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Diabetes-Induced Down-Regulation of β_1-Adrenoceptor mRNA Expression in Rat Heart

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ABSTRACT. The present study addressed the question of whether the number of myocardial β-adrenoceptors in rats with 4- to 6-week streptozotocin-induced diabetes is regulated in a transcriptional or translational manner. Radioligand binding experiments with [^{3}H]CGP 12177 {4-(3-*t*-butylamino-2-hydroxypropoxy)-[5,7-^{3}H]benzimidazol-2-one} showed that the density of β-adrenoceptors fell by 50% with no change in affinity in diabetic rat ventricular myocardium compared with age-matched control myocardium. The relative content of β_1-adrenoceptor mRNA in diabetic myocardium also was reduced from the control level by 57%, as determined by northern blot analysis. The reductions in myocardial β-adrenoceptor number and β_1-adrenoceptor mRNA observed in diabetes were prevented by insulin therapy. These data indicate that the diminished density of myocardial β-adrenoceptors in diabetes occurred, at least in part, at the mRNA level. BIOCHEM PHARMACOL **58**;5:881–885, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. diabetes mellitus; β-adrenoceptors; mRNA; rat myocardium

Autonomic neuropathy, which may underlie abnormalities of cardiac performance, is recognized as a serious complication of chronic diabetes mellitus. An isoproterenol infusion study has shown a decreased β-adrenoceptor responsiveness in type 1 insulin-dependent diabetic patients [1]. A diminished cardiac response to β-adrenoceptor stimulation is also one of the important features found in diabetic animal models. This has been demonstrated in isolated cardiac preparations [2, 3]. In accordance with the diminished functional response, reductions in the number of myocardial β-adrenoceptors have been shown in many prior studies [2, 4–6]. We also have observed that the density of myocardial β-adrenoceptors, which are identified by the use of the radioligand [^{125}I]iodocyanopindolol, is reduced by 50% in streptozotocin-induced diabetic rats compared with age-matched controls [7]. However, it remains to be determined whether diabetes down-regulates the number of myocardial β-adrenoceptors by modulating gene transcription or translation of mRNA. To address this question, we undertook to assess whether the reduced β-adrenoceptor density is accompanied and presumably caused by a drop in mRNA levels in ventricular myocardium from streptozotocin-induced diabetic rats.

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MATERIALS AND METHODS

Induction of Diabetes

Male Wistar rats, 8 weeks old and 180–200 g in body weight, were assigned, at random, to two groups. The rats were anesthetized lightly with diethyl ether, and the diabetic group received a single injection of streptozotocin (45 mg/kg) in citrate buffer into the tail vein. The control group received an equivalent volume of citrate buffer alone. Control and diabetic rats were caged separately but housed under identical conditions. Both groups of animals were given free access to food and water. Some diabetic rats were treated daily with a subcutaneous injection of Ultralente insulin (8 U/day; Novo Nordisk). On the day of the experiments, a blood sample was collected, and the serum glucose level was measured by means of a Rapid Blood Analyzer using a Uni-Kit (Chugai). As well established in our previous report [7], all rats injected with streptozotocin developed severe diabetes as indicated by increased serum glucose levels (>500 mg/dL). The high levels of serum glucose were improved significantly by insulin therapy. The general features of age-matched control, diabetic, and insulin-treated diabetic rats are summarized in Table 1.

Membrane Preparation and Radioligand Binding

Four to six weeks after the injection, rats were anesthetized with diethyl ether. Their hearts were removed and rinsed in ice-cold Tris–HCl buffer. Ventricles were dissected free of connective tissue, major vessels, and atria. The tissues were minced with scissors and homogenized in 5 vol. of ice-cold

TABLE 1. General characteristics of control, diabetic, and insulin-treated diabetic rats

	Body weight (g)	Heart weight (mg)	Serum glucose (mg/dL)
Control	295 ± 8	768 ± 10	153 ± 7
Diabetic	171 ± 4*	515 ± 15*	588 ± 11*
Insulin-treated	278 ± 8	679 ± 14*	152 ± 10

Values are means ± SEM for 7–10 rats.
*Significant difference from the corresponding control value ($P < 0.05$).

Tris buffer by the use of a Polytron for 15 sec. The buffer composition (pH 7.4, 4°) was (mM): Tris–HCl, 75; $MgCl_2$, 25; EDTA, 5; and EGTA, 1. The homogenates were centrifuged at 1000 *g* for 10 min at 4°. The supernatant was filtered through a single layer of cheesecloth and retained. The pellet was suspended in 5 vol. of cold Tris–HCl buffer and centrifuged again. Membrane fractions in the supernatant were concentrated by centrifugation at 100,000 *g* for 30 min at 4°. The final pellets were resuspended in cold Tris–HCl buffer and stored at −80° until used. Protein content was determined by the method of Lowry *et al.* [8] with bovine serum albumin as standard.

β-Adrenoceptors were identified with the use of the radioligand [^{3}H]CGP 12177 {4-(3-*t*-butylamino-2-hydroxypropoxy)-[5,7-^{3}H]benzimidazol-2-one; New England Nuclear} in a saturation isotherm. The membrane suspensions were diluted further in an incubation medium (50 mM Tris–HCl, 10 mM $MgCl_2$, pH 7.4) to give a final protein concentration of ~0.4 mg/mL. [^{3}H]CGP 12177 was prepared in the incubation medium, and an aliquot of membrane suspension (100 μL) was incubated with various concentrations of [^{3}H]CGP 12177 in a final volume of 200 μL. Incubations were carried out for 60 min at 37° and terminated by adding 5 mL of cold incubation medium [4°] to the entire incubation mixture, followed by rapid filtration over Whatman GF/C glass fiber filters. Each filter was washed three times with 5 mL of cold incubation medium [4°] and then air-dried overnight. The radioactivity trapped on each filter was counted in 15 mL of Scintisol EX-H (Dojin) by a liquid scintillation counter. All values in binding experiments are the average of triplicates. Nonspecific binding was defined as binding in the presence of 10 μM propranolol. The equilibrium dissociation constant (K_D) and the maximum binding capacity (B_{max}) were determined by Scatchard analysis.

Total RNA Extraction and Northern Blot Analysis

Total RNA was extracted from ventricular myocardium using a guanidinium thiocyanate-phenol-chloroform method according to the protocol of Chomczynski and Sacchi [9]. Briefly, ventricles were freed from atria, connective tissues, and major vessels in ice-cold 0.9% NaCl, and frozen immediately with liquid nitrogen. The frozen tissue was placed in 1 mL of ISOGEN (Nippon Gene) and homogenized with a Polytron. Subsequently, 200 μL of chloroform was added; the mixture was shaken vigorously for 15 sec and was kept at room temperature for a few minutes. The mixture was centrifuged at 12,000 *g* for 15 min at 4°, the aqueous phase was transferred into a fresh tube, 500 μL of isopropanol was added, and the sample was centrifuged at 12,000 *g* for 15 min. The resulting pellet was washed twice with 75% ethanol by vortexing and subsequent centrifugation at 7500 *g* for 5 min at 4°. Total RNA was resuspended in diethyl pyrocarbonate-treated water. The amount of RNA present was determined by UV absorption. The optical density (OD) ratio of $OD_{260\ nm}$:$OD_{280\ nm}$ was 1.89 to 2.07 in all cases. The amounts of RNA obtained were 533 ± 7 and 365 ±10 μg/heart in control and diabetic groups, respectively (N = 5). However, when quantified by dividing the total mRNA amount by each heart weight, the values were similar in control and diabetic groups (0.71 ± 0.01 vs 0.72 ± 0.01 μg/mg).

RNA (30 μg/lane) was subjected to electrophoresis (75 mA for 120 min) on 1.2% agarose/6.5% formaldehyde gels and then transferred to a Hybond-N$^+$ nylon membrane (Amersham). The membrane was prehybridized in prewarmed rate-enhanced hybridization buffer (Rapid-hyb buffer; Amersham) at 42° for 1 hr. The β_1-adrenoceptor oligonucleotide probe (bases 1536–1565 of the coding region; Biognostik GmbH) was labeled with [α-^{32}P]dATP (6000 Ci/mmol; New England Nuclear) using an oligonucleotide 3′ end labeling system (New England Nuclear). After being hybridized in the buffer containing ^{32}P-labeled probe (~10^7 cpm/mL) at 42° for 2 hr, the membrane was washed with 5 × SSC*/0.1% SDS at room temperature and with 0.1 × SSC/0.1% SDS twice at 42°. The β_1-adrenoceptor mRNA was quantitated by counting the radioactivity using a Fujix BAS 2000 (Fuji Photo Film) and was expressed as PSL per mm^2 [10]. To control for differences in RNA conditions, the membranes were probed sequentially for β-actin, using an oligonucleotide probe (40-mer, New England Nuclear). The membranes were stripped of the β_1-adrenoceptor probe by soaking the membranes twice for 20 min in 0.5% SDS that had been brought to a boil, and subsequent hybridization of the β-actin probe then was performed as described above. Thus, each membrane containing control and diabetic myocardial fractionated RNA samples was probed sequentially for the mRNA of β_1-adrenoceptors and then normalized using the mRNA of the constitutively expressed protein β-actin.

Statistical Analysis

Data are expressed as means ± SEM. Statistical assessment of the data was made by Student's *t*-test or two-way ANOVA with repeated measurements. *P* values of less than 0.05 were considered significant.

* *Abbreviations:* SSC, standard saline citrate; and PSL, photo-stimulated luminescence.

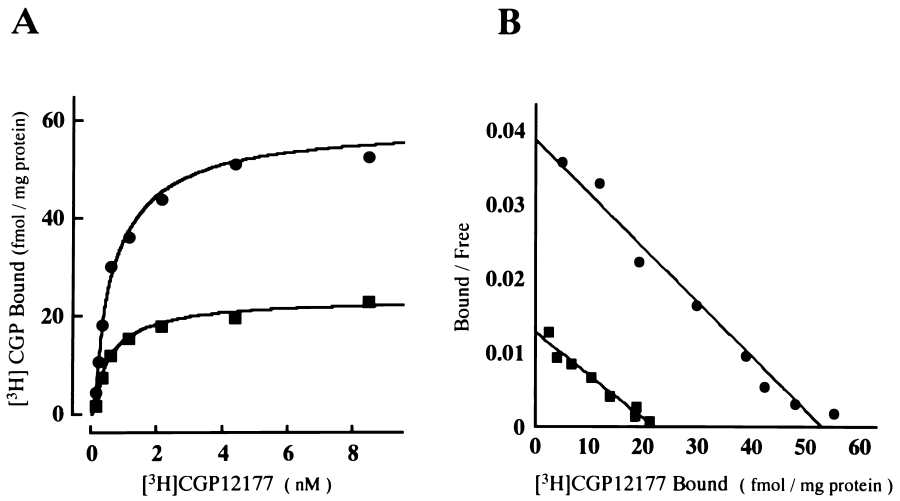

FIG. 1. (A) Specific binding of [^{3}H]CGP 12177 to myocardial ventricular membranes prepared from control (●) and diabetic (■) rats. (B) Scatchard plot of the data. The results shown are representative of four additional experiments.

RESULTS

β-Adrenoceptors in membrane fractions derived from ventricular myocardium of control and diabetic rats were identified using [^{3}H]CGP 12177. The specific binding of [^{3}H]CGP 12177 to myocardial membranes was saturable and showed high affinity in both control and diabetic groups (Fig. 1A). Scatchard analysis of the data revealed that [^{3}H]CGP 12177 bound to a single population of binding sites in both groups (Fig. 1B). The number of β-adrenoceptors was significantly lower in myocardial membranes from diabetic rats (33 ± 7 fmol/mg protein, N = 5) than in controls (67 ± 11 fmol/mg protein, N = 5, $P < 0.01$). The K_D values for [^{3}H]CGP 12177 were similar in control and diabetic groups (0.48 ± 0.03 vs 0.63 ± 0.13 nM). Insulin therapy significantly ($P < 0.05$) prevented the diminished density of myocardial β-adrenoceptors observed in diabetes. Thus, in insulin-treated diabetic rats, the B_{max} was 59 ± 4 fmol/mg protein, and the K_D value was 0.51 ± 0.03 nM (N = 3).

Representative northern blots, which had been hybridized sequentially using probes for β_1-adrenoceptors and β-actin, of total RNA isolated from control and diabetic ventricular myocardium are shown in Fig. 2. The densitometric analysis on the northern blot autoradiograms revealed no significant difference in the absolute integrated optical density values obtained for the β-actin probe between control and diabetic myocardium (185 ± 5 vs 178 ± 9 PSL/mm^2, N = 5). Thus, the steady-state mRNA level for the constitutively expressed protein β-actin remained unchanged in diabetes. When the data were presented as the ratio of the absolute integrated optical density of the β_1-adrenoceptor probe to that of the β-actin probe, the relative content of β_1-adrenoceptor mRNA in diabetes was diminished significantly from the control level, by 57% (Fig. 3). Insulin therapy significantly prevented the diminished level of myocardial β_1-adrenoceptor mRNA observed in diabetes (Fig. 3).

DISCUSSION

In accordance with previous studies from this laboratory and others showing a reduction in myocardial β-adrenoceptor density in experimental animals with diabetes [2, 4–7], the radioligand binding study using [^{3}H]CGP 12177 revealed that the density of β-adrenoceptors was reduced by

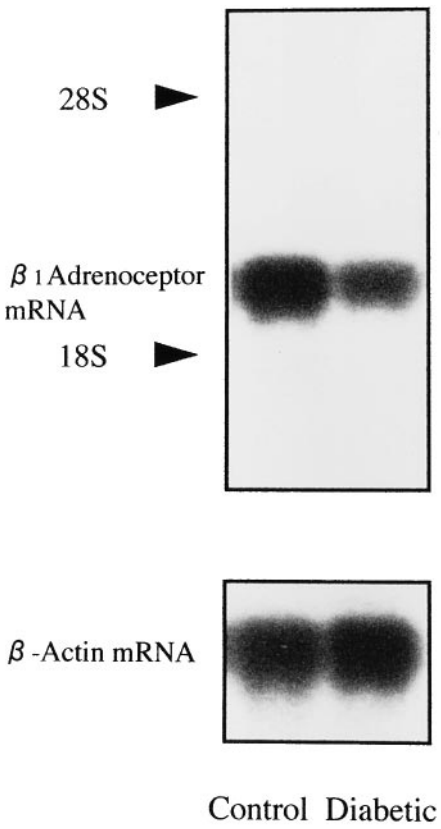

FIG. 2. Autoradiogram of northern blot analysis of the expression of β_1-adrenoceptor mRNA in ventricular myocardium from control and diabetic rats. The blots had been hybridized sequentially using probes for β_1-adrenoceptors and β-actin. The positive signal localized at the molecular size appropriate for the specific mRNA with minimal background. The locations of the 28S and 18S ribosomal RNA are indicated. While there appeared to be no relative change in the abundance of mRNA for β-actin, the relative content of β_1-adrenoceptor mRNA appeared to be decreased in diabetes. The results shown are representative of four additional experiments.

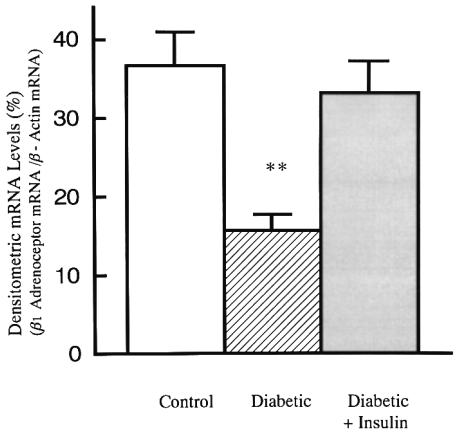

FIG. 3. Quantification of the steady-state levels of β_1-adrenoceptor mRNA in total myocardial RNA from control, diabetic, and insulin-treated diabetic rats. The quantitative analysis was made using a Fujix BAS 2000. The steady-state levels of β_1-adrenoceptor mRNA are normalized to those of β-actin mRNA. Bars are means ± SEM of five experiments. Key: (**) $P < 0.01$ vs the control value (ANOVA for repeated measurements).

50% in ventricular myocardium from rats with 4- to 6-week streptozotocin-induced diabetes. In the present study, we found that this animal model exhibited a significant decrease in the level of myocardial β_1-adrenoceptor mRNA to 43% of control. This parallel behavior of receptor mRNA levels and of receptor expression suggests that reduced mRNA levels may be a reason for a reduction in the β-adrenoceptor density. We assume, therefore, that the reduced mRNA levels result in reduced β-adrenoceptor synthesis in diabetic myocardium.

Although radioligand binding studies have demonstrated the coexistence of β_1- and β_2-adrenoceptors in mammalian ventricular myocardium [11], only β_1-adrenoceptors have been shown to exist in rat ventricular myocytes [12–14]. Even if β_2-adrenoceptors could be detected in membranes from rat ventricle by a binding study, they appear to be localized primarily on non-myocyte cells such as those in coronary arteries [14]. Indeed, β_2-adrenoceptor stimulation does not cause any detectable production of cyclic AMP in rat ventricular myocytes [14]. We also observed that stimulation of adenylate cyclase activity with isoproterenol was mediated entirely by β_1-adrenoceptors in both control and diabetic ventricular myocardium.* Furthermore, in the preliminary experiments, β_2-adrenoceptor mRNA was undetectable in either control or diabetic ventricular myocardium, at least by northern blots using the specific oligonucleotide probe.† Based on these results, it would be reasonable to conclude that the reduced number of myocardial β-adrenoceptors observed in diabetes, as detected by radioligand binding studies, can be traced back to a down-regulation of β_1-adrenoceptor mRNA levels.

It has been reported that *in vivo* insulin treatment is capable of reversing the diminished number of myocardial β-adrenoceptors [6, 15]. We also found that the reduction in the β-adrenoceptor number in diabetic myocardium was prevented significantly by insulin therapy. Furthermore, reversal of reduction in the mRNA level of myocardial β_1-adrenoceptors was observed after insulin replacement. Although insulin is a promoter of the formation of a variety of proteins, whether serum insulin levels are a determining factor in expression of myocardial β_1-adrenoceptor protein and mRNA is not known from this study. Recent experimental evidence has suggested a decrease in the number of myocardial β-adrenoceptors in genetically diabetic rats (WBN/Kob rats), a model of non-insulin-dependent diabetes [16]. Therefore, we interpret the present results obtained in insulin-treated diabetic rats to indicate that streptozotocin-induced reductions in myocardial β_1-adrenoceptor protein and mRNA are a consequence of the resulting diabetic state and are independent of direct cardiac effects of streptozotocin.

Down-regulation of myocardial β_1-adrenoceptor mRNA has been demonstrated in experimental models of heart failure [17] and in human failing hearts [18, 19]. Due to the high sympathetic tone in heart failure, it is believed that this down-regulation is a form of agonist-induced desensitization [20]. In our diabetic models, however, normal levels of plasma and myocardial norepinephrine have been measured [7]. Thus, the mechanisms by which heart failure results in myocardial β_1-adrenoceptor mRNA down-regulation are unlikely to be operative in diabetes. We have found that basal adenylate cyclase activity is enhanced significantly in diabetic myocardium [7]. Activation of adenylate cyclase stimulates a production of cyclic AMP that activates protein kinase A. Protein kinase A clearly can phosphorylate and desensitize β_1-adrenoceptors [20]. However, it is not known whether this process triggers the decrease in β_1-adrenoceptor mRNA.

In conclusion, the present study showed a significant reduction in the β_1-adrenoceptor mRNA levels in diabetic rat ventricular myocardium. This reduction in receptor synthesis at the level of transcriptional regulation would contribute, at least in part, to a decreased number of myocardial β-adrenoceptors in diabetes.

* Gando S, Hattori Y and Kanno M, unpublished results.
† Matsuda N, Hattori Y and Kanno M, unpublished results.

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