are among these complications (Boudina and Abel, 2007). Diabetic cardiomyopathy appears to be critically important, as it increases the susceptibility to ischemic heart disease (Fang et al., 2004).

Three subtypes of β-adrenoceptors are demonstrated in cardiac tissues (Dincer et al., 2001). $\beta_1$- and $\beta_2$-adrenoceptors are coupled to stimulatory G protein ($G_s$). Their stimulation results in a positive inotropic effect by activating adenylyl cyclase-cyclic adenosine monophoshate (cAMP)-protein kinase A pathway. The third subtype, $\beta_3$-adrenoceptors, is coupled to inhibitory *G* protein ($G_i$), and their stimulation results in a negative inotropic effect by activating endothelial nitric oxide synthase (eNOS)-guanylate cyclase-cyclic guanylate monophosphate (cGMP)-nitric oxide pathway (Gauthier et al., 1998). The role of eNOS in the $\beta_3$-adrenoceptor-mediated signaling pathway has been confirmed, as the negative inotropic effect of BRL 37344 was eliminated in eNOS knockout mice (Barouch et al., 2002).

Decreased inotropic and chronotropic responses to *β*-adrenoceptor agonist stimulation have been reported in diabetic hearts (Dincer et al., 1998, 2001). Cardiac $\beta_1$- and $\beta_2$-adrenoceptors are downregulated in streptozotocin-induced diabetes (Bidasee et al., 2008). On the contrary, $\beta_3$-adrenoceptors are upregulated in this pathology (Dincer et al., 2001; Bidasee et al., 2008; Sharma et al., 2008). This redistribution of *β*-adrenoceptor subtypes is thought to contribute to impaired cardiac function in diabetic hearts (Bidasee et al., 2008).

Insulin treatment effectively improves most of these diabetic disturbances. It was reported that 2-week-insulin treatment restored $\beta_1$-adrenoceptor downregulation in 14-week-diabetic rats

* Corresponding author. Tel.: +90 312 203 3145; fax: +90 312 213 1081.
*E-mail address:* arioglu@ankara.edu.tr (E. Arioglu-Inan).

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(Dincer et al., 2001). Furthermore, $\beta_3$-adrenoceptor protein levels were significantly decreased in these animals after insulin treatment (Dincer et al., 2001).

Reduced $\beta$-adrenoceptor-mediated responsiveness in diabetes is thought to be associated with attenuated serum thyroid hormone [3,3′,5-triiodo-L-thyronine ($T_3$) and thyroxine ($T_4$)] levels (Sunderesan et al., 1984). Since insulin treatment improves both $T_3/T_4$ levels and $\beta$-adrenoceptor-mediated responses, it points out a possible relationship between insulin and thyroid hormones (Sunderesan et al., 1984). Haddad et al. (1997) found that insulin could not restore the change in cardiac myosin heavy chain gene in thyroidectomized diabetic rats. The shift in myosin heavy chain α-isoform to $\beta$-isoform was corrected only if the insulin treatment was combined with $T_3$. Furthermore, Karasu et al. (1990) demonstrated that impaired isoprenaline responses in diabetic rat atria were ameliorated with insulin treatment, whereas no improvement was observed in thyroidectomized diabetic rats. Recently, the protective role of thyroid hormone was described in streptozotocin induced diabetes (Falzacappa et al., 2011). In this study, $T_3$ treatment preserved pancreas $\beta$-cell islet morphology in diabetic mice. As a result, hyperglycemia was improved in these animals. All these findings suggest that $T_3$ may have beneficial effects in streptozotocin-induced diabetes. In the present study, we aimed to determine the beneficial effects of $T_3$ in terms of insulin–$T_3$ interaction on $\beta$-adrenoceptor-mediated alterations in diabetic rat hearts.

## 2. Materials and methods

### *2.1. Animals and experimental model*

The experimental protocol and animal care of the present study were approved by the Institutional Ethical Committee of Ankara University (Approval ID:2010-56-283). Ten-week-male Sprague-Dawley rats (250–300 g) provided by Bilkent University (Ankara, Turkey) were used in the study. The rats were housed under the controlled environmental conditions (22 ± 1 °C ambient temperature, 60% relative humidity, and 12:12 h light cycle) and were given free access to rat chow (Purina, Turkey) and tap water. The rats were randomly divided into seven groups: control (C), diabetic (D), insulin treated diabetic (DI), thyroidectomized diabetic (TxD), insulin treated thyroidectomized diabetic (TxDI), insulin+low dose $T_3$ treated thyroidectomized diabetic ($TxDIT_{2.5}$) and insulin+high dose $T_3$ treated thyroidectomized diabetic ($TxDIT_5$). Rats in Tx groups were surgically thyroidectomized, whereas others were sham operated. Hypothyroidism was confirmed by the analysis of serum $T_3$ and $T_4$ by chemical luminescence immunoassay (CLIA; Roche E170, Basel, Switzerland). Diabetes was induced by an intraperitoneal injection of 38 mg/kg of streptozotocin (Sigma, USA), diluted in citrate buffer (pH 4.5). Doses of streptozotocin ranging from 35 to 45 mg/kg were evaluated with preliminary experiments, and 38 mg/kg was chosen as it was the most effective dose with minimum mortality. After 72 h of streptozotocin injection, blood glucose levels were measured with glucose monitor Accuchek (Roche Diagnostics, Mannheim, Germany). Animals having blood glucose levels below 300 mg/dl were not included. At the end of the 6-week-diabetes period, DI group was treated with insulin [5–20 U/kg regular insulin, 8.00 am (Actrapid, Novo Nordisk): 5–20 U/kg isophane insulin, 8.00 pm (Humulin NPH, Eli Lilly)] for 2 weeks. On the other hand; TxDI group was treated with insulin, $TxDIT_{2.5}$ group was treated with insulin+$T_3$ (2.5 μg/kg/day) and $TxDIT_5$ group was treated with insulin+$T_3$ (5 μg/kg/day) for 2 weeks. $T_3$ was administered via osmotic minipumps (Alzet, 1002, Durect, Cupertino, CA). At the end of 2-week treatment period, rats were sacrificed, hearts were quickly excised and weighed, then in vivo or in vitro cardiac experiments were conducted. Blood samples were taken, centrifuged at 4000*g* for 10 min and stored in −20 °C for thyroid hormone measurements. Left ventricle tissues were frozen in liquid nitrogen and stored at −80 °C for subsequent QPCR and western blot experiments.

### *2.2. $T_3$ treatment of rats*

$TxDIT_{2.5}$ and $TxDIT_5$ groups were given $T_3$ in doses of 2.5 μg/kg/day or 5 μg/kg/day by insertion of an osmotic minipump. In all, 2.5 μg/kg/day and 5 μg/kg/day $T_3$ doses were chosen since these doses were shown to be effective as low dose and high dose, respectively (Danzi et al., 2005). Microosmotic pumps delivered 0.25 μl $T_3$ per hour. $T_3$ stock solution (10 mg/kg, Sigma) was prepared in 0.05 N NaOH and diluted in 0.9% Sodium chloride as previously described (Danzi et al., 2005). Pumps filled with diluted $T_3$ solution were inserted into rats under ketamin/xylasine anesthesia.

### *2.3. Hemodynamic assessment*

The rats were anesthetized with an intraperitoneal injection of ketamine–xylasine (90 mg/kg:10 mg/kg) combination. Rats were placed on a heated, temperature-controlled operating table (37 °C) (Harvard apparatus, Holliston, MA, USA). Firstly, the right carotid artery was cannulated with a 1.9 F pressure volume catheter (Advantage PV System, Scisense, Ontario, CA) and arterial blood pressure was recorded. Then the catheter was advanced into the left ventricle. The catheter was connected to a control unit (ADVantage™, Scisense Inc, Ontario) that calculates admittance magnitude and pressure. The outputs from the control unit were recorded and parameters of interest were calculated using Labscribe2 acquisition software (iWorx Systems Inc., Dover, New Hampshire). The calculated hemodynamic parameters were as follows: heart rate (HR), end systolic pressure (ESP), end diastolic pressure (EDP), end systolic volume (ESV), end diastolic volume (EDV), rate of contraction ($+dp/dt_{max}$), rate of relaxation ($-dp/dt_{min}$), time constant of left ventricular pressure decay (tau) according to the Weiss and Glantz method, ejection fraction (EF), cardiac output (CO), and stroke volume (SV). ESV, EDV, SV, and CO were normalized to body weight to exclude the influence of body weight differences [cardiac index (CI), ESV index, EDV index, and SV index] (Dae Hee et al., 2010).

### *2.4. Experiments on isolated papillary muscle*

Rats were anesthesized with ether, and hearts were isolated. Papillary muscles were dissected from left ventricle, and superfused at a flow rate of 5 ml/min with Tyrode's solution (95% O2; 5% CO2; 30 °C). The composition of Tyrode's solution was 116 mM NaCl; 5 mM KCl; 2.7 mM CaCl2; 1.1 mM MgCl2; 0.33 mM NaHPO4; 24 mM NaHCO3 and 5 mM glucose. Pacing cycle length of field stimulation was 1700 ms, stimulus pulse width was 2 ms and amplitude was twice the diastolic threshold (Gauthier et al., 1996). Tension was recorded with a mechanoelectric force transducer. After a 60 min stabilization period, papillary muscles were stretched stepwise (10 μm increments) until the maximal tension and the experiments were performed at 90% of maximal tension. After equilibration, cumulative concentration–response curves of isoprenaline (0.1 nM–30 μM), and a nonselective $\beta$-adrenoceptor agonist were obtained. Then, a cumulative concentration–response curve of BRL37344 (0.1 nM–1 μM), a preferential $\beta_3$-adrenoceptor agonist, was obtained in the presence of forskolin (3 μM).

**Table 1**
Primer sequences used in QPCR experiments.

| Gene | Primer sequence | Accession number | Product size |
|---|---|---|---|
| $\beta_1$-Adrenoceptor sense | 5′-GCT CAC CAA CCT CTT CAT CA-3′ | NM_012701.1 | 158 bp |
| $\beta_1$- Adrenoceptor antisense | 5′-CCG TCA CAC ATA GCA CGT CT-3′ | | |
| SERCA 2A sense | 5′-CTC TGA GAG TTG ACC AGT CGA T-3′ | NM_001110823.2 | 176 bp |
| SERCA 2A antisense | 5′-AGT ATT GAC TCC AGT CGC CA-3′ | | |
| YWHAZ sense | 5′-GATGAAGCCATTGCTGAACTTG-3′ | NM_013011.3 | 117 bp |
| YWHAZ antisense | 5′-GTCTCCTTGGGTATCCGATGTC-3′ | | |

### 2.5. Total RNA isolation, RT PCR, and real-time PCR experiments

Left ventricle tissues were powdered with liquid nitrogen and homogenized with an ultrasonic homogenizer (Bandelin Electronics, Germany). Total RNA was extracted with the Trizol reagent (Sigma-Aldrich Chemical Co., St. Louis, Missouri, USA) according to the manufacturer's protocol. Total RNA samples were treated with DNase1 (Roche Diagnostics GmBH, Mannheim, Germany). The optical density (OD) values and concentrations of RNA were determined spectrophotometrically using Nanodrop (NanoDrop Tech., USA) at wavelength 260 nm ($\lambda_{260}$) and 280 nm ($\lambda_{280}$). cDNA was synthesized by using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmBH, Mannheim, Germany). Quantitative real-time PCR was done with SYBR Green 1 Master (Roche Diagnostics, Mannheim, Germany) on Lightcycler 480 (Roche Applied Science, Indianapolis, IN, USA). All reactions were run in triplicates. Relative gene expression of each sample was normalized to control gene YWHAZ (Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide). YWHAZ was found as the most stable control gene using GeNorm algorithm (BioGazelle, Belgium). Primer sequences of target genes are shown in Table 1. The real-time PCR efficiency rate ($E$) in the exponential phase and gene expression ratio were calculated by using the following formulas (Pfaffl, 2001):

$$E = 10^{[-1/\text{slope}]}$$

$$\text{Ratio} = (E_{\text{Target}})^{\Delta CP}\text{Target}^{\ (\text{control}-\text{sample})} / (E_{\text{Reference}})^{\Delta CP}\text{Reference}^{\ (\text{control}-\text{sample})}$$

### 2.6. Western blot experiments

Left ventricle tissues were powdered with liquid nitrogen, homogenized with RIPA buffer (Sigma, USA) and sonicated. After constant agitation for 2 h on an orbital shaker at +4 °C, samples were centrifuged at 16,000*g* for 30 min at +4 °C. Protein concentrations were determined using the BCA assay (Pierce, Thermo, USA). Protein samples (10–50 ug per lane) were separated on SDS-PAGE gels (6–15%) and transferred to PVDF membrane (Bio-Rad, USA). Membranes were blocked with 2% BSA in Tris-Buffered Saline-0.1% Tween 20 (TBST) and incubated with following primary antibodies overnight at +4 °C; phospholamban (1:2000, Cell signaling, USA); SERCA 2 A (1:5000, Cell signaling, USA); $\beta_3$-adrenoceptor (1:500, Abcam, USA); eNOS (1:500, Cell signaling, USA); α-tubulin (1:10,000, Abcam, USA). Then membranes were washed with TBST and incubated with the following secondary antibodies for 2 h at +4 °C: antirabbit (1:2000, Cell signaling, USA), antirabbit (1:5000, Abcam, USA), antichicken (1:2000, AnaSpec, USA). Membranes were washed with TBST. Blots were detected by enhanced chemiluminescense assay (Pierce ECL, Thermo, USA) and exposed to film (Kodak, Blue, USA). Films were scanned and the bands were quantified by using Image J (NIH, USA). Densitometric values were normalized to control gene α-tubulin.

### 2.7. Statistical analysis

The results are expressed as mean ± S.E.M. Statistical significance was tested by ANOVA followed by Newman Keul's post hoc test for multiple comparisons of group means. The analysis was performed using the statistical software package (Graphpad Prism, USA). The probability level of $P < 0.05$ was considered as the threshold for statistical significance.

## 3. Results

### 3.1. General characteristics of rats

As a characteristic of diabetes, a significant body weight loss was observed in D and TxD groups. It was corrected partially in DI and $TxDIT_5$ groups, whereas weight loss was not improved in TxDI and $TxDIT_{2.5}$ groups (Table 2). Although heart weights seemed to be significantly reduced in D and TxD groups, it might have resulted from the body weight loss, since heart weight to body weight ratios were not changed significantly (Table 2). Blood glucose levels were elevated in all diabetic groups, however they were normalized with insulin treatment (Table 2). As a result of thyroidectomy, serum $T_3$ and $T_4$ levels were attenuated in TxD groups. $T_3$ and $T_4$ levels were increased markedly in the $TxDIT_5$ group compared to other TxD groups (Table 2), but it did not reach control levels.

### 3.2. Hemodynamic changes

Basal hemodynamic parameters of rats were evaluated using pressure-volume catheter. These experiments showed that heart rate was decreased in D and TxD groups compared to C group. It was corrected markedly in DI group. No significant change was observed in $TxDIT_5$ group (Table 3). End systolic pressure was reduced in D and TxD groups. A slight increase in $TxDIT_{2.5}$ group and a marked increase in TxDIT5 group were found. End diastolic pressure did not differ markedly between the groups. Both end systolic and end diastolic volume indices were elevated in D and TxD groups compared to C group. End diastolic volume index was ameliorated partially in $TxDIT_{2.5}$. Both end diastolic and end systolic volume indices were significantly improved in DI and $TxDIT_5$ groups (Table 3). Cardiac output was reduced in D and TxD groups, and it was corrected with insulin treatment in DI group. Cardiac index was attenuated markedly in TxD group and it was not ameliorated after treatment. Stroke volume was reduced in D and TxD groups, and it was increased in DI group. However, there was no statistically significant difference between the groups in terms of stroke volume index (Table 3).

Impairment of the contractility parameter $dp/dt_{max}$ was found to be markedly increased in D group compared to C group. The impairment was even greater in TxD group. This disturbance was successfully ameliorated in DI and $TxDIT_5$ groups. No improvement was observed in TxDI or $TxDIT_{2.5}$ groups (Fig. 1a). However,

**Table 2**
General characteristics of rats at the end of the 8-week diabetes period.

| Parameters | C | D | DI | TxD | TxDI | $TxDIT_{2,5}$ | $TxDIT_5$ |
|---|---|---|---|---|---|---|---|
| **BW (g)** | $377.29 \pm 12.15$ | $284.00 \pm 15.49^{***}$ | $313.43 \pm 11.55^{**}$ | $215.60 \pm 12.62^{***,\varepsilon\varepsilon\varepsilon,\Pi\Pi\Pi}$ | $251.17 \pm 17.16^{***,\Pi\Pi}$ | $250.82 \pm 7.79^{***},^{\Pi\Pi}$ | $311.20 \pm 18.84^{*},^{\#\#\#,\varnothing,\Delta}$ |
| **HW (g)** | $1.48 \pm 0.08$ | $1.23 \pm 0.22^{**}$ | $1.20 \pm 0.05^{**}$ | $0.91 \pm 0.03^{***},^{\varepsilon\varepsilon\varepsilon,\Pi\Pi}$ | $0.90 \pm 0.03^{***},^{\varepsilon\varepsilon\varepsilon,\Pi\Pi}$ | $0.97 \pm 0.04^{***},^{\varepsilon\varepsilon,\Pi\Pi}$ | $1.02 \pm 0.06^{***}$ |
| **HW/BW** | $0.0037 \pm 0.0001$ | $0.0040 \pm 0.0001$ | $0.0040 \pm 0.0002$ | $0.0041 \pm 0.0002$ | $0.0039 \pm 0.0003$ | $0.0038 \pm 0.0002$ | $0.0034 \pm 0.0001$ |
| **BG (mg/dl)** | $103.42 \pm 2.97$ | $414.33 \pm 13.45^{***}$ | $121.14 \pm 14.58^{\varepsilon\varepsilon\varepsilon}$ | $512.60 \pm 27.04^{***},^{\varepsilon\varepsilon\varepsilon,\Pi\Pi\Pi}$ | $129.13 \pm 15.50^{\varepsilon\varepsilon\varepsilon,\#\#\#}$ | $111.94 \pm 8.61^{\varepsilon\varepsilon\varepsilon,\#\#\#}$ | $100.92 \pm 5.64^{\varepsilon\varepsilon\varepsilon,\#\#\#}$ |
| **fT3 (ng/ml)** | $3.27 \pm 0.10$ | $2.76 \pm 0.07$ | $2.98 \pm 0.14$ | $0.95 \pm 0.06^{***},^{\varepsilon\varepsilon\varepsilon,\Pi\Pi\Pi}$ | $1.01 \pm 0.06^{***},^{\varepsilon\varepsilon\varepsilon,\Pi\Pi\Pi}$ | $1.22 \pm 0.09^{***},^{\varepsilon\varepsilon\varepsilon,\Pi\Pi\Pi}$ | $1.77 \pm 0.14^{**},^{\Pi\Pi}$ |
| **tT3 (pg/ml)** | $1.32 \pm 0.05$ | $1.18 \pm 0.11$ | $1.48 \pm 0.06^{*},^{\varepsilon\varepsilon}$ | $0.45 \pm 0.03^{***},^{\varepsilon\varepsilon\varepsilon,\Pi\Pi\Pi}$ | $0.54 \pm 0.05^{***},^{\varepsilon\varepsilon\varepsilon,\Pi\Pi\Pi}$ | $0.64 \pm 0.04^{***},^{\varepsilon\varepsilon\varepsilon,\Pi\Pi\Pi}$ | $0.94 \pm 0.06^{***},^{\varepsilon,\Pi\Pi\Pi\ \#\#\#,\varnothing\varnothing,\Delta\Delta}$ |
| **fT4 (ng/dl)** | $3.23 \pm 0.11$ | $2.29 \pm 0.34^{***}$ | $2.61 \pm 0.07^{***}$ | $0.07 \pm 0.01^{***},^{\varepsilon\varepsilon\varepsilon,\Pi\Pi\Pi}$ | $0.08 \pm 0.02^{***},^{\varepsilon\varepsilon\varepsilon,\Pi\Pi\Pi}$ | $0.22 \pm 0.05^{***},^{\varepsilon\varepsilon\varepsilon\varepsilon,\Pi\Pi\Pi}$ | $1.35 \pm 0.20^{***},^{\varepsilon\varepsilon\varepsilon,\Pi\Pi\Pi,\#\#\#,\varnothing\varnothing\varnothing,\Delta\Delta\Delta}$ |
| **tT4 (ug/dl)** | $5.37 \pm 0.11$ | $3.60 \pm 0.37^{***}$ | $4.30 \pm 0.23^{***},^{\varepsilon\varepsilon}$ | $0.68 \pm 0.02^{***},^{\varepsilon\varepsilon\varepsilon,\Pi\Pi\Pi}$ | $0.66 \pm 0.04^{***},^{\varepsilon\varepsilon\varepsilon,\Pi\Pi\Pi}$ | $0.93 \pm 0.10^{***}$ | $3.27 \pm 0.39^{***},^{\varepsilon\varepsilon,\#\#\#,\varnothing\varnothing\varnothing,\Delta\Delta\Delta}$ |

Values are expressed as mean $\pm$ S.E.M. ($n$=7–12). BW: body weight; HW: heart weight; BG: blood glucose; fT3 and fT4: free T3 and free T4; tT3 and tT4: total T3 and total T4.; $^{*}P<0.05$; $^{**}P<0.001$; $^{***}P<0.001$, compared to C; $^{\varepsilon\varepsilon}$, $P<0.01$; $^{\varepsilon\varepsilon\varepsilon}$, $P<0.001$, compared to D; $^{\Pi\Pi}$, $P<0.01$; $^{\Pi\Pi\Pi}$, $P<0.0001$, compared to DI; $^{\#\#\#}$, $P<0.001$, compared to TxD; $^{\varnothing}$, $P<0.05$; $^{\varnothing\varnothing}$, $P<0.001$; $^{\varnothing\varnothing\varnothing}$, $P<0.001$, compared to TxDI; $^{\Delta}$, $P<0.05$; $^{\Delta\Delta}$, $P<0.001$; $^{\Delta\Delta\Delta}$, $P<0.001$, compared to TxDIT2,5.

as a parameter of systolic function, ejection fraction was not found to be significantly different among the groups (Fig. 1b).

Impairment of relaxation parameter $dp/dt_{min}$ was also remarkable in D and TxD groups compared to C group. Insulin treatment corrected this impairment in DI group, but it was ineffective in TxDI group. A significant improvement was observed in $TxDIT_5$ group but not in $TxDIT_{2.5}$ group (Fig. 1c). Furthermore, tau values were prolonged in D and TxD groups. This increase was greater in TxD group compared to D group. Prolongation of tau was normalized in DI and $TxDIT_5$ groups (Fig. 1d). However, it was still high in TxDI and $TxDIT_{2.5}$ groups (Fig. 1d).

### *3.3. Papillary muscle experiments*

Left ventricular papillary muscles were used to assess $\beta$-adrenoceptor-mediated contractile responses. Basal tension values were not changed significantly among the groups (supplemental Fig. 1). Inotropic effect of isoprenaline was impaired in D and TxD groups (Fig. 2a). It was improved in DI and $TxDIT_5$ groups (fig. 2a). Maximal response to isoprenaline (1 uM) was markedly attenuated in D ($30.94 \pm 5.29\%$) and TxD ($26.46 \pm 3.25\%$) groups compared to C ($100.00 \pm 3.45\%$) group (Fig. 2b). With insulin treatment, contractile response was increased in DI ($82.17 \pm 7.62\%$) and $TxDIT_5$ ($78.23 \pm 3.32\%$) groups (Fig. 2b). On the other hand, no significant improvement was observed in TxDI ($27.59 \pm 4.77\%$) group. The slight increase in $TxDIT_{2.5}$ group ($45.16 \pm 7.41\%$) was insignificant (Fig. 2b). pD2 values of isoprenaline dose–response curve were not changed among groups (C, $7.82 \pm 0.10$; D, $7.47 \pm 0.22$; DI, $7.24 \pm 0.19$; TxD, $7.41 \pm 0.13$; TxDI, $7.78 \pm 0.18$; $TxDIT_{2.5}$, $7.09 \pm 0.19$; TxDIT5, $7.31 \pm 0.17$).

BRL 37344 (preferential $\beta_3$-adrenoceptor agonist) was chosen to evaluate $\beta_3$-adrenoceptor-mediated negative inotropy. We used this drug since it was shown more selective than CL 316243, other $\beta_3$-adrenoceptor agonist (Gauthier et al., 1999). On the other hand, Shen et al. (1996) demonstrated that CL 316243 was more selective. However they found BRL 37344 was more potent (Shen et al., 1996). Although $\beta_1$- and $\beta_2$-adrenoceptor agonistic effects of BRL 37344 were also mentioned, this effect appeared only with higher concentrations of the drug (1 μM and higher doses). Furthermore, in the present study we also confirmed the negative inotropic effect of BRL 37344 as this effect was abolished in the presence of the SR 59230A (0.1 μM), a $\beta_3$-adrenoceptor selective antagonist (Fig. 3).

BRL 37344 caused significant negative inotropic effect in papillary muscle preparations of C group (Fig. 2c). Maximal response to BRL 37344 (0.1 uM) was diminished markedly in D ($40.35 \pm 8.47\%$) and TxD ($40.92 \pm 11.72\%$) groups compared to C ($100 \pm 14.02\%$) group (Fig. 2d). It was increased in DI ($65.04 \pm 6.19\%$) and $TxDIT_5$ ($75.26 \pm 12.13\%$) groups (fig. 2d). No difference was seen in TxDI ($34.99 \pm 9.93\%$) and $TxDIT_{2.5}$ ($30.96 \pm 8.20\%$) groups (Fig. 2d).

### *3.4. $\beta_1$-Adrenoceptor and SERCA 2A mRNA expressions*

QPCR experiments demonstrated that $\beta_1$-adrenoceptor mRNA expression was decreased in D ($0.51 \pm 0.05$) and TxD ($0.45 \pm 0.10$) groups compared to C group ($1.00 \pm 0.01$) (Fig. 4a). Insulin treatment restored this decline in DI ($1.00 \pm 0.12$) and $TxDIT_5$ ($0.84 \pm 0.20$) groups. However, the treatment was ineffective in TxDI ($0.44 \pm 0.08$) and $TxDIT_{2.5}$ ($0.44 \pm 0.07$) groups (Fig. 4a).

SERCA 2A mRNA expression was found to be reduced in D ($0.67 \pm 0.08$) and TxD ($0.70 \pm 0.06$) groups compared to C group ($1.00 \pm 0.04$) (Fig. 4b). It was increased in $TxDIT_5$ ($1.20 \pm 0.11$) group significantly (Fig. 3b). SERCA 2A mRNA expressions in DI ($0.80 \pm 0.04$), TxDI ($0.83 \pm 0.12$) and $TxDIT_{2.5}$ ($0.80 \pm 0.09$) groups were slightly increased, but did not reach statistical significance (Fig. 4b).

**Table 3**
Hemodynamic parameters of rats.

| Parameters | C | D | DI | TxD | TxDI | $TxDIT_{2,5}$ | $TxDIT_5$ |
|---|---|---|---|---|---|---|---|
| **HR (beat/min)** | 255.50 ± 9.97 | 179.00 ± 10.79*** | 246.00 ± 11.08 $^{\varepsilon\varepsilon\varepsilon}$ | 137.67 ± 6.02***, $^{\varepsilon\varepsilon,\ \Pi\Pi\Pi}$ | 130.83 ± 5.21***, $^{\varepsilon\varepsilon,\ \Pi\Pi\Pi}$ | 148.50 ± 5.04***, $^{\varepsilon,\ \Pi\Pi\Pi}$ | 190.83 ± 5.69***,$^{\Pi\Pi\Pi}$, $^{\#\#\#,\ ØØØ,\ \Delta\Delta}$ |
| **ESP (mmHg)** | 102.50 ± 7.31 | 75.00 ± 3.41** | 80.00 ± 2.79** | 60.00 ± 1.79***, $^{\Pi}$ | 64.67 ± 3.52*** | 80.67 ± 7.01**, $^{\#}$ | 108.00 ± 4.68 $^{\varepsilon\varepsilon\varepsilon,\ \Pi\Pi,\ \#\#\#,\ ØØØ,\ \Delta\Delta\Delta}$ |
| **EDP (mmHg)** | 4.18 ± 0.62 | 4.66 ± 0.63 | 4.47 ± 0.60 | 3.47 ± 0.12 | 4.27 ± 0.86 | 4.59 ± 0.91 | 4.68 ± 1.01 |
| **ESV (µl)** | 101.33 ± 11.06 | 116.25 ± 12.88 | 115.80 ± 9.55 | 108.50 ± 4.75 | 146.80 ± 21.51 | 115.17 ± 13.02 | 122.17 ± 13.52 |
| **ESV index (µl/g.10$^3$)** | 277.75 ± 34.30 | 469.95 ± 81.62* | 311.73 ± 50.00 $^{\varepsilon}$ | 499.01 ± 14.31*, $^{\Pi\Pi}$ | 672.72 ± 71.70*** | 471.41 ± 44.74 | 432.87 ± 58.662 $^{Ø}$ |
| **EDV (µl)** | 358.33 ± 12.25 | 309.25 ± 9.08 | 353.33 ± 12.18 | 299.5 ± 7.78*, $^{\Pi}$ | 338.00 ± 21.02 | 314.50 ± 9.31 | 311.33 ± 10.50 |
| **EDV index (µl/g.10$^3$)** | 976 ± 35 | 1463 ± 160** | 1055 ± 78 $^{\varepsilon\varepsilon}$ | 1384 ± 56**, $^{\Pi\Pi}$ | 1467 ± 84***, $^{\Pi\Pi}$ | 1299 ± 14*, $^{\Pi}$ | 1076 ± 58 $^{\varepsilon\varepsilon}$, $^{\#\#\ ØØ,\ \Delta}$ |
| **CO** | 65.96 ± 4.68 | 36.50 ± 3.46*** | 61.31 ± 3.90 $^{\varepsilon\varepsilon\varepsilon}$ | 25.76 ± 1.82***, $^{\Pi\Pi\Pi}$ | 23.56 ± 3.03***, $^{\varepsilon,\ \Pi\Pi\Pi}$ | 29.50 ± 1.09***, $^{\Pi\Pi\Pi}$ | 38.85 ± 3.33***, $^{\Pi\Pi\Pi,\ Ø}$ |
| **CI (ml/min.g.10$^3$)** | 178.69 ± 10.20 | 151.93 ± 11.91 | 180.32 ± 8.63 | 113.70 ± 6.25***, $^{\Pi\Pi\ \Pi}$ | 103.61 ± 15.09***, $^{\varepsilon,\ \Pi\Pi\Pi}$ | 122.65 ± 7.23**, $^{\Pi\Pi\Pi}$ | 130.61 ± 2.70**, $^{\Pi\Pi\Pi}$ |
| **SV (µl)** | 256.67 ± 10.08 | 204.00 ± 14.37* | 250.00 ± 15.63 | 190.83 ± 6.95*, $^{\Pi}$ | 178.67 ± 20.25**, $^{\Pi\Pi}$ | 198.17 ± 6.43*, $^{\Pi}$ | 197.83 ± 10.71*, $^{\Pi}$ |
| **SV index (µl/g.10$^3$)** | 697.64 ± 17.87 | 874.62 ± 113.89 | 741.68 ± 55.02 | 884.48 ± 53.91 | 789.91 ± 109.08 | 822.23 ± 39.66 | 676.53 ± 12.26 |
| **EF (%)** | 71.50 ± 2.57 | 65.60 ± 4.15 | 70.83 ± 4.13 | 62.20 ± 1.16 | 53.17 ± 5.65*, $^{\Pi}$ | 63.83 ± 3.27 | 62.17 ± 3.83 |
| **dP/dt $_{max}$** | 8460 ± 715 | 4953 ± 190*** | 7059 ± 307*, $^{\varepsilon\varepsilon}$ | 2978 ± 180***, $^{\varepsilon\varepsilon,\ \Pi\Pi\Pi}$ | 3194 ± 243***, $^{\varepsilon\varepsilon,\ \Pi\Pi\Pi}$ | 4294 ± 370***, $^{\Pi\Pi\Pi}$ | 6616 ± 299**, $^{\varepsilon\varepsilon}$, $^{\#\#\#,\ ØØØ,\ \Delta\Delta\Delta}$ |
| **dP/dt $_{min}$** | 5865 ± 501 | 3295 ± 252*** | 4759 ± 343 $^{\varepsilon\varepsilon}$ | 2282 ± 116***, $^{\Pi\Pi\Pi}$ | 2589 ± 130***, $^{\Pi\Pi\Pi}$ | 3131 ± 211***, $^{\Pi\Pi}$ | 5005 ± 431 $^{\varepsilon\varepsilon}$, $^{\#\#\#,\ ØØØ,\ \Delta\Delta\Delta}$ |
| **Tau Weiss (ms)** | 12.52 ± 0.37 | 18.00 ± 1.05*** | 12.52 ± 0.49 $^{\varepsilon\varepsilon\varepsilon}$ | 22.68 ± 0.98***, $^{\varepsilon\varepsilon,\ \Pi\Pi\Pi}$ | 21.88 ± 0.95***, $^{\varepsilon\varepsilon,\ \Pi\Pi\Pi}$ | 20.08 ± 0.74***, $^{\Pi\Pi\Pi}$ | 17.85 ± 0.63***, $^{\Pi\Pi\Pi}$, $^{\#\#,\ ØØ}$ |
| **Tau Glantz (ms)** | 13.37 ± 0.85 | 16.86 ± 1.14* | 13.12 ± 0.69 $^{\varepsilon}$ | 21.85 ± 1.30***, $^{\Pi\Pi\Pi}$ | 19.65 ± 1.77**, $^{\Pi\Pi}$ | 19.67 ± 067**, $^{\Pi\Pi}$ | 15.45 ± 0.94 $^{\#\#,\ Ø,\ \Delta}$ |

Values are expressed as mean ± S.E.M. ($n$=5–7). HR: heart rate; ESP: End systolic pressure; EDP: End diastolic pressure; ESV: End systolic volume; EDV: End diastolic volume; CO: Cardiac output; CI: cardiac index; SV: stroke volume; EF: ejection fraction; dP/dt max: Rate of contraction; dP/dt min: Rate of relaxation; Tau: time constant of left ventricular pressure decay. $^*P<0.05$;$^{**}P<0.01$;$^{***}P<0.001$, compared to C; $^{\varepsilon}$, $P<^{0.05}$; $^{\varepsilon\varepsilon}$, $P<0.01$; $^{\varepsilon\varepsilon\varepsilon}$, $P<0.001$, compared to D; $^{\Pi}$, $P<0.05$, $^{\Pi\Pi}$, $P<0.01$; $^{\Pi\Pi\Pi}$, $P<0.001$, compared to DI; $^{\#}$, $P<0.05$; $^{\#\#}$, $P<0.01$; $^{\#\#\#}$, $P<0.001$, compared to TxD; $^{Ø}$, $P<0.05$; $^{ØØ}$, $P<0.01$; $^{ØØØ}$, $P<0.001$, compared to TxDI; $^{\Delta}$, $P<0.05$; $^{\Delta\Delta}$, $P<0.01$; $^{\Delta\Delta\Delta}$, $P<0.001$, compared to TxDIT2,5.

### 3.5. SERCA 2A and phospholamban protein expressions

Western blot studies were performed to evaluate protein expressions. These studies showed a significant reduction in SERCA 2A protein levels in D and TxD groups compared to C group (Fig. 5a). With insulin treatment, an increase was observed in DI group, but it did not reach control levels. Insulin treatment did not cause any change in TxDI group. A slight increase in SERCA 2A expression was seen in $TxDIT_{2.5}$ group, but it was not significant compared to TxDI. On the other hand, a marked increase was observed in$TxDIT_5$ group (Fig. 5a).

Phospholamban protein expression level was evaluated with comparing monomeric forms of this protein between the groups. Protein samples were boiled before electrophoresis to detect monomeric forms of phospholamban. We found that phospholamban protein levels were not significantly affected in diabetic status. There was a slight increase in phospholamban protein levels in D and TxD groups, but this increase did not reach statistical significance (Fig. 5c). SERCA 2A/phospholamban ratio, however, was reduced in diabetic status (Fig. 5b). Insulin treatment ameliorated this decline in DI group slightly, whereas it did not elicit an improvement in TxDI group. In $TxDIT_5$ group SERCA 2A/phospholamban ratio was increased markedly, whereas a slight but insignificant increase was observed in $TxDIT_{2.5}$ group (Fig. 5b).

### 3.6. $\beta_3$-Adrenoceptor and eNOS protein expressions

Western blot studies demonstrated that $\beta_3$-adrenoceptor protein expression levels were significantly higher in D and TxD groups relative to C group (Fig. 6a). This increase was reduced clearly in DI group. In TxDI group, a significant decrease was observed compared to TxD group. Increased $\beta_3$-adrenoceptor protein expression levels were found to be reduced in $TxDIT_{2.5}$ and $TxDIT_5$ groups compared to TxD group (Fig. 6a). eNOS protein levels were demonstrated not to be changed markedly (Fig. 6b). A slight decrease in eNOS protein expression of D group was not significant (Fig. 6b).

## 4. Discussion

In the present study, we demonstrated impaired systolic and diastolic hemodynamic parameters in diabetic rats as reported previously in experimental diabetes models (Dae Hee et al., 2010; Connely et al., 2007; Radovits et al., 2009). $dp/dt_{max}$, one of the parameters of systolic function, was decreased significantly in diabetic and thyroidectomized diabetic rats. Although insulin treatment ameliorated this impairment in diabetic rats, it failed to correct it in thyroidectomized diabetic rats. The improvement in thyroidectomized diabetic rats was accomplished only when insulin was combined with high dose $T_3$ (5 µg/kg/day). This result suggests that the beneficial effect of insulin was lost in the absence of $T_3$. However, another marker of systolic function, ejection fraction was not changed among the groups. A diastolic dysfunction is mentioned in the pathologies, in which some of the systolic parameters are altered but ejection fraction is preserved. Connely et al. (2007) reported impaired diastolic function with preserved ejection fraction in 6-week-Sprague Dawley and Ren2 diabetic rats. Similarly, in the present study we found that $dp/dt_{min}$ and tau parameters of diastolic function were impaired in diabetic and thyroidectomized diabetic rats despite of unaltered ejection fraction. Decreased $dp/dt_{min}$ and prolonged Tau values were ameliorated with insulin treatment in diabetic rats, but not in thyroidectomized diabetic rats. Improvement of these relaxation parameters was achieved by insulin+high dose $T_3$ treatment in

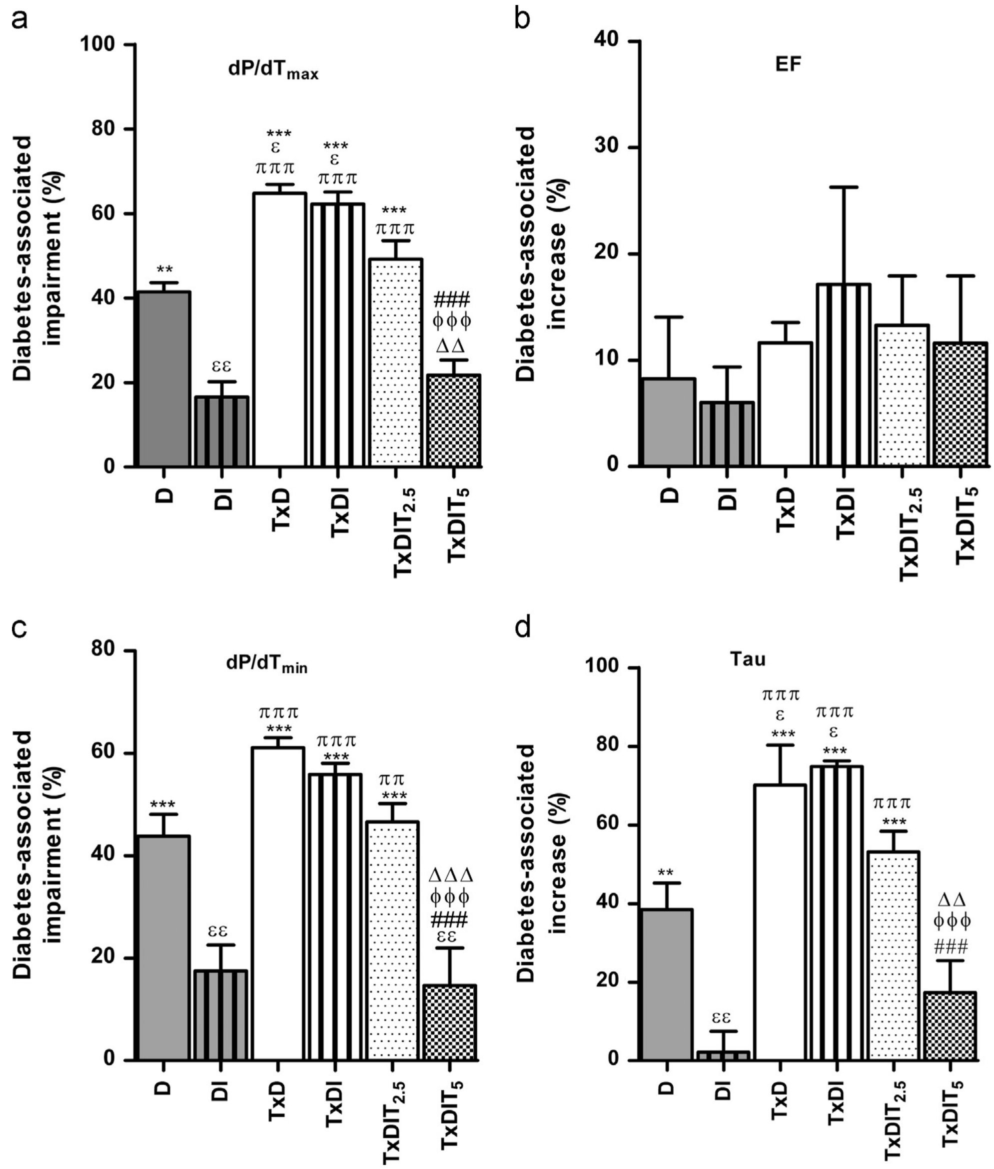


**Fig. 1.** The severity of cardiac dysfunction; diabetes associated impairment of dp/dt$_{max}$ (a), ef (b), and dp/dt$_{min}$ (c), diabetes associated increase of Tau (d). Values are expressed as mean $\pm$S.E.M. ($n$=5–7). dp/dt$_{max}$: rate of contraction, ef: ejection fraction, dp/dt$_{min}$: rate of relaxation, Tau: time constant of left ventricular pressure decay. $^{**}$, $P<0.01$; $^{***}$, $P<0.001$, compared to C; $^{\varepsilon}$, $P<0.50$; $^{\varepsilon\varepsilon}$, $P<0.01$, compared to D; $^{\Pi\Pi}$, $P<0.01$; ΠΠΠ, $P<0.001$, compared to DI; $^{\#\#}$, $P<0.01$; $^{\#\#\#}$, $P<P<0.001$, compared to TxD; $^{ØØ}$, $P<0.01$; $^{ØØØ}$, $P<0.001$, compared to TxDI; $^{\Delta\Delta}$, $P<0.01$; $^{\Delta\Delta\Delta}$, $P<0.001$, compared to TxDIT$_{2,5}$.

thyroidectomized diabetic rats. This result supports our hypothesis that $T_3$ is essential for the effect of insulin.

Since ventricular relaxation during the active phase of diastole is dependent on reuptake of $Ca^{++}$ into sarcoplasmic reticulum by a pump called SERCA 2A (Dhalla et al., 1998), alterations in expression of this pump are predicted to affect ventricular relaxation parameters dp/dt$_{min}$ and Tau. Indeed, diastolic dysfunction accompanied by decreased SERCA 2A expression was demonstrated in 6-week-diabetic rats (Connely et al., 2007). Similarly, SERCA 2A protein levels were reduced in 8-week- and 12-week-diabetic rats (Zhang et al., 2008; Teshima et al., 2000). On the contrary, Watanuki et al. (2004) demonstrated that SERCA 2A levels were unchanged in 4- or 6-week-diabetic rats. In this study, authors claimed that despite the similar results reported earlier (Zarain-Herzberg et al., 1994), their findings may depend on the duration of diabetes, since SERCA 2A protein expression appears to be diminished as the disease progressed (Zhang et al., 2008). These controversial data remain to be elucidated. The most possible explanation for this discrepancy seems to be the duration of the diabetes since diabetes-induced changes worsen as diabetic period increases. In the present study, we found SERCA 2A protein expression decreased in 8-week-diabetic and thyroidectomized diabetic rats. Diastolic dysfunction observed in our study may be relevant to decreased SERCA 2A expression. The lack of diastolic function improvement in thyroidectomized diabetic rats with insulin treatment could be explained by the fact that SERCA 2A protein levels were not increased.

Since SERCA 2A activity is regulated by phospholamban, the changes of this protein is also an important marker for cardiac contractility. Most of the previous studies reveal that phospholamban expression is upregulated in diabetic rat hearts (Zhong et al., 2001; Watanuki et al., 2004; Netticadan et al., 2001). On the contrary, we found that phospholamban protein levels were not significantly changed in diabetic rats. However, the study of Connely et al. (2007) supports our finding as they showed unchanged phospholamban expression in diabetic rats. Teshima et al. (2000) showed that phospholamban mRNA expression levels were unchanged in 12-week-diabetic rats. It has also been

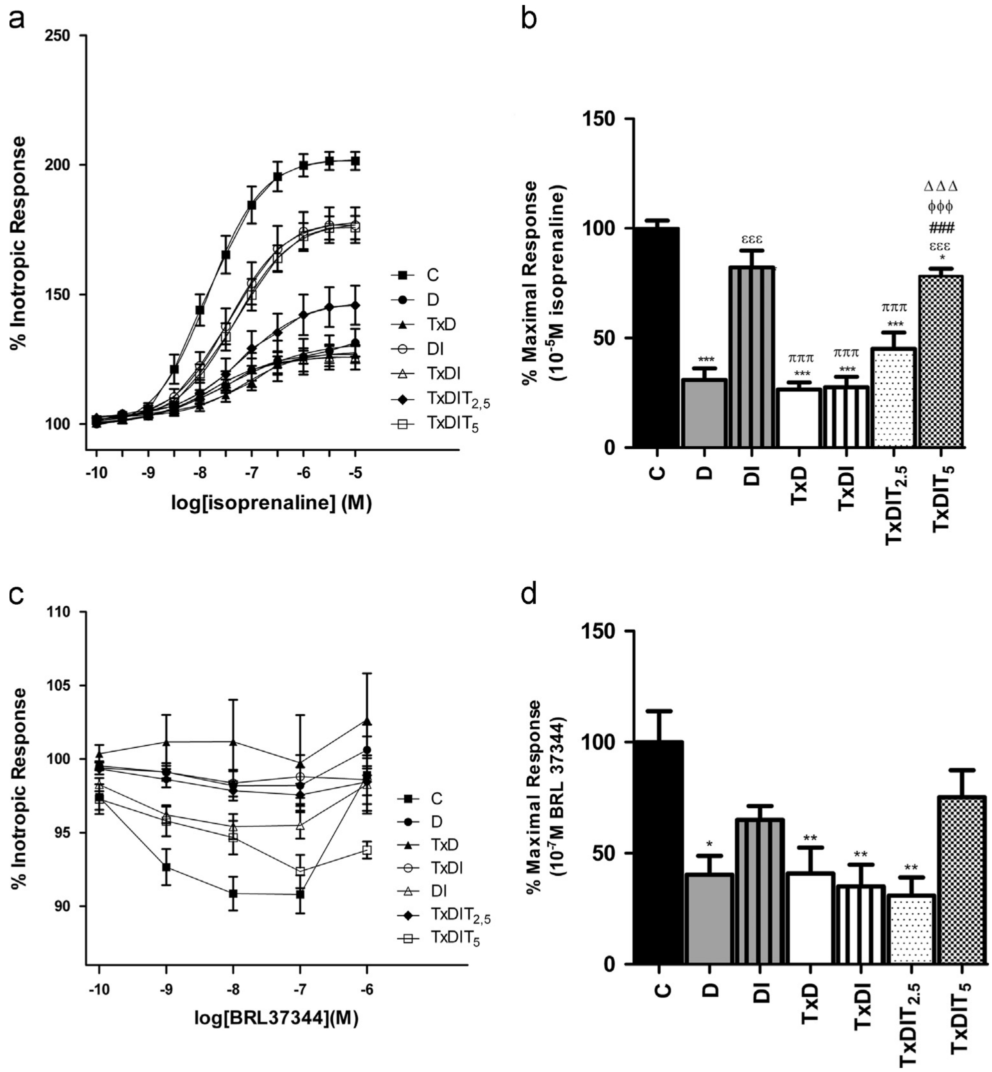


**Fig. 2.** Isoprenaline mediated response; dose–response curve (a), maximal response (1 uM) (b). BRL 37344 mediated response; dose–response curve (c), maximal response (0,1 uM) (d). Values are expressed as mean $\pm$S.E.M. ($n$=4–7). *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$, compared to C; $^{\varepsilon\varepsilon\varepsilon}$, $P<0.001$, compared to D; $^{\Pi\Pi\Pi}$, $P<0.001$, compared to DI; $^{\#\#\#}$, $P<0.001$, compared to TxD; $^{ØØØ}$, $P<0.001$, compared to TxDI; $^{\Delta\Delta\Delta}$, $P<0.001$, compared to TxDIT$_{2,5}$.

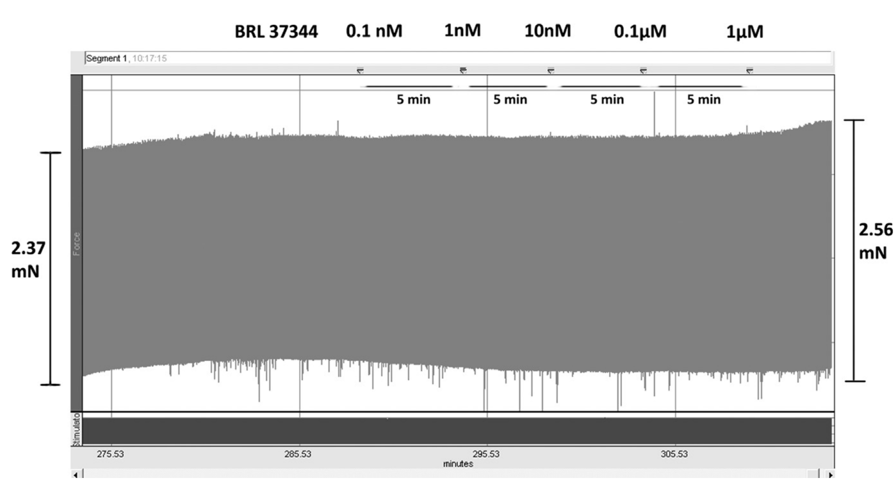


**Fig. 3.** Represantive dose response trace of BRL 37344 (0.1 nM–1 µM) in the presence of SR 59230A (0.1 µM) obtained from papillary muscle of a control rat.

demonstrated that phospholamban protein levels were decreased in diabetic rats (Netticadan et al., 2001; Vasanji et al., 2004). We could not explain this discrepancy with present data.

Diminished inotropic and chronotropic responses to $\beta$-adrenoceptor-agonist stimulation were demonstrated on diabetic heart preparations (Dincer et al., 2001; Connely et al., 2007). This effect

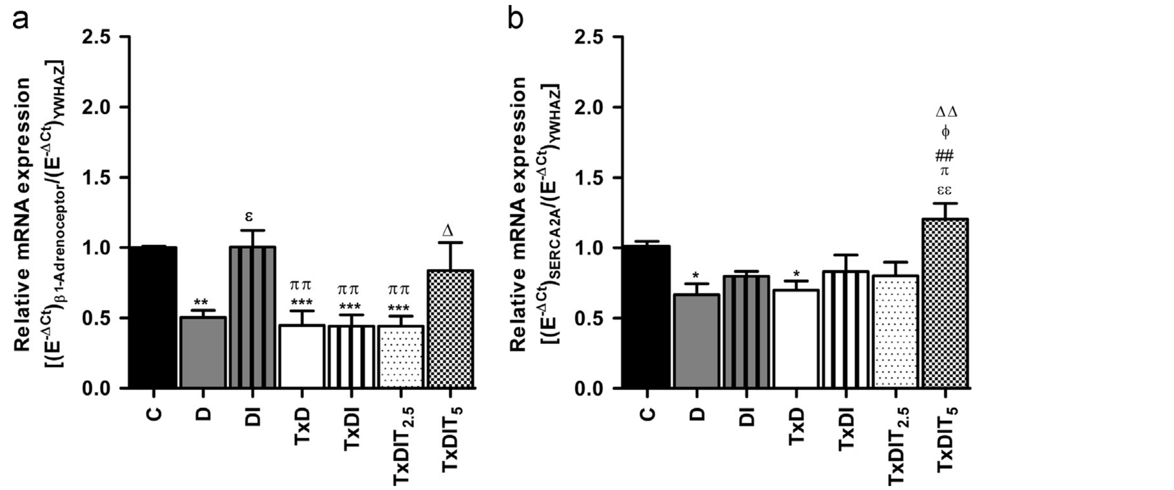


**Fig. 4.** mRNA expressions; $\beta_1$-adrenoceptor (a), SERCA 2 A (b). Values are expressed as mean $\pm$S.E.M. ($n$=5–9). *, $P<0.05$; ***, $P<0.001$, compared to C; $^{\varepsilon}$, $P<0.05$, $^{\varepsilon\varepsilon}$, $P<0.01$ compared to D; $\Pi$, $P<0.05$, $\Pi\Pi$,

is mostly accompanied by decreased $\beta_1$- and $\beta_2$-adrenoceptor expression. It was demonstrated that $\beta$-adrenoceptor-mediated cardiac responses and $\beta_1$-adrenoceptor protein expression were reduced in 14-week-diabetic rats (Dincer et al., 2001). In the present study, we demonstrated that inotropic responses to isoprenaline were reduced in both diabetic and thyroidectomized diabetic rats. $\beta_1$-adrenoceptor mRNA expression was also decreased in these groups. Two-week-insulin treatment corrected both isoprenaline-induced responses and $\beta_1$-adrenoceptor mRNA expression in diabetic rats. No significant improvement was observed with insulin treatment in thyroidectomized diabetic rats. Furthermore, inotropic effect of isoprenaline and $\beta_1$-adrenoceptor mRNA expression were ameliorated in insulin+high dose $T_3$ treated thyroidectomized diabetic rats. Since SERCA 2A/phospholamban ratio is mentioned as an important marker of cardiac contractility (Koss and Kranias, 1996), the improvement of isoprenaline-induced cardiac responses in insulin-treated diabetic and insulin+high dose $T_3$ treated thyroidectomized diabetic rats could be attributed to efficiency of treatment on this ratio.

In the present study, we also investigated $\beta_3$-adrenoceptor mediated relaxation in diabetes. The negative inotropic effect of preferential $\beta_3$-adrenoceptor agonist BRL 37344 was significantly decreased in diabetic and thyroidectomized diabetic rats. Insulin treatment caused a slight increase in BRL 37344-induced responses in diabetic rats, whereas it made no difference in thyroidectomized diabetic rats. On the other hand, $\beta_3$-adrenoceptor-mediated relaxation was improved in thyroidectomized rats when insulin was combined with high dose $T_3$. This results suggests that insulin–thyroid hormone interaction is necessary in terms of $\beta_3$-adrenoceptor-mediated relaxation. However, $\beta_3$-adrenoceptor expression was found to be upregulated in diabetic and thyroidectomized diabetic rats. $\beta_3$-adrenoceptor overexpression in diabetic rats was demonstrated also by other investigators (Amour et al., 2007; Dincer et al., 2001). $\beta_3$-adrenoceptor mRNA expression was found to be increased in other pathologies such as heart failure (Moniotte et al., 2001; Cheng et al., 2001); sepsis (Moniotte et al., 2007) and hypothyroidism (Arioglu et al., 2010). In our previous study (Arioglu et al., 2010) and also in the present study we found that $T_3$ deficiency upregulates $\beta_3$-adrenoceptor expression. However, Germack et al. (2000) demonstrated that hyperthyroidism upregulated $\beta_3$-adrenoceptor protein expression in rat white adipose tissue. This discrepancy could result from the tissue differences, since $\beta_3$-adrenoceptors are coupled to Gi protein in cardiac tissue, whereas this receptor has both Gs and Gi protein coupling in adipose tissue (Soeder et al., 1999). It has been shown that expression of Gi$\alpha$ subunits was diminished in hyperthyroidism and increased in hypothyroidism (Rapiejko et al., 1989; Milligan and Saggerson, 1990). According to these data, Germack et al. (2000) suggest that $\beta_3$-adrenoceptors mainly coupled to Gs$\alpha$ in hyperthyroidism and predominantly to Gi$\alpha$ in hypothyroidism. Thus, increased Gi expression may have resulted in $\beta_3$-adrenoceptor upregulation in hypothyroid status.

Decreased inotropic response to BRL 37344, despite $\beta_3$-adrenoceptor upregulation in diabetic and thyroidectomized diabetic rats, is somewhat surprising. However, Moniotte et al. (2003) demonstrated reduced $\beta_3$-adrenoceptor mediated negative inotropic effect accompanied by $\beta_3$-adrenoceptor upregulation in heart failure. In our study, insulin treatment decreased $\beta_3$-adrenoceptor protein levels significantly in diabetic rats, and it caused a slight decrease in thyroidectomized diabetic rats. Furthermore, both in insulin+low dose $T_3$ and insulin+high dose $T_3$ treated groups, $\beta_3$-adrenoceptor upregulation was reduced.

Activation of $\beta_3$-adrenoceptors is thought to be an adaptive mechanism that protects the myocardium in pathologies in which sympathetic system is overactivated. However, this mechanism becomes maladaptive, as the disease progressed, at least in the case of heart failure (Rozec and Gauthier, 2006). On the contrary, Rasmussen and Bundgaard (2005) suggested that $\beta_3$-adrenoceptor upregulation is a compensatory mechanism in heart failure since stimulation of $\beta_3$-adrenoceptors activates $Na^+$-$K^+$-pump which carries $Na^+$ out of the cell.

$\beta_3$-adrenoceptor stimulation causes negative inotropic effect by activating eNOS-guanylate cyclase-cGMP-nitric oxide pathway (Gauthier et al., 1998). Except for eNOS coupling, $\beta_3$-adrenoceptors have been shown to be coupled also to nNOS (neuronal NOS) and iNOS (inducible NOS) (Amour et al., 2007;Maffei et al., 2007). Amour et al. (2007) have demonstrated that $\beta_3$-adrenoceptors exert their effect mostly through nNOS in diabetic cardiomyopathy. They have reported that nNOS protein expression was predominant, and resulted in nitric oxide production in diabetic cardiomyocytes. Furthermore, Aragon et al. (2011) have suggested that $\beta_3$-adrenoceptors act like a dual activator for eNOS and nNOS. In this study, it was demonstrated that compounds with $\beta_3$-adrenoceptor agonistic activity, such as nebivolol or CL 316243, caused an increase in eNOS activation and nNOS expression (Aragon et al., 2011). In the present study, we demonstrated that eNOS protein expression was not different among the groups, but we were unable to evaluate eNOS activation/coupling or nNOS protein expressions. Therefore, we could not explain decreased $\beta_3$-adrenoceptor mediated responses in diabetic rats despite the

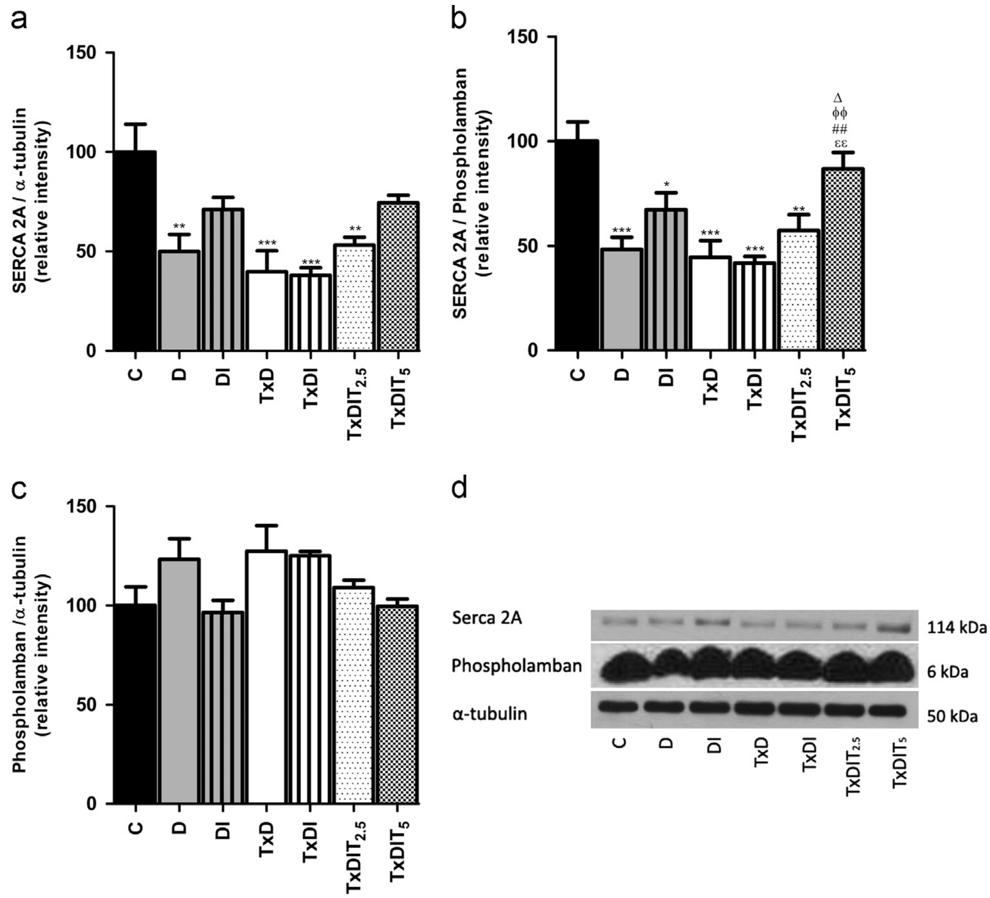


**Fig. 5.** Protein expressions; SERCA 2 A (a), SERCA 2 A/PLN (b), PLN (c), representative western blots for each group (d). Values are expressed as mean ± S.E.M. ($n$=3–4). $^{**}$, $P<0.01$; $^{***}$, $P<0.001$, compared to C; $^{\varepsilon\varepsilon}$, $P<0.01$, compared to D; $^{\#\#}$, $P<0.01$, compared to TxD; $^{ØØ}$, $P<0.01$, compared to TxDI; $^{\Delta}$, $P<0.05$, compared to TxDIT$_{2.5}$.

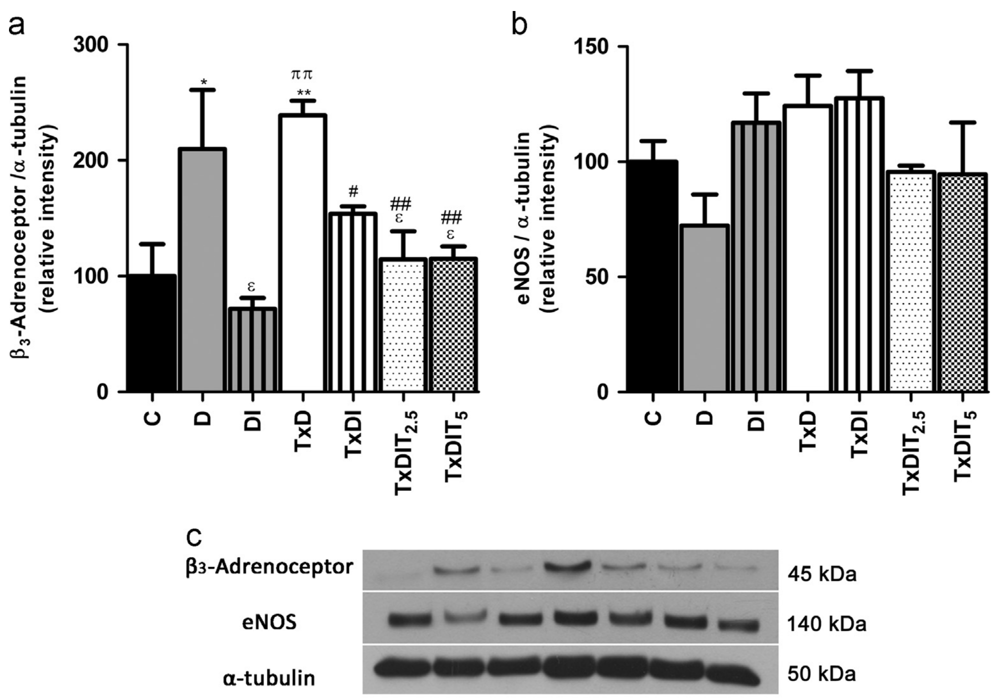


**Fig. 6.** Protein expressions; β$_3$-adrenoceptor (a), eNOS (b), representative western blots for each group (c). Values are expressed as mean ± S.E.M. ($n$=3–4). $^{*}$, $P<0.05$, $^{**}$, $P<0.01$, compared to C; $^{\varepsilon}$, $P<0.05$, compared to D; $^{\Pi\Pi}$, $P<0.01$, compared to DI, $^{\#}$, $P<0.05$, $^{\#\#}$, $P<0.01$, compared to TxD.

$\beta_3$-adrenoceptor upregulation. eNOS uncoupling as a result of increased oxidative stress has been demonstrated in diabetic mice (Youn et al., 2012). Thus, reduced NO bioavailability due to eNOS uncoupling may explain decreased $\beta_3$-adrenoceptor responsiveness in diabetic rats. Alternatively, diminished $\beta_3$-adrenoceptor responsiveness may be related to nNOS overexpression. However, these approaches have to be proven with further experiments.

In summary, in the present study we demonstrated that insulin ameliorated impaired β-adrenoceptor mediated cardiac responses in diabetic rats, but it failed to do so in thyroidectomized rats. The improvement in thyroidectomized diabetic rats was observed only when insulin was combined with high dose $T_3$. Thus, the findings of the present study points out that the beneficial effect of insulin on β-adrenoceptor mediated responses in diabetic rat heart is dependent on $T_3$ levels.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ejphar.2013.06.021.

## References

Amour, J., Loyer, X., LeGuen, M., Mabrouk, N., David, J.S., Camors, E., Carusio, N., Vivien, B., Andriantsitohaina, R., Heymes, C., Riou, B., 2007. Altered contractile response due to increased beta 3 adrenoceptor stimulation in diabetic cardiomyopathy: The role of nitric oxide synthase 1 derived nitric oxide. Anesthesiology 107, 452–460.
Aragon, J.P., Condit, M.E., Bhushan, S., Predmore, B.L., Patel, S.S., Grinsfelder, D.B., Gundewar, S., Jha, S., Calvert, J.W., Barouch, L.A., Lavu, M., Wright, H.M., Lefer, D. J., 2011. Beta 3 adrenoceptor stimulatin ameliorates myocardial ischemia reperfusion injury via endothelial nitric oxide synthase and neuronal nitric oxide synthase activation. J. Am. Coll. Cardiol. 58 (25), 2683–2691.
Arioglu, E., Guner, S., Ozakca, I., Altan, V.M., Ozcelikay, A.T., 2010. The changes in beta-adrenoceptor-mediated cardiac function in experimental hypothyroidism: the possible contribution of cardiac beta3-adrenoceptors. Mol. Cell. Biochem. 335 (1–2), 59–66.
Barouch, L.A., Harrison, R.W., Scaf, M.W., Rosas, G.O., Cappola, T.P., Kobeissi, Z.A., Hobai, I.A., Lemmon, C.A., Burnett, A.L., O'Rourke, B., Rodriguez, E.R., Huang, P.L., Lima, J.A., Berkowitz, D.E., Hare, J.M., 2002. Nitric oxide regulates the heart by spatial confinement of nitric oxide synthase isoforms. Nature 416, 337–339.
Bidasee, K.R., Zheng, H., Shao, C.H., Parbhu, S.K., Rozanski, G.J., Patel, K.P., 2008. Exercise training initiated after the onset of diabetes preserves myocardial function: Effects on expression of beta adrenoceptors. J. Appl. Physiol. 105, 907–914.
Boudina, S., Abel, E.D., 2007. Diabetic cardiomyopathy revisited. Circulation 115, 3213–3223.
Cheng, H.J., Zhang, Z.S., Onishi, K., Ukai, T., Sane, D.C., Cheng, C.P., 2001. Upregulation of functional beta 3 adrenergic receptor in the failing canine myocardium. Circ. Res. 89, 599–606.
Connely, K.A., Kelly, D.J., Zhang, Y., Prior, D.L., Martin, J., Cox, A.J., Thai, K., Feneley, M.P., Tsoporis, J., White, K.E., Krum, H., Gilbert, R.E., 2007. Functional structural and molecular aspects of diastolic heart failure in the diabetic (mRen-2) 27 rat. Cardiovasc. Res. 76, 280–291.
Dae Hee, K., Yong-Jin, K., Hyung-Kwan, K., Sung, A.C., Min-Seok, K., Dae-Won, S., Byung-Hee, O.H., Young-Bae, P., 2010. Usefulness of mitral annulus velocity for the early detection of left ventricular dysfunction in a rat model of diabetic cardiomyopathy. J. Cardiovasc. Ultrasound 18 (1), 6–11.
Danzi, S., Dubon, P., Klein, I., 2005. Effect of serum triiodothyronine on regulation of cardiac gene expression: Role of histone acetylation. Am. J. Physiol. Heart Circ. Physiol. 289, 1506–1511.
Dhalla, N.S., Liu, X., Panagia, V., Takeda, N., 1998. Subcellular remodeling and heart dysfunction in chronic diabetes. Cardiovasc. Res. 40, 239–247.
Dincer, U.D., Bidasee, K.R., Guner, S., Tay, A., Ozçelikay, A.T., Altan, V.M., 2001. The effect of diabetes on expression of beta 1, 2 and 3 receptors in rat hearts. Diabetes 50, 455–461.
Dincer, U.D., Onay, A., Ari, N., Ozcelikay, A.T., Altan, V.M., 1998. The effects of diabetes on beta adrenoceptor mediated responsiveness of human and rat atria. Diabetes Res. Clin. Pract. 40, 113–122.
Falzacappa, C.V., Mangialardo, C., Madaro, L., Ranieri, D., Lupoi, L., Stigliano, A., Torrisi, M.R., Bouché, M., Toscano, V., Misiti, S., 2011. Thyroid hormone T3 counteracts STZ induced diabetes in mouse. Plos One 6 (5), e19839.
Fang, Z.Y., Prins, J.B., Marwick, T.H., 2004. Diabetic Cardiomyopathy: evidence, mechanism and therapeutic implications. Endocr. Rev. 25, 543–567.
Fowler, M.J., 2008. Microvascular and macrovascular complications of diabetes. Clin. Diabetes 26, 77–82.
Gauthier, C., Leblais, V., Kobzik, L., Trochu, J.N., Khandoudi, N., Bril, A., Balligand, J.L., Le Marec, H., 1998. The negative inotropic effect of beta 3 adrenoceptor stimulation is mediated by activation of a nitric oxidase synthase pathway in human ventricle. J. Clin. Invest. 102, 1377–1384.
Gauthier, C., Tavernier, G., Trochu, J.N., Leblais, V., Laurent, K., Langin, D., Escande, D., LeMarec, H., 1999. Interspecies differences in the cardiac negative inotropic effects of beta(3)-adrenoceptor agonists. J Pharmacol Exp Ther 290 (2), 687–693.
Gauthier, G., Tavernier, G., Charpentier, F., Langin, D., Le Marec, H., 1996. Functional $\beta_3$-adrenoceptor in the human heart. J. Clin. Invest. 98 (2), 556–562.
Germack, R., Starzec, A., Perret, G.Y., 2000. Regulation of $\beta_1$-and $\beta_3$-adrenergic agonist-stimulated lipolytic response in hyperthyroid and hypothyroid rat white adipocytes. Br. J. Pharmacol. 129, 448–456.
Haddad, F., Masatsugu, M., Bodell, P.W., Qin, A., McCue, S.A., 1997. Role of thyroid hormone and insulin in control of cardiac isomyosin expression. J. Mol. Cell Cardiol. 29, 559–569.
Karasu, C., Ozturk, Y., Altan, N., Yıldızoglu-Arı, N., Ikizler, C., Altan, V.M., 1990. Thyroid hormone mediated effect of insulin on alloxan diabetic rat atria. Gen. Pharmacol. 21 (5), 735–740.
Koss, K.I., Kranias, E.G., 1996. Phospholamban, a prominent regulator of myocardial contractility. Circ. Res., 1059–1063.
Maffei, A., Di Pardo, A., Carangi, R., Carullo, P., Poulet, R., Gentile, M.T., Vecchione, C., Lembo, G., 2007. Nebivolol induces nitric oxide release in the heart through inducible nitric oxide synthase activation. Hypertension 50, 652–656.
Milligan, G., Saggerson, E.D., 1990. Concurrent upregulation of guanine-nucleotide-binding proteins, Gi1α, Gi2α and Gi3α in adipocytes of hypothyroid rats. Biochem. J. 270, 765–769.
Moniotte, S., Kobzik, L., Feron, O., Trochu, J.N., Gauthier, C., Balligand, J.L., 2003. Upregulation of beta 3 adrenoceptors and altered contractile response to inotropic amines in human failing myocardium. Circulation 103, 1649–1655.
Moniotte, S., Belge, C., Sekkali, B., Massion, P.B., Rozec, B., Dessy, C., Balligand, J.L., 2007. Sepsis is associated with an upregulation of functional beta 3 adrenoceptors in the myocardium. Eur. J. Heart Fail. 9, 1163–1171.
Moniotte, S., Vaerman, J.L., Kockx, M.M., Larrouy, D., Langin, D., Noirhomme, P., Balligand, J.L., 2001. Real time RT-PCR for the detection of beta adrenceptor Messenger RNAs in small human endomyocardial biopsies. J. Mol. Cell. Cardiol. 33, 2121–2133.
Netticadan, T., Temsah, R.M., Kent, A., Elimban, V., Dhalla, N.S., 2001. Depressed levels of Ca+2 cycling proteins may underlie sarcoplasmic reticulum dysfunction in the diabetic heart. Diabetes 50, 2133–2138.
Pfaffl, M.W., 2001. A new mathematical model for relative quantification in Real time RT-PCR. Nucleic Acids Res. 29 (9), 45.
Radovits, R., Korkmaz, S., Sivakkanan, L., Barnucz, E., Bömicke, T., Arif, R., Karck, M., Szabo, G., 2009. Comparative investigation of the left ventricular pressure-volume relationship in rat models of type 1 and type 2 diabetes mellitus. Am. J. Physiol. Heart Circ. Physiol. 297, 1125–1133.
Rapiejko, P.J., Watkins, D.J., Ros, M., Malbon, C.C., 1989. Thyroid hormones regulate G-protein β-subunit mRNA expression in vivo. J. Biol. Chem. 264, 16183–16189.
Rasmussen, H., Bundgaard, H., 2005. Methods and Compositions for the Treatment of Myocardial Conditions. Northern Sydney Area Health Service.
Rozec, B., Gauthier, C., 2006. Beta 3 adrenoceptors in the cardiovascular system: the putative roles in human pathologies. Pharmacol. Therapeut. 111 (3), 652–673.
Sharma, V., Parsons, H., Allard, M.F., McNeill, J.H., 2008. Metoprolol increases expression of beta 3 adrenoceptors in the diabetic heart: Effects of nitric oxide signaling and forkhead transcription factor-3. Eur. J. Pharmacol. 595, 44–51.
Shen, Y.T., Cervoni, P., Claus, T., Vatner, S.F., 1996. Differences in beta 3-adrenergic receptor cardiovascular regulation in conscious primates, rats and dogs. J. Pharmacol. Exp. Ther 278 (3), 1435–1443.
Soeder, K.J., Snedden, S.K., Cao, W., Della Rocca, G.J., Daniels, K.W., Luttrell, L.M., Collins, S., 1999. The $\beta_3$-adrenergic receptor activates mitogen-activated protein kinase in adipocytes through a Gi-dependent mechanism. J. Biol. Chem. 274 (17), 12017–12022.
Sunderesan, P.R., Sharma, V.K., Gingold, S.L., 1984. Decreased beta adrenergic receptors in rat heart in streptozotocin induced diabetes: Role of thyroid hormones. Endocrinology 114, 1358–1363.
Teshima, Y., Takahashi, N., Saikawa, T., Hara, M., Yasunaga, S., Hidaka, S., Sakata, T., 2000. Diminished expression of sarcoplasmic reticulum Ca+2 ATPase and ryanodine sensitive Ca+2 channel mRNA in streptozotocin-induced diabetic rat heart. J. Mol. Cell. Cardiol. 32, 655–664.
Vasanji, Z., Dhalla, N.S., Netticadan, T., 2004. Increased inhibition of SERCA '-2 by phospholamban in the type 1 diabetic heart. Mol. Cell. Biochem. 261 (1-2), 245–249.
Watanuki, S., Matsuda, N., Sakuraya, F., Jesmin, S., Hattori, Y., 2004. Protein kinase C modulation of the regulation of sarcoplasmic reticular function by protein kinase A-mediated phospholamban phosphorylation in diabetic rats. Br. J. Pharmacol. 141, 347–359.
Youn, J.Y., Gao, L., Cai, H., 2012. The $p47^{phox}$- and NADPH oxidase organiser 1 (NOXO1)-dependent activation of NADPH oxidase 1 (NOX1) mediates

endothelial nitric oxide synthase (eNOS) uncoupling and endothelial dysfunction in a streptozotocin-induced murine model of diabetes. Diabetologia 55, 2069–2079.
Zarain-Herzberg, A., Yano, K., Elimban, V., Dhalla, N.S., 1994. Cardiac sarcoplasmic reticulum Ca+2 ATPase expression in streptozotocin-induced diabetic rat heart. Biochem. Biophys. Res. Commun. 203, 113–120.
Zhang, L., Cannell, M.B., Phillips, A.R.J., Cooper, G.J.S., Ward, M.L., 2008. Altered calcium homeostasis does not explain the contractile deficit of diabetic cardiomyopathy. Diabetes 57, 2158–2166.
Zhong, Y., Ahmed, S., Grup, I.L., Matlib, M.A., 2001. Altered SR protein expression associated with contractile dysfunction in diabetic rat hearts. Am. J. Physiol. Circ. Physiol. 281, 1137–1147.