

Cardioprotective effect of propranolol on diabetes-induced altered intracellular Ca^{2+} signaling in rat

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Abstract We have previously shown that chronic treatment with propranolol had beneficial effects on heart function in rats during increasing-age in a gender-dependent manner. Herein, we hypothesize that propranolol would improve cardiac function in diabetic cardiomyopathy and investigated the benefits of chronic oral administration of propranolol on the parameters of Ca^{2+} signaling in the heart of streptozotocin-diabetic rats. Male diabetic rats received propranolol (25 mg/kg, daily) for 12 weeks, 1 week after diabetes induction. Treatment of the diabetic rats with propranolol did not produce a hypoglycaemic effect whereas it attenuated the increased cell size. Basal and β -agonist response levels of left ventricular developed pressure were significantly higher in propranolol-treated diabetic rats relative to untreated diabetics while left ventricular end diastolic pressure of the treated diabetics was comparable to the controls. Propranolol treatment normalized also the prolongation of the action potential in papillary muscles from the diabetic rat hearts. This treatment attenuated the parameters of Ca^{2+} transients, depressed Ca^{2+} loading of the sarcoplasmic reticulum, and of the basal intracellular Ca^{2+} level of diabetic cardiomyocytes. Furthermore, Western blot data indicated that the diabetes-induced alterations in the cardiac ryanodine receptor Ca^{2+} release channel's hyperphosphorylation decreased the FKBP12.6 protein level. Also, the high phosphorylated levels of PKA and CaMKII were prevented with propranolol treatment. Chronic treatment with propranolol seems to prevent diabetes-related changes in heart function by

controlling intracellular Ca^{2+} signaling and preventing the development of left ventricular remodeling in diabetic cardiomyopathy.

Keywords Beta-blockers · Diabetic cardiomyopathy · Sarcoplasmic reticulum dysfunction · Heart · Intracellular calcium ion homeostasis

Introduction

Cardiac dysfunction is one of the hallmarks of diabetes, and it is manifested as a markedly depressed contractility in late stages. Diabetic heart is also rendered more susceptible to hypertensive or ischemic injury as a result of a number of pathological changes that are collectively referred to as “diabetic cardiomyopathy” which has no evidence of coronary atherosclerosis (Rubler et al. 1972). These include cell death, oxidative stress, impaired calcium handling and decreased calcium sensitivity of myofilaments (Fein et al. 1980; Pierce et al. 1983; Choi et al. 2002). In myocardium related to diabetes mellitus, both electrical and mechanical properties are significantly impaired. Ventricular myocardium from diabetic rats exhibits a reversible decrease in the speed of contraction, prolongation of contraction, and a delay in relaxation (Fein et al. 1980; Magyar et al. 1992; Choi et al. 2002).

The defects identified in the mechanical activity of the hearts from type 1 diabetic animals include alteration of Ca^{2+} signaling via changes in critical processes that regulate intracellular Ca^{2+} concentration (Ganguly et al. 1983; Guatimosim et al. 2002). These defects result partially from a dysfunction of cardiac ryanodine receptor calcium release channel (RyR2) (Ganguly et al. 1983; Guatimosim et al. 2002). Global increase in the amount of intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in cardio-

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myocyte during depolarization consists of the summation of these unitary Ca^{2+} release events in cardiomyocytes (López-López et al. 1995). Yet, there is a general consensus that any alteration of Ca^{2+} signaling could be a major source of cardiomyopathy (Wehrens and Marks 2003).

Depression in contraction and relaxation of myocytes isolated from STZ-induced diabetic rats have been found in parallel with the reduced rate of rise and decline of $[\text{Ca}^{2+}]_i$ transient elicited by electrical stimulation (Choi et al. 2002). These effects have been attributed, in part, to RyR2 protein expression. Other studies, however, supported a current hypothesis on a dysfunctional RyR2 observed in diabetic hearts which could be, in part, due to the formation of disulfide bonds between adjacent sulfhydryl groups (Bidasee et al. 2003; Ulusu and Turan 2005).

Although β -adrenergic receptor (β -AR) blocker therapy improves the chances of survival in patients and animals with heart failure (Reiken et al. 2003; Ahmet et al. 2008; Metra et al. 2010; Shan et al. 2010) and diabetes models (Sharma et al. 2008; Sozmen et al. 2011), the mechanism which improves the cardiac function by this class of drugs has not been determined yet. Both experimental and clinical studies demonstrated that this class of drugs could reduce mortality in patients undergoing high-risk non-cardiac surgery (Angeli et al. 2010). In addition, our previous study has evinced gender-related differences in the basal (Yaras et al. 2007) and β -AR-mediated responses of hearts to the same class of two β -AR blockers, either timolol or propranolol in healthy male and female rats (Tuncay et al. 2009). Our previous data clearly demonstrated that these two β -AR blockers had different effects on both the baseline mechanics and action potential parameters of the left ventricle isolated heart preparations.

The mechanism underlying β -blocker therapy, which improves cardiac function and reduces mortality in chronic heart failure due to its effect via RyR2 in particular, is still not known in its entirety. It is clear that a better understanding of the mechanisms underlying the favorable effects of long-term β -AR blockade on animal models may lead to a more appropriate use of this therapy and facilitate the identification of novel therapeutic targets. Therefore, the purpose of the present study is to evaluate the hypothesis that chronic diabetes-related changes in $[\text{Ca}^{2+}]_i$ homeostasis and receptor-mediated system could be prevented through long-term treatment with propranolol via controlling the $[\text{Ca}^{2+}]_i$ in the heart.

Materials and methods

Experimental animals and induction of diabetes

Three-month-old male Wistar rats, weighing 200–250 g, were subjected to single doses intraperitoneal injection of

streptozotocin (STZ; 50 mg/kg, Sigma) dissolved in 0.1 M citrate buffer (pH 4.5) following an overnight fast. Age-matched control rats received an injection of citrate buffer alone. One week after STZ injection, tail vein blood glucose levels were found during the fasting period using a glucose analyzer (Glucotrend, Roche). A blood glucose concentration >3-fold those of age-matched controls for 7 days and 12 weeks post-STZ injection was the criterion for experimental diabetes. All animals were analyzed for 12 weeks following the injection. The diabetic rats were divided into two groups: the untreated diabetic rats (DM group) and the diabetic rats treated with propranolol (DM+PROP group, 25 mg/kg, daily) for 12 weeks. Age-matched normal rats formed one group of control rats (CON group) which were administered saline while another group of normal rats were treated with the same amount of propranolol (CON+PROP). All animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85–23, revised 1996). The protocol was approved by Ankara University with the approval reference number 2007-11-38.

Langendorff perfusion and measurement of cardiac function

The left ventricular developed pressure (LVDP) and left ventricular end diastolic pressure (LVEDP) of isolated hearts were measured as described previously (Tuncay et al. 2007). Briefly, isolated hearts were electrically stimulated (DCS, Harvard Instruments) at 300 beats per minute by a square wave of twice the threshold voltage of 1.5 ms duration.

Action potential recording

Intracellular action potential recordings in the papillary muscle strips isolated from left ventricle were performed as described in a previous study (Magyar et al. 1992). Muscle strips were stimulated, and intracellular action potential was measured using a conventional glass microelectrode connected to a preamplifier. The action potential data were transferred to a PC through an A/D converter and evaluated by a homemade program. The action potential parameters such as amplitude of action potential, membrane potential, and repolarisation phases of action potential (APD₇₅, ₉₀) were found and compared amongst the groups.

Isolation of ventricular cardiomyocytes

Cell isolation was carried out as described previously (Turan et al. 1996). Briefly, ventricles were removed from rapidly excised hearts and minced into small pieces and gently passed through a nylon mesh. Following the

collagenase digestion, dissociated cardiomyocytes were washed with collagenase-free solution. Subsequently Ca^{2+} in the medium was gradually increased to a final concentration of 1.3 mM. Cells were kept in this solution at 37 °C, and only Ca^{2+} tolerant cells were used in the experiments.

Measurement of cytosolic global Ca^{2+} transients

The intracellular free Ca^{2+} concentration changes, $[\text{Ca}^{2+}]_i$ transients and basal level of $[\text{Ca}^{2+}]_i$ were measured from fura-2 loaded (4 μM fura-2 AM) cardiomyocytes at room temperature (21 ± 2 °C) as described in a former study (Turan et al. 1996). Cells were excited at 340/380 nm and emission was measured at 510 nm. The fluorescence ratio $F_{340/380}$ of the emitted light (with a frequency of 10 Hz) on excitation at 340 and 380 nm was calculated and used as an indicator of $[\text{Ca}^{2+}]_i$. The composition of the bath solution used in $[\text{Ca}^{2+}]_i$ measurement was as follows (mmol/L): 117 NaCl, 5.4 KCl, 1.7 MgCl_2 , 1.8 CaCl_2 , 10 HEPES, and 10 glucose (pH 7.4). Following the monitoring $[\text{Ca}^{2+}]_i$ for 20 s at rest, field-stimulation pulses of 20–30 V with 8 ms duration were applied at 0.2 Hz frequency for optimum peak amplitude. The peak amplitude (difference between basal and peak $F_{340/380}$ ratios; ΔF), the time to peak fluorescence (TP) and the half-decay time of fluorescence (DT_{50}) shifts between groups were estimated by a trend fit to whole Ca^{2+} transients evoked by field stimulation. The background fluorescence measured from a cell-free field was subtracted from all recordings prior to calculation of ratios.

Caffeine-induced Ca^{2+} transients were induced by a rapid application of 10 mM caffeine. Caffeine was applied 30 s after cessation of field stimulation (for 2 min at 0.2 Hz stimulation frequency) to ensure a stable SR Ca^{2+} load. Baseline ratios of sample Ca^{2+} traces were initially set to the same constant value prior to the experiments.

Biochemical analysis

Frozen hearts were crushed at liquid N_2 temperature and then homogenized as described previously (Turan et al. 1996). The homogenate was centrifuged at 10,000g at 4 °C for 10 min, and the supernatant was collected as a cytosolic part of the cell and stored at -80 °C. The protein content in homogenates was analyzed using the Bradford Protein Assay (Bio-Rad), and bovine serum albumin was used as the protein standard.

The protein expression levels were determined by Western blot analysis. Briefly, an equal amount of protein from samples were loaded and separated on 8–15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels under reducing conditions.

CaMKII, pCaMKII, PKA, and pPKA measurements were carried out in the cytosolic part of the homogenates,

while FKBP12.6, RyR, pRyR, and SERCA measurements were performed in cells' membranes. The samples (30 μg) were subjected to 10% SDS-PAGE, except for the RyR protein which was subjected to 5% SDS-PAGE. Following the electrophoresis (150 V, for 3 h, at 20°C), samples were electroblotted onto a PVDF membrane through wet transfer in Towbin buffer (25 V, for 2 h). FKBP12.6, RyR, pRyR, SERCA, CaMKII, pCaMKII, PKA, and pPKA contents in the samples were identified using specific antibodies.

Chemicals, data analysis and statistics

Unless otherwise specified, the reagents used were obtained from Sigma (Sigma-Aldrich Chemie, Steinheim, Germany). The molecular weight markers and PVDF membranes were purchased from Bio-Rad. ECL plus was purchased from GE healthcare.

The collected data were presented as the mean \pm standard error of the mean (SEM). One-way ANOVA followed by Tukey-test was used to determine statistical significance ($P < 0.05$).

Results

General characteristics of the treated and untreated diabetic rats

Diabetes was induced by STZ injection in 80% of the rats and the mortality rate of diabetic rats was found to be less than 20% over the course of 12 weeks. The diabetic group had high blood glucose level and less body weight in comparison to the age-matched non-diabetic group (Aydemir-Koksoy et al. 2010) on the 7th day following the injection. With progressive diabetes during 12 weeks, blood glucose level remained high and body weight kept low in both the propranolol treated and untreated diabetic rats with respect to the untreated or treated control rats (Table 1). In sum, the propranolol treatment for 12 weeks did not affect the hyperglycemia and weight loss in STZ-injected rats, and it did not affect any parameters of the normal control group rats either.

The cell capacitance of the isolated cardiomyocytes from the diabetic rats was markedly larger than that of the control ($p < 0.05$). The propranolol treatment prevented this hypertrophic situation in the cardiomyocytes of the 12-week-old diabetic rats without affecting their high blood glucose level.

The effects of the propranolol treatment on the action potential duration in the left ventricular papillary muscle

The action potential duration (APD) in isolated papillary muscle strips isolated from left ventricles of the 12-week-

Table 1 General characteristics of the rats on 12th week following injection

	CON group (n=25)	CON+PROP group (n=22)	DM group (n=28)	DM+PROP group (n=26)
BW (g)	300±10	311±9	210±10*	215±11*
BGL (mmol/L)	7.0±0.6	6.9±0.7	28.1±1.3*	26.2±1.2*
CC (pF)	203.1±12.9	195.1±11.3	233.5±10.7*	219.1±10.1

Values are given as mean ± SEM. *BW*, body weight; *BGL*, blood glucose level; *CC*, cell capacitance; *CON*, untreated control group; *DM*, untreated diabetic group; *+PROP*, propranolol treated groups. Significant at * $P < 0.05$ vs. CON group

old diabetic rat hearts was found to be significantly longer than that of the age-matched rats. As shown in Fig. 1a, the propranolol treatment significantly decreased the prolongation in the action potential duration. In addition, this treatment induced a significant shortening in APD measured in the similar heart preparations from the propranolol treated normal control group rats. Parameters of the

repolarization phase of the action potentials at 75 and 90 (APD_{75,90}) were markedly shorter in both of the propranolol treated control and diabetic groups than that of the untreated groups. The propranolol treatment did not induce any significant alterations in the other parameters of AP such as resting membrane potential and peak depolarization potential (data not shown).

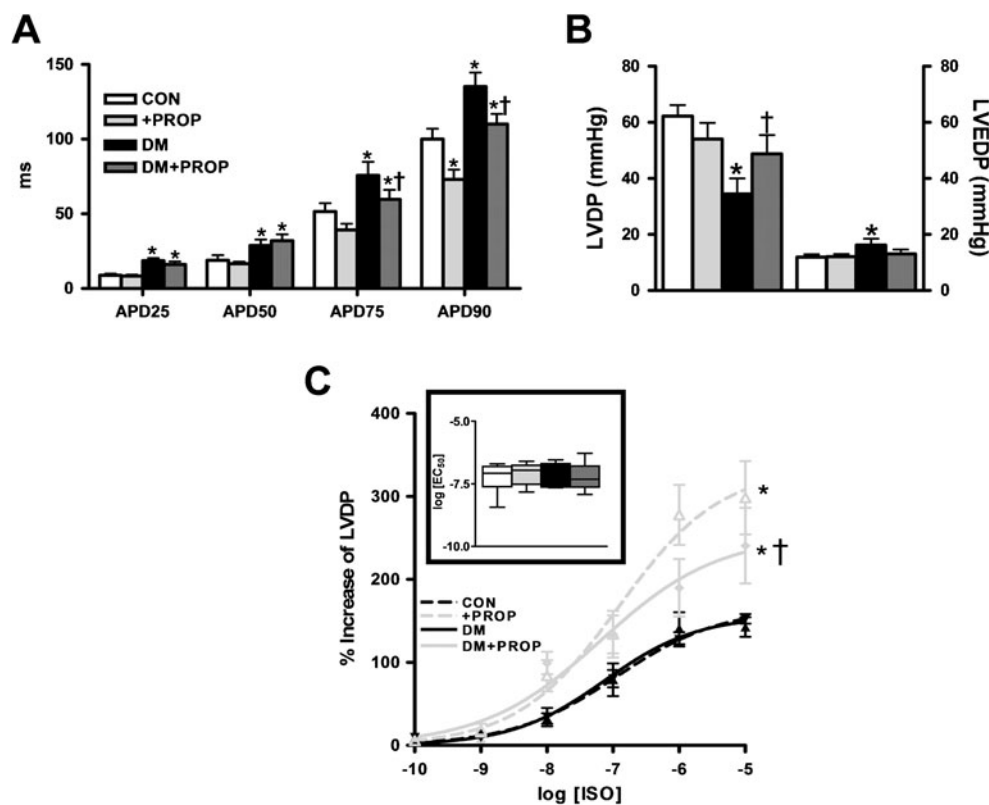


Fig. 1 Effects of chronic propranolol-treatment on action potential duration of the papillary muscle strips and basal cardiac contractile function and responses to β -adrenergic agonist stimulation of left ventricle in diabetic rats. The prolongation in the repolarization phases of the action potential at 25, 50, 75, and 90 (APD_{25,50,75,90}) recorded in papillary muscle strips were prevented by chronic propranolol (+PROP)-treatment. (a) Bar graphs represent mean ± SEM values from PROP-treated, 6 months old control (CON+PROP) and diabetic (DM+PROP) groups ($n_{control}=7$, $n_{diabetic}=7$) and untreated age matched controls (both diabetics and controls, $n_{control}=6$ and $n_{diabetic}=6$). The changes in the basal activity of the heart as both left ventricular developed pressure (LVDP) and left ventricular end

diastolic pressure (LVEDP) (b) in the diabetic group were abolished by chronic PROP-treatment. Bar graphs represent mean ± SEM values from the groups (CON+PROP and DM+PROP, $n_{heart}=8$ and $n_{heart}=10$; CON and DM, $n_{heart}=8$ and $n_{heart}=10$). (c) Effect of submaximal doses of isoproterenol (ISO) on LVDP values. Dose–response curves represent the inotropic responses as % of their initial values. LogEC₅₀ values, which were estimated by fitting dose–responses data with a four parametric logistic equation as -7.27 ± 0.21 , -7.08 ± 0.17 , -7.16 ± 0.28 , and -7.21 ± 0.34 for CON, CON+PROP, DM, and DM+PROP groups, respectively (inset). Number of rat hearts used for this group experiment is 8, 7, 8, and 7 respectively. * $p < 0.05$ vs. untreated controls, and † $p < 0.05$ vs. untreated diabetics, by ANOVA

The propranolol treatment improves basal cardiac contractile function and responses to the β -adrenergic agonist stimulation

The effects of chronic propranolol treatment on LVDP and LVEDP in the 12-week-old diabetic male rats were examined in isolated Langendorff-perfused hearts. The 12-week-old diabetic rats induced significant decreases in both basal LVDP and LVEDP and these depressions were abolished not fully but significantly by the chronic propranolol treatment (Fig. 1b).

In the untreated diabetic group, the maximum contractile responses to ISO (10^{-10} – 10^{-5} M) stimulations were found to be similar to those of the untreated control group (Fig. 1c). The maximum responses in both treated groups, however, were markedly higher than the untreated groups. The cumulative concentration-response curves of LVDP to ISO in these groups, in terms of the sensitivity of the hearts to ISO calculated as LogEC_{50} , were found to be similar for those of the four groups without any shift in any group (Fig. 1c, inset).

The effects of the propranolol treatment on global $[\text{Ca}^{2+}]_i$ in left ventricular cardiomyocytes

To examine whether chronic propranolol treatment had an effect on altered cellular Ca^{2+} signaling, which could underlie diabetes-related cardiac functional changes, we investigated intracellular Ca^{2+} transients in isolated cardiomyocytes from the left ventricle. Figure 2a (left) demonstrates the original Ca^{2+} transients in the propranolol treated and untreated normal control and diabetic group rats. The fluorescence intensity ($\Delta F_{340/380}$) of Ca^{2+} transients of the untreated diabetic group was markedly smaller than the aged-matched treated and untreated control groups while the chronic propranolol treatment of the diabetic group prevented this decrease, significantly without any important effect on the control group (Fig. 2a, right).

In our previous study, we showed that the time courses of transient changes in $[\text{Ca}^{2+}]_i$ such as the time to peak fluorescence changes and the half-decay time of the fluorescence changes were markedly longer in 6-month-old rat cardiomyocytes compared to those of 3-month-old ones (Kandilci et al. 2011). Therefore, the time course of 12-week-old diabetic rats was found to be similar to that of the 6-month-old normal control rats. The propranolol treatment did not affect this parameter in both treated groups as well. In addition, we have also demonstrated that the propranolol treatment did not affect this parameter (data not shown) or any parameters of L-type Ca^{2+} -current in both of the treated groups significantly (data not shown).

Comparison of sarcoplasmic reticulum Ca^{2+} content of cardiomyocytes

The size of the caffeine-induced Ca^{2+} -transient has been used to assess the sarcoplasmic reticulum (SR) Ca^{2+} load of the propranolol treated and untreated diabetic and normal control group rats. To assure a stable SR Ca^{2+} load, the cells were first stimulated and then caffeine (10 mM) was rapidly applied 30 s after the cessation of electrical stimulation. Figure 2b (left) shows the original Ca^{2+} transients from these four groups in response to caffeine application. The caffeine-induced Ca^{2+} -transients from the untreated diabetic group was smaller than that of the control group. The averaged caffeine responses ($\Delta F_{340/380}$) and their basal $[\text{Ca}^{2+}]_i$ in these groups are given in Fig. 2b (right). The parameters related with time courses of the caffeine responses were found to be similar in these four groups (data not shown).

The effects of the propranolol treatment on biochemical analysis of Ca^{2+} handling proteins

An early study by Conlon et al. (1995) demonstrated that the subcutaneous infusion of propranolol for 7 days was accompanied by a marked increase in β_1 - and β_2 -AR densities in 6-month-old male Wistar rats while in β_1 -agonist affinity and adenylate cyclase response to isoprenaline, GTP, Gpp(NH)p, Mn^{2+} and forskolin were not affected by the propranolol infusion. In the present study, total β -AR levels of the groups measured in the left ventricle homogenates were measured as described previously (Bilginoglu et al. 2009). We found that the total β -AR levels of these four groups were similar without any significant difference in the groups (data not given).

In a later study, Doi et al. (2002) showed that propranolol prevented the development of heart failure by restoring FKBP12.6-mediated stabilization of RyR2 in canine left ventricular muscles. Therefore, we hypothesized that the benefits observed in the propranolol treated diabetic rat hearts, in part, were related to its effect on the phosphorylation status of RyR2 (pRyR2) in these heart preparations. The pRyR2 and RyR2 protein levels in the rat hearts were evaluated by using specific antibodies directed against RyR2 and pRyR2. There was a markedly higher RyR2 hyperphosphorylation in the heart of the 12-week-old diabetic rats compared to that of the age-matched controls. In our previous study, we observed 25% hyperphosphorylation of RyR2 in the 6-month-old male Wistar rat hearts while no apparent band was observed in that of the 3-month-old ones (Kandilci et al. 2011). Here there is a two-fold hyperphosphorylation of RyR2 in the 12-week-old diabetic group compared to the age-matched controls while the protein level of RyR2 in the diabetic group was 50% less than the age-matched control groups. Analysis of pRyR2

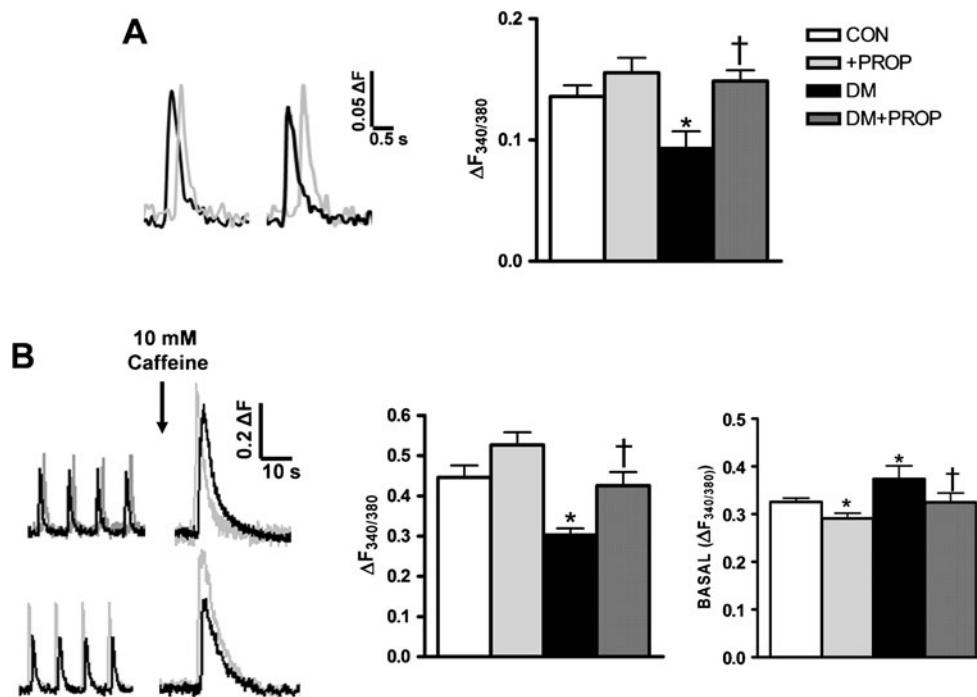


Fig. 2 Protective effect of chronic propranolol treatment on altered parameters of $[Ca^{2+}]_i$ transients, caffeine responses, and basal $[Ca^{2+}]_i$ levels in diabetic rat hearts. $[Ca^{2+}]_i$ transients in freshly isolated cardiomyocytes are obtained with electrical field stimulation at 0.2 Hz. **(a)** Representative tracings of $[Ca^{2+}]_i$ transients elicited in cardiomyocytes isolated from four groups (*left*). *Bar graphs* on the right, represent the changes in the peak amplitude of the fluorescence $[Ca^{2+}]_i$ transients ($\Delta F_{340/380} = F_{340/380} \text{ Peak} - F_{340/380} \text{ Basal}$) of untreated controls (CON; $n_{\text{rat}}=6$, $n_{\text{cell}}=28$), propranolol treated controls (CON+PROP; $n_{\text{rat}}=5$, $n_{\text{cell}}=19$), untreated diabetics (DM; $n_{\text{rat}}=7$, $n_{\text{cell}}=30$),

and propranolol treated diabetics (DM+PROP; $n_{\text{rat}}=6$, $n_{\text{cell}}=28$). **(b)** Representative tracings of caffeine-induced peak $[Ca^{2+}]_i$ transients elicited in cardiomyocytes (*left*). Traces have been shifted for sake of clarifies. *Bar graphs* represent mean caffeine responses of the fluorescence $[Ca^{2+}]_i$ transients (*middle*) and basal $[Ca^{2+}]_i$ (*right*) of the four groups ($n_{\text{rat}}=6$ and $n_{\text{cell}}=28$, $n_{\text{rat}}=5$ and $n_{\text{cell}}=25$, $n_{\text{rat}}=6$ and $n_{\text{cell}}=30$, $n_{\text{rat}}=5$ and $n_{\text{cell}}=24$ for CON, CON+PROP, DM, DM+PROP, respectively). Values are expressed as mean \pm SEM. * $p < 0.05$ vs. untreated controls, and † $p < 0.05$ vs. untreated diabetics, by ANOVA.

expressed as per unit protein level of RyR2 reveals a marked difference between these two groups (Fig. 3a; $p < 0.001$). The propranolol treatment of the diabetic rats significantly prevented both the loss of protein level and the high hyperphosphorylation level in RyR. In addition, as can be seen from Fig. 3a, the propranolol treatment significantly affected both the protein and hyperphosphorylation levels of RyR in the normal control group rat hearts.

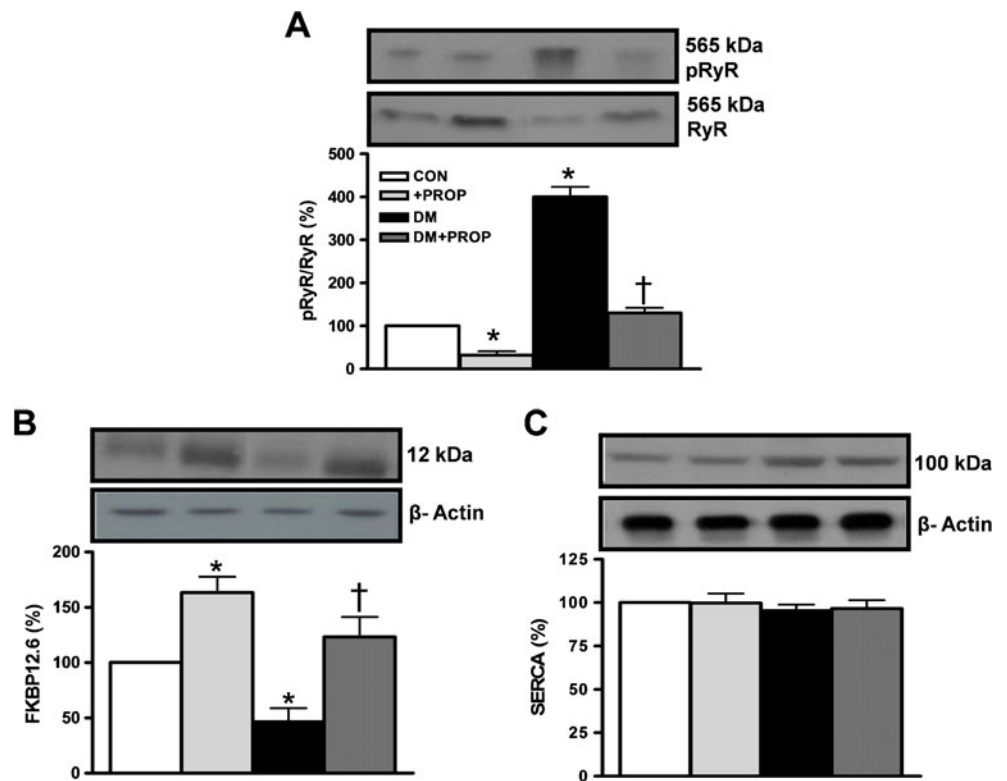
A regulator protein of RyR2, FKBP12.6 was also evaluated. The FKBP12.6 protein level in the 12-week-old diabetic rat hearts was less than the age-matched controls (Fig. 3b, $p < 0.05$). The propranolol treatment of the diabetic rats prevented considerably the loss of protein level in FKBP12.6. Further, as can be seen from Fig. 3b, the propranolol treatment significantly affected the protein level of FKBP12.6 in the normal control group rat hearts.

It has been well documented by now that, any change in contraction and relaxation of left ventricles from pathological hearts can be, in part, attributed to the anomalous SR pump-activity (SERCA) (Choi et al. 2002), and therefore, we measured SERCA levels in the diabetic and control

group rat hearts. We found no significant differences between the protein levels of SERCA of these two groups, and there was no significant effect of the propranolol treatment on these two groups either (Fig. 3c).

To test whether this apparent pRyR2 level in the diabetic group and its prevention with the propranolol treatment corresponds to changes in the PKA or/and Ca^{2+} /calmodulin-dependent kinase II (CaMKII) activity, we analyzed the phosphorylation and protein levels of both PKA and CaMKII in these heart preparations. Diabetes specifically caused marked increases in the phosphorylation levels of both kinases without altering their total protein levels ($p < 0.05$, for both parameters). Analysis of both pPKA and pCaMKII, which were expressed as per unit protein level, revealed significant differences between these two groups (Fig. 4a and b, respectively). The propranolol treatment of the diabetic rats for 12 weeks prevented the loss of protein level as well as the phosphorylation of both PKA and CaMKII notably. Further, as can be seen from Fig. 4a and b, the propranolol treatment significantly decreased the phosphorylation level of both PKA and CaMKII in the 6-month-old control group rat hearts.

Fig. 3 Propranolol effect on RyR2 hyperphosphorylation and protein levels of SERCA and FKBP12.6 in diabetic rat hearts. Top of the *bar graphs*: representative Western blotting for phosho-RyR2 (pRyR2) and 565 kDa RyR2 protein level, 100 kDa SERCA and 12 kDa FKBP12.6 protein levels. *Bars graphs* represent the ratio of 565 kDa pRyR2s to 565 kDa RyR2s protein level (a), the ratio of 12 kDa FKBP12.6 to β -Actin (b), and the ratio of 100 kDa SERCA to β -Actin (c) in the heart homogenates. *Bar graphs* represent mean \pm SEM, $n=5-6$ homogenates/group/protocol. * $p<0.05$ vs. untreated controls, and † $p<0.05$ vs. untreated diabetics, by ANOVA



Discussion

The present study demonstrates that systemic propranolol treatment of STZ-induced diabetic rats exerts a pronounced protective action against cardiac dysfunction. As shown previously (Yaras et al. 2005; Choi et al. 2002; Fein et al. 1980), our study indicates that the induction of diabetes causes alterations in mechanical, biochemical, and electrical functions in the heart. The treatment of the diabetic rats with a β -blocker, propranolol improved cardiac contractile

function, prevented the changes in the repolarization phase of the action potentials, and restored the contractile responses to ISO stimulation without affecting the total β -AR density. It also prevented the changes both in the intensity of Ca^{2+} transients obtained by electrical-field stimulation and the basal level of $[Ca^{2+}]_i$. The inhibition in the caffeine-induced Ca^{2+} -transients observed in the diabetic rat cardiomyocytes was prevented with the propranolol treatment. In addition to the improvement in cardiac function, the propranolol treatment also restored

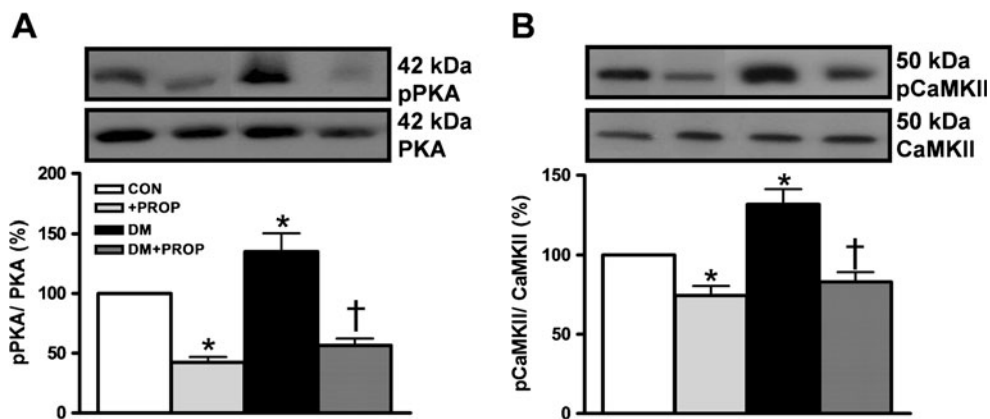


Fig. 4 Propranolol effect on PKA and CaMKII phosphorylation in diabetic rat hearts. Top of the *bar graphs*: representative Western blotting for 42 kDa phosho-PKA (pPKA) and 50 kDa phosho-CaMKII (pCaMKII) in heart homogenates. Ratio of 42 kDa pPKA to

42 kDa PKA protein level (a) and ratio of 50 kDa pCaMKII to 50 kDa CaMKII protein level (b). *Bar graphs* represent mean \pm SEM, $n=5-6$ homogenates/group/protocol. * $p<0.05$ vs. untreated controls, and † $p<0.05$ vs. untreated diabetics, by ANOVA

the normal macromolecular complex composition and function to the RyR2 channel in the diabetic rat hearts. Therefore, there was an associated restoration of myocardial β -AR agonist response and reverse structural remodeling of the left ventricle in the diabetic rat hearts. Taken together, these data suggest that one of the beneficial effects of β -blocker, propranolol therapy is to improve cardiac muscle function by reversing a maladaptive defect in Ca^{2+} signaling in cardiomyocytes in diabetic rat hearts.

It has been previously indicated that the sympathetic nervous system activation resulted in PKA phosphorylation of RyR2 and led to the activation of the channel in animal pathological heart models including the diabetic heart of humans (Yaras et al. 2005; Marx et al. 2000; Yoshida et al. 1992). The use of β -blockers such as carvedilol, metoprolol, or atenolol reduced left ventricular volume, improved cardiac function, restored β -AR response and channel in addition to FKBP12.6 regulation in RyR2 macromolecular complex and RyR2 channel function (Reiken et al. 2003; Doi et al. 2002). In an early study carried out with 6-month-old mature male rats, the propranolol use was accompanied by an increase in both β_1 and β_2 -AR densities of hearts. (Reiken et al. 2003; Doi et al. 2002). Indeed, we recently studied the effect of the propranolol treatment on heart function in male and female hearts. In line with the above finding, our study has found gender-related differences in β -AR-mediated responses of hearts to the same class of two β -AR blockers in healthy male and female rats (Tuncay et al. 2009).

In diabetic hearts, altered local Ca^{2+} signaling with increased basal Ca^{2+} level including reduced amplitude and prolonged time courses of Ca^{2+} transients, depressed Ca^{2+} loading of SR, and altered spatio-temporal properties of the Ca^{2+} sparks in cardiomyocytes are closely associated with hyperphosphorylation of RyR2 and depleted level of FKBP12.6 (Yaras et al. 2005). In the present study, the propranolol treatment improved the Ca^{2+} signaling in cardiomyocytes including altered parameters of $[\text{Ca}^{2+}]_i$ transients, decreased Ca^{2+} loading of SR, increased basal $[\text{Ca}^{2+}]_i$, and decreased L-type Ca^{2+} currents (I_{CaL}) as well as RyR2 hyperphosphorylation without any significant effect on the hyperglycemia and weight loss in the diabetic rats. Accounting for the combined findings, our data demonstrated that the altered heart function in the diabetic rats was closely associated with the marked changes observed in related components of Ca^{2+} signaling in cardiomyocytes during diabetes. The chronic propranolol treatment fully normalized the impairment of Ca^{2+} homeostasis in left ventricular cardiomyocytes in the diabetic rats which also included an increase in reponsiveness of LVDP to β -AR stimulation in the treated groups without affecting their β -AR densities. In the diabetic animal model study, Sharma et al. (2008) showed that metoprolol treatment

improved the cardiac function and modulated cardiac metabolism in the STZ-diabetic rats. The propranolol treatment, here, exerted a cardioprotective action in the diabetic rat hearts via affecting most of the components of Ca^{2+} signaling in cardiomyocytes including the RyR2 hyperphosphorylation level. This finding is backed up with the data by Doi et al. (2002) who demonstrated a restoring effect of propranolol in FKBP12.6-mediated stabilization of RyR2 in heart failures. However, not only the basis of the adrenergic cascade-related myocyte abnormalities but also β -blocker-mediated cardiac benefits in pathological conditions remained largely unclear.

Since the last decade, our understanding of the role of β -AR in the development of heart failures have significantly increased, and β_1 -AR blockade has become the chosen therapy in the treatment of this type of heart dysfunction (Hjalmarson et al. 2000). It has been shown that the stimulation of β_1 -AR activated the G_s -protein pathway and could promote apoptosis of cardiomyocytes, whereas β_2 -AR couples not only with G_s , but also with G_i -protein, and, on the basis of the latter, their stimulation is antiapoptotic and cardioprotective (Communal et al. 1999). However, despite growing experimental and clinical evidence of beneficial effects of β -AR blockers in heart failures, this evidence has not been translated into diabetic cardiomyopathy.

The important new finding proposed by this study is that in diabetes-induced diabetic heart dysfunction indicated with very low plasma insulin level, high oxidative stress and low antioxidant defense markers; (Zeydanli et al. 2011), the propranolol treatment restores intracellular Ca^{2+} signaling in cardiomyocytes via preventing RyR2 hyperphosphorylation in diabetic rat hearts. A similar restored channel regulation in RyR2 which improved the cardiac function in tachycardia-induced canine heart failure with the propranolol treatment was previously shown by Doi et al. (2002). In that study and also in our present study, as opposed to the previously published findings with β -blockers such as enhancement of SERCA expression and activity in heart failure models (Kubo et al. 2001), the treatment with propranolol had no effect on the protein level of SERCA or on Ca^{2+} uptake function. This discrepancy might be attributed to the different experimental dose of β -blockers used or a different regulation of β -AR antagonism.

Beta-blockers are widely used for the treatment of cardiovascular and noncardiovascular diseases. Nevertheless, their mechanism of action is not fully known and is significantly different from other agents in this class. Non-selective β -blockers such as propranolol, metoprolol, or carvedilol exert adrenoceptor-independent effects including scavenging of free radicals and inhibition of PKC leading to controlled cellular redox status and consequently functional recovery in organs including the heart (Djanani et al. 2003; Mochizuki et al. 2007). A direct radical scavenging effect of

the β -blockers was also demonstrated (Djanani et al. 2003). In a cell-line study, Miyamoto et al. (2009) put forward that both nipradilol and timolol possessed a novel mechanism of action and function as potent protective agents against increased oxidative stress. In other studies carvedilol demonstrated a marked beneficial effect in heart failure via scavenging free radicals, preventing Ca^{2+} leak due to stabilization of RyR2s in hearts with a marked failure (Reiken et al. 2003; Mochizuki et al. 2007). They showed that the incubation of the failing cardiomyocytes with a low-dose carvedilol significantly improved cardiomyocyte function (i.e., increases in cell shortening and peak of Ca^{2+} transients), concurrent with a reduction of ROS level in failing cardiomyocytes. Furthermore, Miyamoto et al. (2009) showed an antioxidant-action with timolol due to its protection of trabecular cells from oxidative stress whereas results of Wang et al. (2006) demonstrated the pleiotropic effects of carvedilol on Ca^{2+} regulation during oxidative stress-induced damage in cardiomyocytes. These results combined with the present findings strongly suggest that an antioxidant-action of β -blockers is enough to improve cardiomyocyte function during aging-related alterations as a chronic effect, even without exerting a β -blocking action.

Although we did not measure any parameters related to antioxidant action of propranolol in our study, in our previous work we found a high level of oxidative stress in our diabetic animals and its reversibility with antioxidants (Aydemir-Koksoy et al. 2010). Moreover, in this study the increased cell size (a sign of hypertrophy) of the diabetic rat hearts was prevented with the propranolol treatment. Further, in a recent study, we demonstrated a beneficial action with the chronic timolol treatment on age-related alterations in heart function of the 12-month-old female rats via regulation of cellular redox status (Sozmen et al. 2011). Taking these into consideration, we clarified that the chronic diabetes-related early myocardial impairment was primarily brought into association with the impairment of Ca^{2+} homeostasis, which could be prevented with the chronic propranolol treatment. It is clear that a better understanding of the mechanisms underlying the favorable effects of long-term β -AR blockade on normal controls may lead to a more appropriate use of this therapy and facilitate the identification of novel therapeutic targets.

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No potential conflicts of interest relevant to this article were reported.

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