The Effect of Diabetes on Expression of β_1-, β_2-, and β_3-Adrenoreceptors in Rat Hearts

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Diabetic hearts exhibit decreased responsiveness to stimulation by β-adrenoreceptor (β-AR) agonists. This decrease in activity may be due to changes in expression and/or signaling of β-AR. Recently we showed that right atrial strips from 14-week streptozotocin (STZ)-induced diabetic rat hearts exhibit decreased responsiveness to β_1-AR agonist stimulation, but not to β_2-AR agonist. In the present study, we investigated the effects of long-term diabetes on the expression of cardiac β_1-, β_2-, and β_3-ARs and looked at whether these changes could be restored with insulin treatment. Using reverse transcription-polymerase chain reaction (RT-PCR), PAGE, and Western blot analysis, we found that β_1-AR mRNA and protein levels decreased by 34.9 ± 5.8 and $44.4 \pm 5.8\%$, respectively, in 14 week–STZ-treated diabetic rat hearts when compared with age-matched controls. On the other hand, mRNA levels encoding β_2- and β_3-ARs increased by 72.5 ± 16.6 and $97.3 \pm 26.1\%$, respectively. Although the latter translated into a proportional increase in β_3-AR protein levels ($100.0 \pm 17.0\%$), β_2-AR protein levels decreased to $82.6 \pm 1.1\%$ of control. Insulin treatment for 2 weeks, after 12 weeks of untreated diabetes, partially restored β_1-AR mRNA and protein levels to 60.1 ± 8.4 and $83.2 \pm 5.0\%$, respectively, of control. Although insulin treatment minimally attenuated the rise in mRNA levels encoding β_2- and β_3-ARs, the steady-state levels of these proteins returned to near control values. These data suggest that the decreased responsiveness of diabetic hearts to stimulation of β-AR agonists may be due to a decrease in β_1-AR and an increase β_3-AR expression. *Diabetes* **50:455–461, 2001**

As is well known, cardiac disease is one of the most important complications associated with chronic diabetes. Alloxan and streptozotocin (STZ) are two drugs that are commonly used to experimentally reproduce this pathology in animals, including rats. Among the prominent defects observed in these models are the decreased responsiveness of cardiac preparations to the inotropic and chronotropic effects of β-adrenoreceptor (β-AR) agonist stimulation (1–3). Although the mechanisms underlying depressed cardiac responses to stimulation of β-AR agonists are poorly understood, of interest are studies demonstrating that the density of cardiac β-ARs decreases in diabetic rats (4–7). Almost 20 years ago, Savarase and Berkowitz (8) reported a 28% reduction in the number of β-ARs accompanied by a 24% decrease in the heart rate of STZ-induced diabetic rats when compared with controls. However, in these studies, no attempt was made to distinguish between the various subtypes of β-ARs. In a recent study, we demonstrated that β_1-AR–mediated chronotropic responses in right atria of STZ-induced diabetic rats are impaired, but those mediated via β_2-ARs are preserved (9). It has also been demonstrated that in the human atrium, both β_1- and β_2-ARs are functionally coupled to the adenylate cyclase system (10), and both subtypes of β-ARs are reported to contribute to the cardiac responses of β-AR agonists (11). Thus it has been suggested that, in the human right atrium, both β_1- and β_2-ARs are involved in the physiological regulation of the force of contraction and/or heart rate.

On the other hand, evidence has been provided for the functional expression of β_3-ARs in the human heart, stimulation of which, in contrast to β_1- or β_2-ARs, decreases contractile force (12). Thus we have become interested in the role of β-AR subtypes in diabetes-induced cardiac problems. Our interest stems from reports demonstrating that several pathological states, including diabetes and heart failure, alter the density, sensitivity, and responsiveness of ARs in the heart (13–14). Moreover, no data, to our knowledge, are available on the influence of diabetes on β_3-AR expression. Therefore, in this study we investigated the effects of long-term diabetes on the expression of the three subtypes of cardiac β-ARs in rats and looked at whether changes could be restored and/or reversed with insulin treatment.

RESEARCH DESIGN AND METHODS

Chemicals and drugs. Thiopental sodium was purchased from Abbott Laboratories (Istanbul, Turkey), STZ and ethidium bromide were obtained from Sigma-Aldrich (St Louis, MO), and NPH Iletin II (long-acting insulin) was obtained from Eli Lilly (Indianapolis, IN). Antibodies against rat β_1-AR (sc 568), β_2-AR (sc 570), and β_3-AR (sc 1473) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents and solvents used were of the highest grade commercially available.

Induction and verification of experimental diabetes. This study was approved by the Ankara University Animal Care and Use Committee. Male Wistar rats weighing 200–250 g were purchased from Baskent University Animal Care Unit (Ankara, Turkey). The rats were housed two animals per cage in a room with controlled temperature (22°C) and 12-h light:12-h dark cycles. Diabetes was induced with 45 mg/kg STZ (Sigma-Aldrich) dissolved in citrate buffer (pH 4.5) administered as a single intravenous tail-vein injection under

TABLE 1
Primers used in PCR reactions for amplification and quantitation of mRNA encoding β-AR in rat hearts

Primer	Primer sequence 5′–3′	PCR product size (bp)	Annealing temperature (°C)
β_1-ARs (sense)	$_{307}$GCCGATCTGGTCATGGGA$_{324}$	327	58
β_1-ARs (antisense)	$_{635}$GTTGTAGCAGCGGCGCG$_{617}$		
β_2-ARs (sense)	$_{591}$ACCTCCTCCTTGCCTATCCA$_{610}$	560	60
β_2-ARs (antisense)	$_{1150}$TAGGTTTTCGAAGAAGACCG$_{1131}$		
β_3-ARs (sense)	$_{465}$AGTGGGACTCCTCGTAATG$_{483}$	444	62
β_3-ARs (antisense)	$_{908}$CGCTTAGCTACGACGAAC$_{891}$		
β-actin (sense)	$_{2750}$CGTAAAGACCTCTATGCCAA$_{2768}$	387	60
β-actin (antisense)	$_{3222}$AGCCATGCCAAATGTGTCAT$_{3203}$		

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-AR (accession number NM-012701.1); β_2-AR (accession number NM-012492.1); β_3-AR (accession number NM-013108.1); β-actin (accession number VO-1217-J00691). Subscript numbers refer to positions of bases within the published cDNA sequences.

light ether anesthesia. Control rats were injected with an equivalent volume of the vehicle only. Rats were checked for glycosuria semiquantitatively using Urine-Glucostix reagent strips (Diastix; Bayer Diagnostics, U.K.) 3 days after STZ injection. Rats exhibiting glycosuria were then analyzed for hyperglycemia with Glucostix reagent strips (Peridochrom Glucose GOD-PAP Assay Kit; Boehringer Mannheim, Indianapolis, IN), read by a Glucometer II (Accu-Check; Boehringer Mannheim). Those with a blood glucose level ≥ 300 mg/dl were considered diabetic.

Insulin treatment protocol. After 12 weeks of STZ injection, diabetic rats were randomly divided into treated and nontreated groups. Treated rats were given daily subcutaneous insulin injections (NPH Iletin II) for 2 weeks. Insulin dosages were individually adjusted based on each animal's blood glucose level to maintain the euglycemic state (8–15 U · kg^{-1} · day^{-1}), given once per day between 9:00 and 10:00 A.M. For this, blood glucose levels were monitored every 2 days using Glucostix reagent strips. Then 14 weeks after the induction of diabetes, control, STZ-induced diabetic, and insulin-treated diabetic rats were killed under thiopental sodium (60 mg/kg, i.p) anesthesia. Final blood glucose levels were measured on samples taken when the rats were killed.

Sample collection. After rats were killed, their abdomens were opened and 4–5 ml blood was collected via the left renal artery (15). Blood samples were centrifuged at 3000g for 20 min, and plasma fractions were removed and stored at –20°C. Plasma glucose and insulin levels were later determined using Peridochrom Glucose GOD-PAP assay and DPC kits (Coat-A-Count; Diagnostic Products, Los Angeles, CA), respectively.

Isolation and quantitation of total RNA. Hearts were removed from killed rats, quick-frozen by embedding in dry ice, and stored at –80°C. Total RNA was extracted from whole hearts using a Quick Prep total RNA extraction kit (Amersham Pharmacia Biotech, Piscataway, NJ). At the end of the isolation, RNA samples were dissolved in 1 ml diethylpyrocarbonate (DEPC)-treated water (pH 7.5). The optical density (OD) values of each sample were determined spectrophotometrically using ultraviolet (UV)-visible spectrophotometer (UV-1601, Shimadzu) at wavelength 260 nm (λ_{260}). The amount of RNA in each sample was then determined using the following formula: [RNA] = ODλ_{260} × dilution factor × 40 μg/ml. OD values of RNA samples were also determined at λ_{280}, and the ODλ_{260}/ODλ_{280} ratio was used as a cursory estimation of RNA quality. Formamide/formaldehyde agarose gels were later used to evaluate RNA quality. At the time this study was being conducted, we did not have cRNA probes specific for β_1-, β_2-, and β_3-ARs. As such, we decided to use the more sensitive reverse transcriptase–polymerase chain reactions (RT-PCR) over Northern blot analysis for detection and quantitation of these receptor subtypes.

Preparation of first strand cDNA via RT reactions. RNA samples of acceptable quality were then used as templates for the synthesis of first strand cDNA. Briefly, 1 μl oligo dT$_{12–18}$ (Life Technologies-Gibco BRL, Gaithersburg, MD) was added to equivalent amounts of total RNA (4–10 μl) from control, diabetic, and insulin-treated diabetic rat hearts. The mixtures were then placed into a thermocycler (Hybaid, PCR Express, U.K.) and held at 70°C for 10 min. At the end of this time, the samples were transferred into an 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 10 mmol/l deoxynucleotide triphosphate (dNTP), 2 μl 0.1 mol/l dithiothreitol, 4 μl 5 × first strand buffer, 1 μl Superscript II, and 1 μl RNAsin were added; water was then added to a final volume of 20 μl. The tubes were again placed into the thermocycler and heated for 45 min at 42°C for RT, 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_1-, β_2-, and β_3-ARs. Segments of the cDNA encoding each of the three major subtypes of rat β-ARs were amplified in PCR reactions using gene-specific primers as a way of determining the amount of transcripts present in each sample. For this, 5 μl Tfl DNA polymerase 10× reaction buffer; 2.2 μl 25 mmol/l MgSO$_4$; 1 μl 100 mmol/l dNTP; 0.2 μl Tfl DNA polymerase (5 U/μl) (Promega, Madison, WI); 2 μl control, diabetic, or insulin-treated diabetic heart cDNA; and 2 μl (from 25 μmol/l stocks) of respective sense and antisense 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 min at 94°C. Thereafter, segments of β-AR cDNAs were amplified using the sequence 45-s denaturation (94°C) followed by 45-s annealing (56°C) and 2-min extension (72°C); this sequence was repeated for a total of 35 cycles, except for β_3-AR samples, which were amplified using 40 cycles. β-Actin was amplified in each set of PCR reactions, and these genes served as internal references during quantitation to correct for operator and/or experimental variations. At the end of the reactions, 5 μl of each PCR product were mixed with 5 μl 2 × blue/orange loading dye. The samples were then loaded onto a 2% agarose gel containing ethidium bromide and electrophoresed for 2 h at 100 V (Sci-Plas, U.K.). The resulting gels were then visualized using an UV transilluminator (Viber Loumat TFX 20M UV) and photographed using UV gel camera (Polaroid GH 10; U.K.). Images of the gels were scanned into Adobe Photoshop 3.0 (Adobe Systems, Mountain View, CA) and then imported into Scion Imaging Software (Version 1.62; Frederick, MD). Areas under the curves were measured and used as mRNA concentrations.

Confirmation of the identities of PCR products. The products obtained from the PCR reactions were experimentally verified using restriction endonucleases to digest them into specific fragments. For this, banked nucleotide sequences for β_1-, β_2-, and β_3-ARs were digested in silico using a Web-based restriction analysis program (http://darwin.bio.geneseo.edu/~yin/WebGene/RE.html). The endonucleases *Acc*I and *Sac*I were selected for β_1-ARs, *Pst*I and *Nci*I for β_2-ARs, and *Sma*I and *Acc*I for β_3-ARs. Restriction maps are shown in Fig. 1.

Preparation of membrane vesicles from rat hearts. Membrane vesicles (MVs) were prepared from rat hearts using procedures previously described (16,17), except that the tissue was homogenized for 6 × 10 s instead of 3 × 30 s. Protein concentrations of each of the three preparations were determined using the method of Lowry et al. (18).

Quantitation of β_1-, β_2-, and β_3-AR protein in MV preparations

SDS-PAGE. The amount of β_1-, β_2-, and β_3-AR protein present in plasma membrane preparations from control, 14-week–STZ-induced, and 12-week–STZ-induced/2-week–insulin-treated diabetic rat hearts were assessed using SDS-PAGE and Western blot analysis. Briefly, 75 μg MV from each sample were dissolved in 20 μl gel dissociation medium (62.5 mmol/l Tris base, 6% SDS, 20% glycerol, and 0.002% bromophenol blue) and electrophoresed for 3.5 h using 4–20% linear gradient gels (BioRad, Burlingame, CA). At the end of this time, the gels were stained with Coomassie solution and destained overnight. Images of destained gels were captured using the Kodak Digital Science Electrophoresis Documentation and Analysis System 120 (Eastman-Kodak, Rochester, NY); intensities of β_1-, β_2-, and β_3-AR protein bands were used as indexes of protein amount.

Western Blot analysis. Linear gradient gels (4–20%) containing samples of interest were run as described above. At the end of electrophoresis, separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes
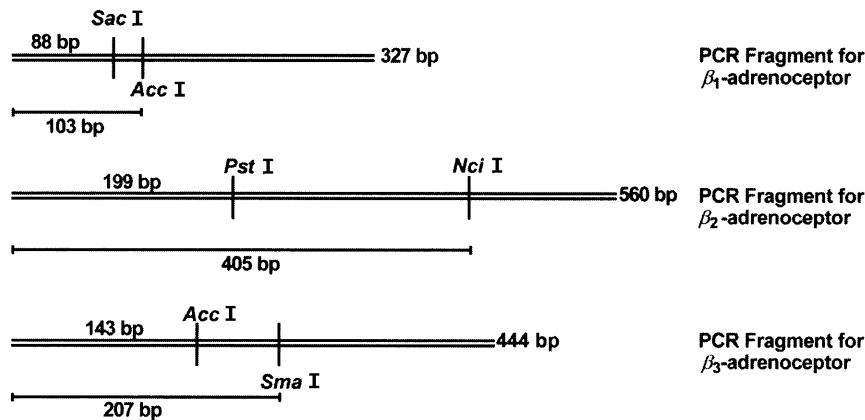
FIG. 1. Restriction maps used for digestion of segments of β_1-, β_2-, and β_3-AR cDNAs. Maps were generated using a Web-based restriction analysis program available at http://darwin.bio.geneseo.edu/~yin/WebGene/RE.html.

RESULTS

Induction of experimental STZ-induced diabetes. All rats injected with STZ had high blood glucose levels within 3 days. At the end of the in vivo experimental protocol, analysis of plasma from diabetic rats showed increases in glucose levels compared with controls (348 ± 14 vs. 105 ± 5 mg/dl; $P < 0.001$) and parallel decreases in insulin levels (26.4 ± 4 vs. 103 ± 10 pmol/l; $P < 0.01$). Insulin treatment for 2 weeks returned plasma glucose levels to near control values (102 ± 18 mg/dl). Mean body weights were significantly lower in diabetic rats than in controls (197 ± 7 vs. 347 ± 8 g; $P < 0.001$). These results were expected, as loss of body mass is a characteristic feature of type 1 diabetes. Insulin treatment of diabetic rats partially corrected the changes in body weight (277 ± 11 g). Heart weights were found to be similar in all groups (0.9–1.2 g). However, heart weight–to–body weight ratios were higher in diabetic rats than in controls (4.56 vs. 3.42 mg/g; $P < 0.001$) and similar between insulin-treated diabetic rats and controls (3.97 vs. 3.42 mg/g).

Quantitation of total RNA isolated from rat hearts. OD values at λ_{260} and ratios of ODλ_{260}/ODλ_{280} were used to quantitate as well as estimate the quality of total RNA isolated from the three groups of rat hearts. With similar overall quality (ODλ_{260}/ODλ_{280} ratios ~ 1.7), total RNA isolated from diabetic hearts was less than that of control animals (536 ± 80

vs. 992 ± 160 µg). These data are consistent with the observations that several proteins are downregulated in diabetic hearts (19). When RNA samples were electrophoresed using formamide/formaldehyde agarose gels, two distinct bands representing 28S and 18S ribosomal RNA were observed (data not shown). The latter suggests that minimal degradation of RNA occurred during the isolation procedure.

Quantitation of β-AR transcripts. After converting the mRNAs into more stable cDNAs, PCRs were used to determine the amounts of β-AR transcripts in hearts of control, diabetic, and insulin-treated diabetic rats. As shown in Fig. 2A, chronic diabetes significantly decreased mRNA levels of β_1-ARs to $34.9 \pm 5.9\%$ of control levels ($P < 0.001$). All data points were normalized to β-actin, as its mRNA levels did not change significantly in this experimental paradigm (data not shown). Treatment of diabetic rats with insulin partially restored mRNA levels of β_1-AR, to $60.1 \pm 8.4\%$ of control levels. In contrast to β_1-ARs, chronic diabetes significantly increased mRNA levels of cardiac β_2-ARs to $173.5 \pm 16.6\%$ of control levels ($P < 0.001$) (Fig. 2B). Insulin treatment did not reverse this increase in β_2-AR mRNA ($168.8 \pm 13.9\%$ of controls). We also found that 14 weeks of untreated diabetes almost doubled β_3-AR mRNA levels in rat hearts, increasing them to $197.3 \pm 26.1\%$ of age-matched control levels (Fig. 2C). Insulin treatment of diabetic rats only partially attenuated this rise in β_3-AR mRNA levels, lowering it to $162.2 \pm 18.9\%$ of control levels.

Characterization of PCR products. Specific restriction endonucleases were used to confirm the identities of the PCR products obtained. PCR products generated for each subtype were digested completely and resulted in fragments of predicted sizes (data not shown). These data thus confirm that the products generated in the PCR reactions resulted from specific amplification of cDNA encoding β_1-, β_2-, and β_3-ARs.

Quantitation of β_1-, β_2-, and β_3-proteins in plasma membrane preparations from control, diabetic, and insulin-treated diabetic rat hearts. SDS-PAGE as well as Western blot analysis was used to quantitate the amount of β_1-, β_2-, and β_3-proteins in MVs from control, diabetic, and insulin-treated diabetic rat hearts. As shown in Fig. 3A, the density of β_1-AR protein in plasma membrane fraction prepared from diabetic rat hearts was significantly less than that from age-matched controls ($44.5 \pm 5\%$ of control; $P < 0.001$).

The methods text (left column continued):

(Immobilon; Millipore, Bedford, MA) at 400 mA using 10 mmol/l cyclohexylamino-1-propane sulfonic acid in 10% methanol (pH 9.0) as the transfer buffer. After transfer, the membranes were treated for 1 h with block solution (0.01 mol/l Tris-HCl, 0.05 mol/l NaCl, 5% skim milk, and 0.04% Tween 20, pH 7.4), and then washed for three 10-min washes in phosphate-buffered saline (PBS), pH 7.4. The membranes were then incubated overnight at 4°C with antibodies against β_1-, β_2-, or β_3-AR protein using the manufacturer's recommended dilutions. The next day, the membranes were washed with PBS (3×10 min) and incubated for 2 h at room temperature with the appropriate secondary antibody (IgG–horseradish peroxidase). At the end of the incubation, the membranes were again washed with PBS and then treated for 1 min with enhanced chemiluminescence (Amersham Pharmacia). Membranes were then wrapped in cellophane and placed into cassettes with X-ray films (Hyperfilm; Amersham Pharmacia). Autoradiograms were developed after 2–10 min. Intensities of β_1-, β_2-, and β_3-AR signals on autoradiograms were measured and used as indexes of β-AR protein content in each MV preparation.

Data analysis and statistics. Differences among group values were evaluated by one-way analysis of variance followed by a Newman-Keuls test. Data are presented as means ± SE. Results were considered significantly different at $P < 0.01$.
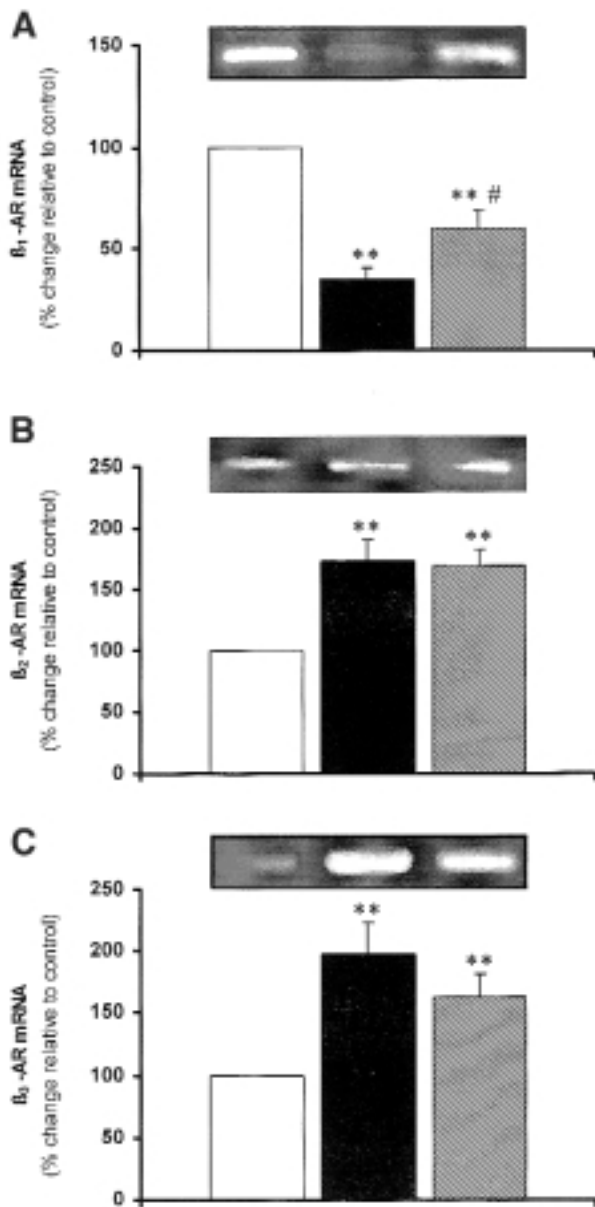
FIG. 2. RT-PCR products obtained from control (□), 14-week–STZ-induced diabetic (■), and 12-week–STZ-induced/2-week–insulin-treated diabetic (▧) rat hearts. Total RNA was reverse-transcribed using oligo dT_{12-18}, and the first strand cDNA was subjected to amplification by PCR. Digestion of PCR products was carried out by mixing 5 μl of respective cDNA with 5 μl 10 × enzyme buffer, 2 μl restriction endonuclease, and 38 μl distilled water. The samples were then lightly vortexed and digestion was allowed to proceed for 2 h at 37°C. At the end of this time, the samples were loaded onto 2% agarose gel and electrophoresed for 2 h at 100 V. Shown are examples and quantitation of signals for β_1-AR (*A*), β_2-AR (*B*), and β_3-AR (*C*) obtained using RT-PCR reactions. Data are means ± SE obtained from five experiments. ** $P <$ 0.001 vs. control group; # $P < 0.001$ vs. diabetic group.

FIG. 3. Quantitation of the amount of β_1-, β_2-, and β_3-AR in plasma membrane preparations from control (□), 14-week–STZ-induced diabetic (■), and 12-week–STZ-induced/2-week–insulin-treated diabetic (▧) rat hearts. Briefly, 75 μg membranes from each of the three samples were dissolved in 20 μl gel dissociation medium, loaded onto 4–20% linear gradient polyacrylamide gels, and electrophoresed at 150V for 3.5 h. At the end of this time, proteins were transferred onto PVDF membranes and probed for β_1-, β_2-, or β_3-AR protein using isoform-specific antibodies. *A*: Example and quantitation of signals for β_1-AR obtained using sc 568 anti-β_1-AR antibodies. *B*: Example and quantitation of signals for β_2-AR obtained using sc 570 anti-β_2-AR antibodies. *C*: Example and quantitation of signals for β_3-AR obtained using sc 1473 anti–β_3-AR antibodies. Data are means ± SE obtained from five experiments. * $P < 0.01$; ** $P < 0.001$ vs. control group; # $P <$ 0.001 vs. diabetic group.

Plasma membrane fractions from diabetic rat hearts showed a smaller but still significant decrease in β_2-AR protein content (17.4. ± 1.1% of control; $P < 0.01$) (Fig. 3*B*). On the other hand, untreated diabetes doubled protein levels of β_3-AR, to 200.0 ± 17% of control (Fig. 3*C*). Insulin treatment partially restored expression of β_1-AR to 84.4 ± 4.8% of controls and completely restored expression of β_2-AR to 100.9 ± 4.8% of

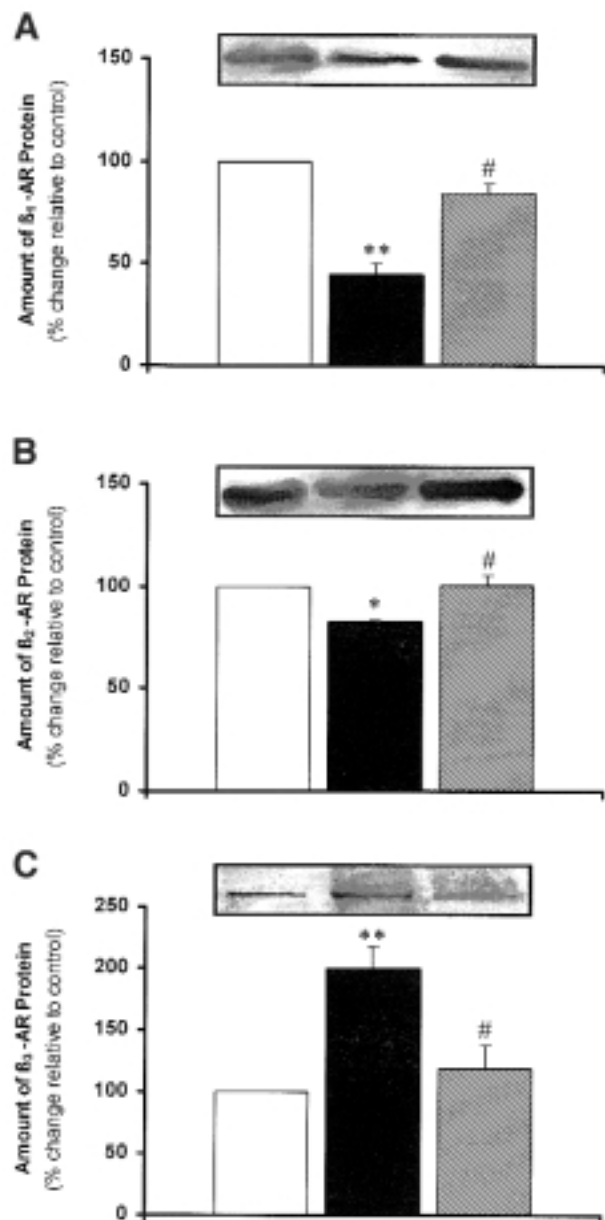controls. Insulin-treatment also significantly attenuated the increase in the expression of β_3-AR induced by long-term diabetes (118.9 ± 18.7% of controls).

Based on the intensity of Coomassie-stained proteins corresponding to β_1-, β_2-, and β_3-AR, we estimated the ratio of

β_1-AR:β_2-AR:β_3-AR in control rat hearts to be approximately 62:30:8. After 14 weeks of untreated diabetes, this ratio changed to approximately 40:36:23. Insulin treatment for 2 weeks restored the complement of β-ARs to 57:33:10.

DISCUSSION

One of the major causes of morbidity and mortality in diabetic patients is cardiovascular disease. Diabetes is also frequently associated with the development of heart failure, even in the absence of coronary artery complications or hypertension (20). The mechanisms involved in diabetes-induced cardiac problems are not clearly understood, but bradycardia (2,21) and decreased chronotropic and inotropic responses to β-AR–agonist stimulation (1–3) have been demonstrated. Thus a reduction in the number of cardiac β-ARs has been questioned as one of the causative factors for diabetic cardiomyopathy. In fact, reduced density of cardiac β-ARs in diabetic rats has been demonstrated in previous studies (4–7). In those studies, however, β-ARs were not differentiated according to their subtypes. β-ARs of both the β_1 and β_2 subtypes have been reported to coexist in certain cardiac tissues (10,11). Human cardiac β_1- and β_2-ARs are coupled to adenylate cyclase through the G_s protein. In the human heart, both receptors mediate the increases in contractile force and sinoatrial rate (14). A growing body of recent evidence, on the other hand, suggests that β_3-ARs that couple to the G_i protein and mediate cardiodepressant effects are also present in human heart (12,14,22). Therefore, in the present study we investigated the influence of diabetes on mRNA and protein levels of selective β_1-, β_2-, and β_3-ARs in rat heart.

A principal finding of the present study was that the complement of β-ARs expressed in the heart is altered with long-term STZ-induced diabetes. These levels were restored to close to those of age-matched controls with 2 weeks of insulin treatment, begun after 12 weeks of untreated diabetes. We observed that the levels of mRNA encoding β_1-AR decreased by 65% in hearts of diabetic rats, and the density of β_1-AR protein on the plasma membrane decreased by 55%. Our findings are in accordance with the those of Matsuda et al. (23), who reported a parallel decrease in myocardial β-AR density and β_1-AR mRNA levels in 6-week diabetic rats. Although those investigators did not detect β_2-ARs in their experiments, they suggested that the reduced number of myocardial β-ARs that they observed in diabetes could be due to a downregulation of β_1-AR mRNA levels. Matsuda et al. (23) also stated that β_2-AR mRNA was undetectable in control or diabetic ventricular myocardium, at least by Northern blot using the specific oligonucleotide probe. Indeed, downregulation of myocardial β_1-AR mRNA has been demonstrated in human failing hearts (24,25). β_2-ARs, on the other hand, appear to be somewhat uncoupled from adenylate cyclase (26), with no detectable change in β_2-AR mRNA levels in patients with advanced heart failure (24). Moreover, heterogeneous β_1- plus β_2-AR populations have been identified by radioligand binding in rat heart (27), but only β_1-ARs appear to mediate the rate and tension responses in rat atria (28). Hence, under normal physiological conditions, it seems unlikely that β_2-ARs have a functional role in the regulation of heart rate and contractility in this species, in contrast to the situation in humans. Based on these findings, a decrease in the density of cardiac β-ARs in diabetic rats might be attributable to a decrease in the number of β_1-ARs only. In the pres-

ence of a pathological state, however, the function of the β_2-AR stimulation might be changed.

Indeed, in the present study, we found that levels of mRNA encoding β_2-ARs were increased 74% in the hearts of diabetic rats, but that the density of this protein on the plasma membrane was decreased by 17.4% when compared to controls. It should be pointed out that the failure of Matsuda et al. (23) to detect β_2-AR mRNA in diabetic ventricular myocardium might have been attributable in part to the use of the less-sensitive Northern blot analysis. However, the reasons for diversity between our mRNA and protein findings of β_2-ARs in diabetic rats are uncertain. An increase in mRNA levels is not necessarily associated with an increase in steady-state levels of protein. Therefore, the decrease in protein levels, despite the increase in mRNA, raises the possibility that the rate of β_2-AR protein degradation might be elevated in diabetes. It is also possible that there could be some post-translational modifications in diabetic heart so that all of the β_2-AR protein is not being delivered from the endoplasmic reticulum to the membrane fraction, which we analyzed.

Because diabetes frequently leads to cardiac pump failure, further leading to congestive heart failure (29), the changes in cardiac β-AR levels in heart failure might be comparable with those of the heart in diabetes. The alterations in failing human hearts have been suggested to be, at least in part, a consequence of the increased stimulation of β_1-ARs by noradrenaline released from the sympathetic nerves in an attempt to restore cardiac function (30). Similarly, it has been demonstrated that cardiac noradrenaline content is increased in diabetic rats (31). In addition, it was reported that noradrenaline turnover, uptake, synthesis, and release are all enhanced in diabetic cardiomyopathy (32,33). Therefore, chronically high concentrations of noradrenaline in diabetes may contribute to the selective downregulation of β_1-ARs, as the affinity of noradrenaline is lower for β_2-ARs. From these data, in contrast to β_1-ARs, one would not expect a decrease in the expression of cardiac β_2-ARs in diabetic rats. As a matter of fact, in paced pigs and dogs, β_2-AR protein and mRNA levels were found to be unchanged despite the reduced number of β_1-ARs and mRNA content (34,35). Therefore, the decrease that we observed in β_2-AR protein levels in diabetic rat hearts is somewhat surprising. However, this slight decrease does not necessarily result in a significant decrease in functional responses mediated by this β-AR subtype, because cardiac β_2-ARs are more effectively coupled to adenylate cyclase than are β_1-ARs. Indeed, cross-regulation between G protein–coupled receptors through G protein function has been demonstrated (36). Strips of right atrial appendage from patients treated with β_1-AR blockers have been reported to exhibit sensitization of β_2-AR–mediated inotropic responses (36). Interestingly, it has been reported that single contracting ventricular myocytes from patients with severe heart failure responded to noradrenaline predominantly through β_2-ARs (37). Thus the subtype density and coupling of cardiac β-ARs might be complicated in certain disease states, such as heart failure and diabetes. These studies lead us to hypothesize that the stimulation of cardiac responses might be brought about predominantly through β_2-AR stimulation when the functional responses mediated by β_1-ARs are depressed. Thus, in an attempt to gain insight into the role of β_2-ARs in diabetes, we previously studied the effect of diabetes on selective β_1- and β_2-AR stimulation in the

right atria of STZ-induced diabetic rats (9). We found a significant decrease in the chronotropic responses of the right atria from 14-week diabetic rats to noradrenaline, although the responsiveness to fenoterol was similar to that of controls. These findings suggest that β_1-AR– but not β_2-AR–mediated chronotropic responses were reduced in the right atria of diabetic rats. On the other hand, it is important to note that in 14-week–STZ-induced diabetic rats, β_1-AR protein in heart was decreased by almost 55% in the present study, whereas the decrease in maximum chronotropic response of the right atria to β_1-AR stimulation was only 29% in our previous study (9). Taken together, these results suggest an abundance of spare receptors in this system. Therefore, by analogy, it might be speculated that the 17.4% decrease in β_2-AR protein observed in this study was not sufficient to cause a significant change in β_2-AR–mediated cardiac responses. As is well known, abnormal β-AR signal transduction appears to be one of the major causes of systolic and diastolic dysfunction in humans with heart failure (38) or diabetes (39). Thus our previous findings demonstrating that there are defective β_1-AR–mediated chronotropic responses yet preserved β_2-AR–mediated chronotropic responses, coupled with data from the present study demonstrating an increase in mRNA levels encoding β_2-ARs, may have some physiological importance. However, at present our results do not allow us to determine whether β_2-ARs might compensate the decrease in the heart rate of rats when β_1-AR–mediated responses are impaired to a certain extent in those pathological states. This point remains to be determined.

In the present study, we found a 97% increase in mRNA–encoding β_3-ARs and a 100% increase in plasma membrane density of β_3-ARs when compared with controls. β_3-AR mRNA has recently been detected in human heart ventricular myocytes (12,14,22). It has also been shown in human ventricular endomyocardial biopsies that isoprenaline produces consistent negative inotropic effects in the presence of the β_1- and β_2-AR antagonist nadolol (12). The negative inotropic effect is antagonized by the nonselective β-AR antagonist bupranolol. A similar negative inotropic effect elicited by β_3-AR selective agonist BRL 37344 is sensitive to treatment of the preparations with pertussis toxin. These results indicate the involvement of inhibitory G proteins in the β_3-AR signaling pathway, thereby producing negative inotropic effects. β_3-ARs differ from β_1- and β_2-ARs in that they lack the phosphorylation sites for the β-AR kinases and the cAMP-dependent protein kinase (40), and may not be downregulated in heart failure. Thus it has been proposed that cardiodepressant effects mediated through β_3-ARs contribute to the impaired cardiac function in patients with heart failure (12). To our knowledge, we are the first to demonstrate an increased expression of β_3-AR mRNA in diabetic rat hearts. Because the contribution of β_3-ARs to the cardiac responses of β-AR agonists in rat heart is not clear at present, questions arise as to the pathophysiological implications of our data. Thus, to comment on the role of increased expression of cardiac β_3-AR mRNA in diabetes-induced cardiac dysfunction, these results should be confirmed in the human heart in future studies.

In the present study, we also found that 2-week insulin treatment of diabetic rats increased the expression of β_1-AR mRNA, but to a level that was still lower than that of controls. No significant effect of insulin, on the other hand, was observed on the expression of β_2- and β_3-AR mRNAs. Insulin appears to be the most effective compound not only in preventing but also reversing the diabetes-induced cardiac changes. In addition, it was reported that the decreased number of cardiac β-ARs returned to normal in diabetic rats after insulin treatment (4). Insulin treatment, however, seems to be less effective at the more chronic stages of the disease (41). In this regard, one would conclude that the inability of insulin to normalize completely decreased β_1-AR mRNA in diabetic rats may be due to the duration of diabetes. Alternatively, 2-week treatment of insulin may not be enough for complete normalization. However, our findings that insulin reverses the changes in β-AR protein levels do not support these possibilities. Studies of the heart in vitro demonstrate that insulin stimulates protein synthesis and inhibits protein degradation (42,43). The effect of insulin, hence, could be more prominent at the level of protein turnover.

In conclusion, the present study demonstrated that the expression of β_1-ARs decreases, whereas that of β_3-ARs increases, in hearts of long-term diabetic rats, . Our results thus suggest that a decrease in β_1-AR together with an increase in β_3-AR expression might be involved in the development of diabetes-induced cardiac dysfunction. The physiological relevance of our findings demonstrating a decrease in cardiac β_2-AR protein despite the increased β_2-AR mRNA in diabetic rats is not clear. Further studies are needed to delineate the precise role of cardiac β-ARs in health and disease.

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