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Decreased expression of β_1- and β_2-adrenoceptors in human diabetic atrial appendage

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Abstract

Background: Using the streptozotocin-induced diabetic rat model, we have recently showed that the expression and function of β_1-adrenoreceptor were decreased in the diabetic rat heart. However, the effect of diabetes on expression of β-adrenoreceptors in human cardiac tissue remains undefined. Therefore, the focus of the present study was to investigate the effect of diabetes on mRNA encoding β_1- and β_2-ARs in human atrial tissues.

Methods: Right atrial appendages from five diabetic (mean age 65 ± 4.5; 4 female, 1 male) and five nondiabetic patients (mean age 56.2 ± 2.8; 4 male, 1 female) undergoing coronary artery bypass grafting were collected and assayed using reverse transcriptase-polymerase chain reaction (RT-PCR) for their mRNA content. No patient from these two groups suffered from acute myocardial infarction and/or failure. All diabetic patients received insulin for at least two years and had been diagnosed as diabetics for at least five years.

Results: When compared with levels in nondiabetics, steady state levels of mRNA encoding β_1-adrenoreceptor decreased by 69.2 ± 7.6 % in diabetic patients while β_2-adrenoreceptor mRNA decreased by 32.2 ± 5.5 % ($p < 0.001$).

Conclusions: Our findings show a decreased expression of β_1- and β_2-adrenoreceptors in human diabetic atrial appendage.

Background

During the last two decades, significant efforts have been made by several laboratories for a better understanding of the molecular basis underlying cardiovascular complications in diabetics. As it is well known, these complications are responsible for the increased incidence of morbidity and mortality in this patient group and are brought about by metabolic and biochemical shifts as well as by ultrastructural alterations [1,2]. A substantial body of literature indicated that β-adrenoreceptors (AR)s are involved in altered cardiac contraction and/or velocity in different types and stages of heart disease. At early stages, the heart compensate by increasing its neurohumoral and neuroendocrine system activity. However, at later stages,

excessive amounts of catecholamine stimulation could have harmful effects on the already failing myocardium [3]. Changes in expression and function of β-ARs depend on the type and stage of heart failure and also depend on the region of the heart [4].

Some of the hallmarks of diabetes induced cardiomyopathy are bradycardia, nonhomogeneity of atrial conduction and prolongation of sinus node recovery times [5]. Our laboratory previously demonstrated that diabetes has altered the responsiveness, function and expression of the β-ARs in the STZ-diabetic rat heart [6–8]. In addition to STZ-diabetic rat model, we also studied the inotropic responses to β-AR stimulation using atrial appendages from diabetic and nondiabetic humans. In those studies we demonstrated that the full agonist potency order was isoprenaline = fenoterol > noradrenaline [8]. However, no data is currently available on the levels of β-ARs in human diabetic atria. Thus, the aim of the present study was to compare the relative levels of β-AR subtypes in diabetic and nondiabetic human atrial appendages.

Methods

Patient Characteristics

Protocol for collection, storage and analysis of human tissues was reviewed and approved by the Başkent University School of Medicine Ethics Committee. Age and sex dispersion as well as medical history of subjects were prospectively obtained from 51 diabetic and nondiabetic patients from undergoing coronary bypass operation in cardiovascular department for two month period. However, only 10 atria selected and collected (5 of each group) to analyze mRNA expression. For the purpose of this study, samples for analysis based on the following criteria; they should (i) be angiographically proven coronary artery disease. The point that all patients presented with coronary artery disease is important because it allows for the interpretations that differences between nondiabetic and diabetic tissues most likely reflect the presence of diabetes, not just due to the consequences of ischemia (ii) have not suffered from prior acute myocardial infarction and/or heart failure (iii) the nondiabetic group has no history of cardiac diseases (they were sudden angina pectoris and then needed by-pass operation), and (iv) all diabetic patients have been diagnosed for at least five years and receiving insulin therapy (24 ± 5 U/day) for at least two years. Using these criteria, five diabetic (insulin-treated) samples (age; 65 ± 4.5, sex; 4F/1M, n = 5) and five nondiabetic samples (age, 56.2 ± 2.8; 4M, 1F) were chosen for mRNA analysis. The diabetic group had been treated with insulin (n = 5), calcium antagonists (n = 2), nitrovasodilators (n = 2) and aspirin (n = 5), on the other hand nondiabetic patients had received calcium antagonists (n = 2), ACE inhibitors (n = 2) and aspirin (n = 2). None of the patients received β-AR blocking agents for their medication before the operation. All diabetic patients had normal glucose concentration before the operation. Dolantin, promethazine and atropine were given as premedication and operation was carried out under balanced anaesthesia with fentanyl and isoflurane. Heparin, prednisolone, dopamine, nitroglycerin and anti-arrhythmic were also given to some patients.

Isolation and quantitation of total RNA

Atrial appendages (≈ 100 mg tissues) removed, placed in liquid N_2 and then stored at -80°C. Total RNA were extracted using the procedure provided with Quick Prep® total RNA extraction kit (Amersham Pharmacia Biotech, Piscataway, New Jersey) as described before [6,9]. At the end of the isolation, RNA samples were dissolved in diethylpyrocarbonate (DEPC)-treated water (pH 7.5) and the optical density (OD) values of each sample were determined spectrophotometrically using UV-visible spectrophotometer (UV-1601, Shimadzu, Japan) at wavelength 260 nm (λ_{260}) and 280 nm (λ_{280}). The amount of RNA in each sample was then determined using the formula, [RNA] = ODλ_{260} X dilution factor X 40 μg/ml. OD values of RNA samples were also determined at λ_{280} and the ODλ_{260}/ ODλ_{280} ratio were used as cursory estimations of RNA quality (6). RNA samples were electrophoresed using denaturing (formamide/ formaldehyde) agarose gels to qualitatively assess for any degradation that may have occurred during the isolation.

Preparation of first strand cDNA via reverse transcriptase reactions

RNA samples with distinct 18S and 28S ribosomal RNA bands on denaturing agarose gels were then used as templates for synthesis of first strand cDNAs as described previously (6, 9). Briefly, 1 μl of oligo dT_{12-18} (Life Technologies-Gibco BRL, Gaithersburg, MD, USA) was added to equivalent amounts of total RNA from control and diabetic human atrial appendages. The mixtures were then placed into a thermocycler (Hybaid, PCR Express, UK) and held at 70°C for 10 min. At the end of this time, the samples were transferred into ice bath for 5 min to permit selective binding of the oligo dT_{12-18} to the poly-A tail of the mRNA. Thereafter, 1 μl of 10 mM deoxynucleotide triphosphate (dNTP), 2 μl of 0.1 M dithiothreitol (DTT), 4 μl of 5 X 1st strand buffer, 1 μl Superscript II and 1 μl RNasin were added followed by water for a final volume of 20 μl. The tubes were again placed into the thermocycler and heated for 45 min at 42°C for reverse transcription followed by 5 min at 94°C for denaturation. First strand cDNA samples were then cooled to 4°C and stored at -80°C until use.

Amplification of cDNA encoding β-AR subtypes

PCR reactions using gene specific primers were used to amplify segments of cDNA encoding β_1- and β_2-ARs in

Table 1: Primers used in PCR reactions.

Primer	Primer sequence 5'-3'	PCR product size (bp)	Annealing Temperature (°C)	$MgCl_2$ (mM)
β_1-ARs (sense)	$_{236}$CGAGCCGCTGTCTCAGCAGTGGACA$_{260}$	201	54	1,2
β_1-ARs(antisense)	$_{436}$GGTGGCCCCGAACGGCACCACCAGCA$_{412}$			
β_2-ARs (sense)	$_{2135}$ACTGCTATGCCAATGAGACC$_{2154}$	463	59	1,2
β_2-ARs(antisense)	$_{2597}$TGGAAGGCAATCCTGAAATC$_{2578}$			
β-actin (sense)	$_{1079}$AAGTACTCCGTGTGGATCGG$_{1098}$	286	54–59	1,2–1,3
β-actin(antisense)	$_{1364}$CACCTTCACCGTTCCAGTTT$_{1345}$			
β-actin (sense)	$_{854}$CTCTTCCAGCCTTCCTTCCT$_{873}$	513	54–59	1,2–1,3
β-actin(antisense)	$_{1366}$GTCACCTTCACCGTTCCAGT$_{1347}$			

Primers were designed based on published sequences in the National Center for Biotechnology Information GenBank database http://www3.ncbi.nlm.nih.gov/entrez/: β_1-ARs accession number **NM_000684**) [24]; β_2-ARs (accession number **XM_004030**); β-actin (accession number **NM_001101**). Subscript numbers refer to positions of bases within the published cDNA sequences.

each sample. For this, 5 μl of 10 X Tfl buffer, 25 mM $MgCl_2$ (Table 1), 1 μl of 100 mM dNTP, 0.2 μl of Taq DNA polymerase (5 U/μl) (Promega, Madison, WI, USA), 3 μl of either control or diabetic human heart cDNA and 2 μl (from 25 μM stocks) of respective sense and anti-sense primers were added to PCR tubes (Table 1). DEPC water was then added to each tube for a final volume of 50 μl. The samples were then mixed, placed in the thermocycler and denatured for 3 minutes at 94°C. Amplified were carried out using the program: 1 min denaturation (94°C) followed by 1 min annealing and 2 min extension (72°C), repeated for a total of 35. β-actin was amplified in each set of PCR reactions and this gene served as internal references during quantitation to correct for operator and/or experimental variations. At the end of the reactions, 25 μl of each PCR product was mixed with 5 μl of 2 X Blue/Orange loading dye and the samples were loaded onto a 2 % agarose gel containing ethidium bromide and electrophoresed for 2 hr at 100 V (Sci-plas, England). The resulting gels were then visualized using an ultraviolet transilluminator (Viber Loumat TFX 20 M UV) and photographed using UV gel camera (Polaroid GH 10, UK). Images of the gels were scanned into Adobe Photoshop® 3.0 (Adobe Systems Incorporated, Mountain View, CA, USA) and then imported into Scion Imaging Software, Version 1.62 (Frederick, MD, USA, http://scioncorp.com). Areas under the curves were measured and used as mRNA concentrations.

Data analysis and statistics

Differences between values of all groups were evaluated by student t test. The experimental data are mean ± standard error of mean (S.E.M) of n experiments. Results were considered significantly different at $P < 0.01$.

Results

Age and sex dispersion of subjects

Right atrial appendages were obtained from a total of 51 patients undergoing coronary by-pass operation in Department of Cardiovascular Surgery at Baskent University, Ankara Turkey for two month period. Analysis of data prospectively evaluated from 51 patients revealed that 35 patients (68.63%) were nondiabetic and 16 were diabetic (31.37%) (Figure 1B). Of the 35 nondiabetic patients, 27 patients were males (53.0% of total, mean age of 59.4 ± 9.8 years) and 8 patients were females (15.7% of total; mean age of 62.4 ± 7.5 years) (see Figure 1A,1B). These data indicate that more than three times as much nondiabetic males underwent coronary by-pass surgery at Baskent University during this period than non-diabetic females (Figure 1B). In the diabetic group, 9 patients were males (17.6% of total; mean age of 62.2 ± 7.9 years) and 7 were females (13.7% of total; mean age of 66.1 ± 9.0 years), Figure 1A,1B. Therefore, during the period approximately similar amounts of males and females diabetic patients underwent coronary by-pass surgery at Baskent University (Figure 1B).

For this study only ten of the 51 samples satisfied the selection criteria (nondiabetic group; mean age, 56.2 ± 2.8 and sex 4M/1F, n = 5, diabetic group; mean age, 65 ± 4.5 and sex, 4F/1M, n = 5) and collected for mRNA expression experiment.

Quantitation of total RNA isolated from human hearts

Optical density (OD) values at λ_{260} and ratios of $OD\lambda_{260}/OD\lambda_{280}$ were used to quantitate as well as to estimate the quality of total RNA isolated from the diabetic and nondiabetic human atria. All samples used for analysis were of similar quality ($OD\lambda_{260}/OD\lambda_{280}$ ratios ~ 1.7) and showed distinct 18 and 28S bands on denaturing agarose gels.

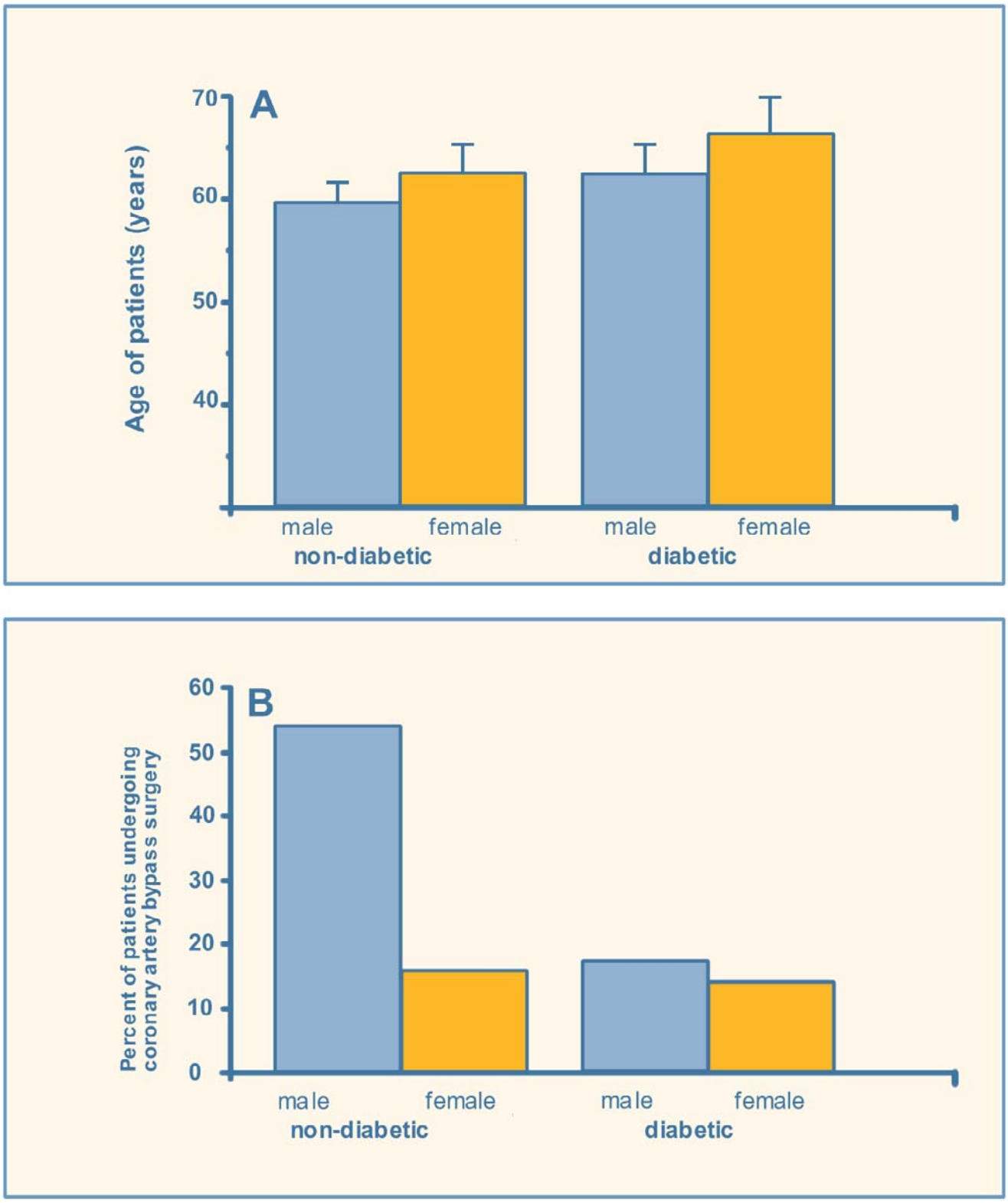

Figure 1
Patients undergoing coronary arterial by-pass grafting during a two month period at Baskent University, Ankara, Turkey. (A) mean age of patients (B) Percentage distribution of patients according to gender and diabetic state.

Quantitation of β-AR transcripts

After converting mRNAs into more stable cDNAs, polymerase chain reactions were used to determine the amounts of β-AR transcripts in hearts of control and diabetic human atria. As shown in Figure 2, diabetes significantly decreases ($P < 0.001$) mRNA levels of β_1-ARs to 69.2 ± 7.6 (Figure 1A) and β_2-ARs to 32.2 ± 5.5 % (Figure 2B) of control in diabetic human atria. Also β_1/β_2 mRNA ratio was 67% in nondiabetic and this ratio was lowered to 43% in diabetic human atrial appendages (Figure 3). All data points were normalized to β-actin as its mRNA levels did not change significantly in this experimental paradigm (Figure 2C).

Discussion

We have previously demonstrated that β_1-ARs mediated chronotropic responses decreased by 29%, but β_2-ARs mediated responses preserved in 14-week diabetic rat atria [8]. In the same study the inotropic responses to β-AR agonists were also studied on diabetic and nondiabetic human atrial tissues. The full agonist potency order was isoprenaline > or = fenoterol > noradrenaline. We have also previously demonstrated that β_1-ARs mRNA decreased to 65.1 % but β_2-ARs mRNA expression increased to 72.5 % in 14 week STZ-diabetic rat heart [6].

In this study we used human atrial appendages obtained from highly selected group of patients. Unfortunately, this lead to a very small final population. Unlike the STZ-induced diabetic rat model, it is very difficult to find out large sample size of patients. Nevertheless, our present result (decreased to 69.2 ± 7.6%) in human atrial appendage related with β1-ARs mRNA expression is very similar if compared with our previous results in the 14-week STZ diabetic rat heart.

As a matter of fact, β_2-ARs expression is still indefinite, contrarily to the β_1-AR subtype in different model of heart failure. Bristow et al. demonstrated that β_1- but not β_2-ARs are downregulated by 50% in the human ventricles, not specifically in diabetic heart but during CHF [10]. Decreased expression of β1-ARs and stimulatory protein Gs and increased expression of inhibitory protein Gi have extensively been investigated in different types of human heart failure by many investigators [10–15]. Like the other types of heart failure, high levels of circulating catecholamine levels lead to decreased expression of cardiac β-ARs and to diminished β-ARs mediated inotropic and chronotropic responses in the diabetic heart [16,17]. The hazardous effects of elevated catecholamine levels are mediated primarily by β_1-ARs, contrary to β_2-ARs stimulation, which may be adaptive in some cases [15]. Nevertheless, in contrast to other types of heart failure, the diabetes mellitus is a complex metabolic disorder and the elevation of circulating blood glucose level possibly alters the structures of many proteins in the heart. These structural and ultrastructural alterations could lead to transcriptional or posttranslational modifications of these proteins. However, if insulin therapy is applied, the cardiac disturbances could be restored partially or completely in the early stage of diabetic heart even if catecholamine levels are still considerably high [6,9]. In the early stage, the cardiac disturbances can return to almost normal levels by insulin therapy, unfortunately, in more chronic stages this is mostly irreversible. For this reason, probably the blood glucose variations shift the present disturbances to the irreversible side and/or trigger the initiation of new pathologies in the diabetic heart.

As it is well known, β_1- and β_2-ARs each couple to Gs. However, a growing body of recent evidence suggests that β_2-ARs, but not β_1-ARs also couple to the inhibitory protein G_i [14,15]. Brodde et al. indicated that β_2-ARs are more effectively coupled to adenylate cyclase than are β_1-ARs in the human right atrium [18]. They also suggested that isoprenaline and adrenaline cause almost same increases in force of contraction via β_1- and β_2-ARs stimulation because of the more effective coupling of β_2 ARs to adenyly cyclase in vitro on isolated human right atrium in spite of the predominance of β_1-ARs density [18]. Similarly, we have previously demonstrated that β_2-selective agonist fenoterol was more potent than β_1-selective agonist noradrenaline on the human right atrium obtained from coronary artery by-pass grafting diabetic and nondiabetic patients [8].

At the same time, we used PCR reactions that simultaneously amplify cDNAs encoding for β_3-AR in different $MgCl_2$ concentrations as well as annealing temperatures. Different set of gene specific primers were used for β_3-AR transcripts: sense $_{839}$CCTTCCTCTTCTCGTGATGC$_{858}$ and anti-sense-$_{1492}$TCTGAACAGAGGCCAGAGGT$_{1473}$, sense $_{1659}$AGTGGTAGTGTCCAGGTGCC$_{1678}$ and anti-sense $_{2156}$AAGCCAGCGCAGAGTAGAAG$_{2137}$, (Primers were designed based on published sequences in the National Center for Biotechnology Information GenBank database; http://www3.ncbi.nlm.nih.gov/entrez/, accession number NM_000025) and sense AGGTTATCCTGGATCACATG and anti-sense CTGGCTCATGATGGGCGC (Last primers based on the previous report; Gauthier, 1996) [22]. Consequently we could not detect the presence of β_3-AR mRNA expression in human atrial appendage from both patient groups. It may depend on very small amount of β_3-AR mRNA expression in human atrial appendage.

In our present study we also found that β_1/β_2 mRNA ratio was 67% in nondiabetic however, 43% in diabetic human atrial appendages (Figure 3). Brodde et al. also demonstrated that atrial and ventricular β_1- and β_2-ARs density

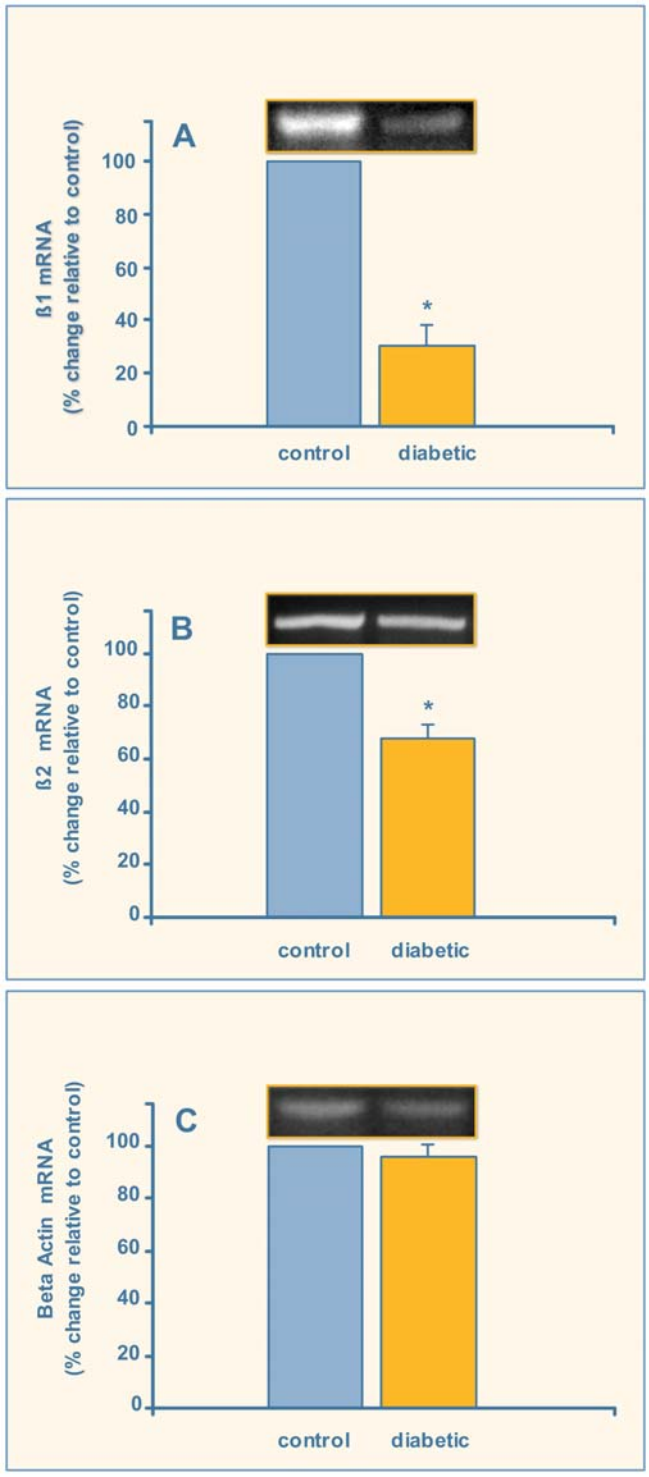

Figure 2
Reverse transcription-polymerase chain reaction (RT-PCR) products obtained from diabetic (5) and nondiabetic (5) human atrial appendages. Total RNA was reverse-transcribed using oligo dT_{12-18} and the first strand cDNA was subjected to amplification by PCR. The samples were loaded onto 2% agarose gel and electrophoresed for 2 hr at 100 V. **A.** Example and quantitation of signals for β_1-AR obtained using RT-PCR reactions. **B.** Example and quantitation of signals for β_2-AR obtained using RT-PCR reactions. **C.** Example and quantitation of signals for β-actin obtained using RT-PCR reactions. Values shown are mean ± SEM obtained from five experiments. *P < vs. control group.

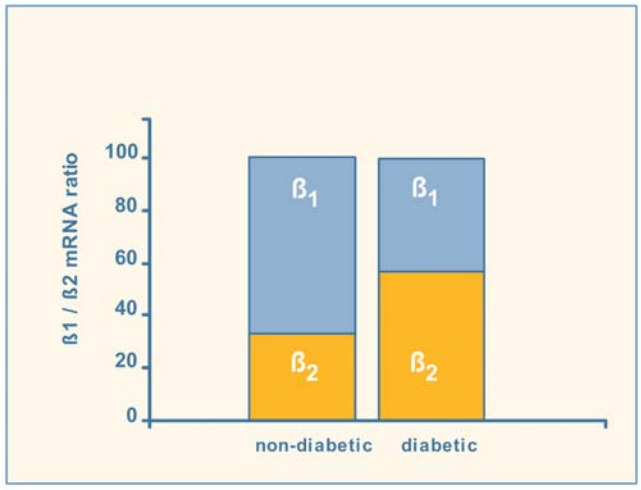

Figure 3
β_1/β_2 mRNA ratio in diabetic and non-diabetic human atrial appendages.

was different in human myocardium [the β_1/β_2 ratio is about 60/70:40/30 % in the atria ; 70/80:30/20 % in the ventricles] [20]. Furthermore, Rodefeld et al. demonstrated that in human sinoatrial nodes β_1-ARs densities were 3 times and β_2-ARs densities were 2.5 times higher than right atria. However β_1-AR subtypes predominate in sinoatrial node [21]. We can also speculate that β_1- and β_2-ARs mRNA expression and β_1/β_2 ratio could be attenuated in sinoatrial node in diabetic patients and it could be one of the reason to decreased chronotropism seen in diabetic patients. Further studies are necessary to reveal the disturbances of sinoatrial β-ARs subtypes in diabetic atria.

Limitations

Study results are obtained from very small final population. This mainly depends on highly selected group of patient samples. Due to the same reason, age differences between diabetic and non diabetic subgroups look quite different and this may influence the results. Age-associated diminution in myocardial β-ARs has been widely demonstrated. However, Brodde et al. demonstrated that β-AR function with increasing age is not due to alterations in receptor density but involves an impairment of the activity of the catalytic unit of the adenylyl cyclase in human right atrium [23].

Conclusions

The principal finding of the present study is that in diabetic human atria, β1-ARs mRNA expression is extensively decreased, while β2-ARs mRNA expression is moderately decreased.

List of abbreviations used

STZ, streptozotocin; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; DEPC, diethylpyrocarbonate; OD, optical density; β-AR, β-adrenoreceptor; CHF, congestive heart failure; ACE, angiotensin converting enzyme; DC, diabetic cardiomyopathy

Authors' contributions

U.D.D. from Ankara University undertook to design, analysis and interpretation of the study and also wrote the manuscript; Ş.G., A.T. and E.A. from Ankara University participated tRNA extraction, RT-PCR and PCR experiments; A.T. from Ankara University prepared all the figures and table as well as analyzed the data; A.T. and S.A. from Başkent University helped to collection and selection of the human tissues; K.R.B. from Nebraska University provided technical advice, supplied some chemicals as well as helped interpretation of the results

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