Altered K⁺ channel gene expression in diabetic rat ventricle: isoform switching between Kv4.2 and Kv1.4

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Nishiyama, Atsushi, Douglas N. Ishii, Peter H. Backx, Bruce E. Pulford, Barbara R. Birks, and Michael M. Tamkun. Altered K⁺ channel gene expression in diabetic rat ventricle: isoform switching between Kv4.2 and Kv1.4. Am J Physiol Heart Circ Physiol 281: H1800–H1807, 2001.—Expression of voltage-gated K⁺ channels encoding the K⁺ independent transient outward current in the streptozocin-induced diabetic (DM) rat ventricle was studied to determine the basis for slowed cardiac repolarization in diabetes mellitus. Although hypertrophy was not detected in diabetic rats at 12 wk after streptozocin treatment, ventricular Kv4.2 mRNA levels decreased 41% relative to nondiabetic controls. Kv1.4 mRNA levels increased 179% relative to controls, whereas Kv4.3 mRNA levels were unaffected. Immunohistochemistry and Western blot analysis of the diabetic controls, whereas Kv4.3 mRNA levels were unaffected. IGF-II treatment did not change Kv1.4, or Kv4.3 mRNA levels were unaffected by diabetes. Myosin heavy chain (MHC) gene expression was altered with a 32% decrease in α-MHC mRNA and a 259% increase in β-MHC mRNA levels in diabetic ventricle. Low-dose insulin-like growth factor-II (IGF-II) treatment during the last 6 of the 12 wk of diabetes (DM + IGF) protected against these changes in MHC mRNAs despite continued hyperglycemia and body weight loss. IGF-II treatment did not change K⁺ channel mRNA levels in DM or control rat ventricles. Thus IGF treatment may prevent some, but not all, biochemical abnormalities in the diabetic heart.

Cardiac contractility (expressed as ventricular pressure development and peak pressure) is reduced, and relaxation time and velocity are markedly prolonged in experimental diabetes (15, 44, 45). In concordance with these mechanical changes, changes in electrocardiogram and action potential duration are often observed in diabetic patients (20, 37). The most prominent electrophysiological alteration is the prolongation of the Q-T interval due to an increase in ventricular action potential duration (APD) (24, 38, 49). Recent electrophysiological findings have shown that the density of the K⁺ independent transient outward current (I_TO) is reduced and the recovery kinetics of I_TO slowed in diabetic myocardium relative to normal tissue. These changes in I_TO properties are likely to be responsible for the APD prolongation in diabetic rats (48, 50).

However, the molecular mechanisms responsible for these changes in I_TO have not been examined.

Cardiac Kv1.4 and Kv4.2 channels were first proposed to be responsible for the I_TO based solely on functional parameters (3, 41). Recently, Kv1.4, Kv4.2, and Kv4.3 were confirmed as responsible for the native I_TO by gene knockout, antisense, and dominant negative approaches (19, 62). The primary difference between Kv1.4 and the two Kv4 channels is in the recovery from inactivation, with Kv1.4 recovering much more slowly than Kv4.2. Changes in outward current observed in diabetic animals could result from a decrease in the expression of Kv4.2 and an increase in the more slowly recovering Kv1.4 isoform. One aim of this study was to test the hypothesis that Kv4.2 expression is reduced in diabetes mellitus, along with a compensatory increase in Kv1.4 expression.

The second aim was to determine whether insulin-like growth factor (IGF) treatment can prevent or reverse the biochemical pathology associated with the diabetic ventricle. Circulating and tissue IGF levels are reduced in clinical (52, 59) as well as experimental (4, 11, 39) diabetes. IGF gene expression is reduced in diabetic nerves (60), and IGF administration can protect against diabetic neuropathy (21, 22, 64). Because

THE DIABETIC HEART is attended by increased mortality. The cardiovascular complications are well known in both human and experimental diabetes mellitus (16, 25, 43, 54). There are distinct alterations in both the mechanical and electrophysiological properties of the myocardium (13, 14, 32). The purpose of this study was to examine the biochemical pathology that may contribute to the mechanical and electrophysiological dysfunction in the diabetic heart.

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IGF mRNA is reduced in cardiac muscle in diabetes (28), these observations together prompted us to determine whether IGF treatment could be cardioprotective. The expression of myosin heavy chain (MHC) isoforms is altered in diabetic cardiac muscle (35). Because altered isoform expression may contribute to mechanical dysfunction, the hypothesis that IGF treatment can prevent abnormal expression of MHC genes as well as Kv channel genes was tested.

MATERIALS AND METHODS

**Materials.** The affinity-purified Kv1.4 antibody, affinity-purified Kv4.2 antibody, and Kv4.2-specific antiserum have been previously characterized (6, 58). Immunological reagents were purchased from Jackson Immunoresearch Laboratories.

**Induction of diabetes and tissue preparation.** All studies involving rats were done in accordance with the principles set forth in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, 1996). Male Sprague-Dawley rats (12 wk old) were randomly assigned to nondiabetic (control), diabetic (DM), and DM + IGF-II treatment (DM + IGF) groups. Each group contained four rats. After the rats were fasted overnight, diabetes was induced in rats with a single intraperitoneal injection of 50 mg/kg streptozocin (STZ; Sigma) and maintained for 12 wk with free access to rat chow and water. At the end of 6 wk, the STZ-treated animals were implanted subcutaneously in the midback with osmotic minipumps (50 μl/h) that released either vehicle (1 mM acetate, pH 6) or IGF-II (5 μg·rat −1·day −1) for the remaining 6 wk. Serum glucose concentrations were determined at the end of the first 6-wk period and on the day of euthanasia with the use of a glucose diagnostic kit (model 510A; Sigma). The cardiac ventricle and brain in each animal were removed, and small pieces of the left ventricular free wall near the left descending artery were embedded in Tissue Tek (Baxter) and slowly frozen at −80°C for immunohistochemical analysis. The remaining ventricular tissue was used for both membrane preparation and RNA extraction. One brain hemisphere (except for the olfactory bulb) was used for RNA extraction.

**Northern blot analysis.** Total RNA was extracted from freshly isolated ventricles and brains using the guanidinium thiocyanate method (1). Ethidium bromide (40 μg/ml) was added to RNA samples before electrophoresis to enable visual confirmation of RNA integrity. After fractionation of 10 μg of total RNA through a 1% agarose-3% formaldehyde gel in 20 mM MOPS and 1 mM EDTA, pH 7.4, the RNA was transferred to a Nytran filter (Schleicher and Schuell) by capillary action in 20× saline sodium citrate. RNA was cross-linked to the filter by ultraviolet irradiation using a Stratalinker (Stratagene). The filter was hybridized with a rat α- or β-MHC oligonucleotide probe (Calbiochem). Oligonucleotide probes were 5’ end labeled with [γ-32P]ATP (6,000 Ci/mmol; ICN) using T4 polynucleotide kinase. The hybridization and wash protocol was performed according to standard protocols. After hybridization and wash, the filter was exposed to a PhosphorImager screen (Molecular Dynamics) for quantification and then exposed to XAR-5 film (Kodak).

**Ribonuclease protection assay.** The sequence of the cDNA templates used for the production of the radioactive antisense RNA probes for Kv1.4, Kv4.2, and Kv4.3 and the detailed procedure of ribonuclease protection assay (RPA) have been described previously (36). In brief, plasmids containing Kv channel cDNAs were linearized with the appropriate restriction enzyme and antisense cRNA probes were synthesized using the MAXIscript kit (Ambion) with [α-32P]UTP (ICN Radiochemicals). The cychrophilin cRNA probe was synthesized from cDNA (pTRI-cyclophilin rat antisense control template) purchased from Ambion to detect cyclophilin mRNA as an internal control. RPA was performed using a RPAII kit (Ambion) according to the manufacturer’s protocol. Hybridization of the Kv channel and cyclophilin probes with 10 μg total sample RNA was carried out at 42°C overnight, followed by digestion with a RNase A and T1 mix at 37°C for 30 min. The reaction was terminated by the addition of SDS and proteinase K. After ethanol precipitation, the protected RNA fragments were subjected to electrophoresis in a 5% polyacrylamide-8 M urea gel. The gel was dried on 3M paper and subjected to quantitative analysis using a PhosphorImager. The mRNA levels were normalized to the levels of cyclophilin mRNA and expressed in relative arbitrary units.

**Immunofluorescence.** Cryosections of the rat ventricle (10 μm) were incubated with primary rabbit (anti-rat IgG [Jackson Immunoresearch] and CY3-conjugated streptavidin as previously described in detail (34). Samples were analyzed using a Nikon E800 microscope equipped with standard epifluorescence and a Princeton Instruments charge-coupled device camera. Antigen-blocking experiments were conducted to confirm Kv4.2 antiserum specificity. Here, the antibody staining was performed as described above except that cryosections were stained with antiserum that had been preincubated for 2 h at room temperature with 200 nmol of the Kv4.2 peptide per milliliter of diluted antiserum (34). Blocking experiments were not necessary with the other two antibodies because they were affinity purified.

**Western blot analysis.** Approximately 0.5 g of each thawed rat ventricle was homogenized with a high-shear homogenizer (Tekmar) in 5 ml of 0.32 M sucrose-5 mM Na2HPO4 with the following protease inhibitors (Sigma, unless otherwise noted): 0.31 mg/ml benzamidine, 0.62 mg/ml N-ethylmaleamide, 1 mg/ml bacitracin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 0.31 mg/ml pefabloc (Boehringer Mannheim), and 0.8 μg/ml calpain inhibitor (Calbiochem). Tissue was homogenized on ice at high speed with a small (1.0 cm) diameter probe for 60 s. The homogenate was centrifuged at 4°C for 10 min at 3,000 rpm (Beckman JA 25.5 rotor) to remove large debris and nuclei. The supernatant was further homogenized by 10 strokes in a 7-ml Wheaton dounce tissue grinder on ice and centrifuged for 1 h at 4°C and 12,000 rpm (JA 25.5; Beckman Instruments). The resulting membrane pellet was resuspended in 100 μl of ice-cold PBS and stored at −80°C. Membrane proteins were fractionated by SDS-PAGE and transferred overnight to nitrocellulose membrane (Schleicher & Schuell) using the standard Laemmli method as previously described (33). Briefly, SDS sample buffer was added to the isolated membranes before electrophoretic separation on a 10% polyacrylamide gel using a minigel system (Bio-Rad). After overnight transfer onto nitrocellulose membrane, the samples were stained with 0.1% Ponceau S Solution (Sigma) (to visualize the quality of the transfer), rinsed with deionized water, and incubated overnight at 4°C in solution 1 (S1) [50 mM Tris (pH 7.5), 150 mM NaCl, and 0.1% Tween-20, and 5% nonfat dry milk] to block nonantigenic sites. The blots were then incubated in S1 at room temperature for 1 h with either Kv1.4 or Kv4.2 polyclonal antibodies (diluted 1:1,000). The blots were washed 3 times for 15 min each with solution 3 (S3) [50 mM Tris (pH 7.5), 500 mM NaCl, and 0.1% Tween 20] and then incubated for 1 h at room temperature in S1 containing a 1:3,000 dilution of horseradish peroxidase

**Immunoperoxidase.** Cardiac sections were stained for Kv4.2 and cyclophilin using a PhosphorImager. The mRNA levels were normalized to the levels of cyclophilin mRNA and expressed in relative arbitrary units.

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and DM + IGF: 17.7 ± 2.2, 18.5 ± 2.7, and 18.3 ± 3.6 μm, respectively) among the groups (data not shown) 
(n = 40 myocytes total per group, each group consisting of four animals). These data indicate that diabetes, with or without IGF treatment, was not associated with cardiac hypertrophy using standard morphological measurements.

MHC isoform switching from the α- to β-isoform occurs in the hypertrophied or diseased rat heart (23, 46). Therefore, although gross ventricular hypertrophy was not detected, we tested for altered myosin gene expression. The ventricular α- and β-MHC mRNA levels as a function of treatment are shown by the Northern blot analysis of Fig. 1, A and B. Diabetic treatment
resulted in a significant decrease of α-MHC mRNA to 32 ± 11% of control (P < 0.01), and IGF treatment restored the mRNA level to 51 ± 3% (P < 0.01 vs. DM) (Fig. 1A). Diabetic treatment increased the β-MHC mRNA level to 259 ± 8% of control (P < 0.01), and IGF treatment reduced this change to 217 ± 31% of control (P < 0.05 vs. DM) (Fig. 1B). Thus hypertrophy may have developed with a longer time period, for myosin isoform expression did change.

Effect of diabetes and IGF treatment on potassium channel α-subunit mRNA levels in heart and brain. To examine the effect of diabetes and IGF treatment on Kv1.4, Kv4.2, and Kv4.3 gene expression in the heart, mRNA levels were measured in ventricles using an RNase protection assay with cyclophilin mRNA used as an internal control. As shown in Fig. 2, A and B, Kv1.4 mRNA level increased to 179 ± 24% of control (P < 0.01), whereas Kv4.2 mRNA level decreased to 41 ± 5% of control (P < 0.01) in the diabetic rat ventricles. IGF treatment did not reverse these changes. In contrast, Kv4.3 mRNA levels showed no significant change with any treatment (Fig. 2C).

To determine whether change in K⁺ channel mRNA levels in diabetes was restricted to the heart, mRNA levels of Kv1.4, Kv4.2, and Kv4.3 were examined also in the brain. As shown in Fig. 3, A–C, diabetic animals had the same relative mRNA levels for all three channels in the brain. Two alternatively spliced variants of Kv4.3 are present in the rat brain, whereas only a single isoform is found in the ventricle (36, 51). Diabetics had no effect in relative levels of these two splice variants in the brain.

Immunoreactive Kv4.2 and Kv1.4 protein levels in diabetic ventricle. Kv α-subunit protein expression levels do not always correlate with mRNA levels in rat heart (61). In addition, altered expression could be occurring in cell types other than ventricular myocytes. Therefore, Kv4.2 immunostaining of ventricle cryosections was performed to address the location and amount of ventricular K⁺ channel protein levels in the diabetic state. In the control rat ventricle, Kv4.2 immunoreactivity was distributed evenly over myocytes, while being slightly concentrated at the ends of the cells [Fig. 4A, consistent with previous reports (6)]. In the diabetic rat ventricle, the staining level was reduced compared with control (compare Fig. 4, A and B) as predicted from the changes in mRNA levels. The antibody staining of IGF- and vehicle-treated diabetic rat ventricles was similar (data not shown), again as predicted from the mRNA analysis. Figure 4, C and D, shows the staining observed with no primary antibody or with antiserum previously adsorbed with the pep-
tide used for antiserum production. Only background staining was observed in the absence of primary antibody, and the peptide treatment reduced the staining to near-background levels. Together these data indicate antibody specificity. Also supporting the antibody specificity are the results shown in Fig. 4, E and F. Here, a different and affinity-purified anti-Kv4.2 antibody was used to stain control and diabetic myocardium, Fig. 4, E and F, respectively. Again, antibody staining was reduced in the diabetic tissue. Figure 4, G and H, illustrates the immunostaining observed with an affinity-purified anti-Kv1.4 antibody. As predicted from the changes in mRNA levels, the diabetic state caused an increase in Kv1.4 protein as detected by the immunofluorescence. Figure 4G shows staining to con-

control tissue, whereas Fig. 4H represents diabetic tissue. Although gradients of Kv1.4 and Kv4.2 expression exist across the ventricular wall (6, 8, 58), a protein gradient was not observed for either channel in this study. Perhaps this is because the tissue sections employed in this study were from the left ventricular free wall base and the gradient is more apparent in the middle and apex of the ventricle (6). Together, these immunostaining data argue for altered expression in the left ventricular myocytes as opposed to cells derived from, for example, vascular beds.

Western blot analysis of Kv4.2 and Kv1.4 protein levels in diabetic ventricle. We also confirmed via Western blot analysis that Kv4.2 and Kv1.4 protein levels in the ventricle changed in parallel to the observed changes in mRNA. As shown in Fig. 5, Kv1.4 protein was dramatically increased with the diabetic state, whereas Kv4.2 was markedly reduced. Because of the limited depth of film, quantitation was not attempted. Still, these data confirm the mRNA analysis and thus further support the idea that the change from $I_{To fast}$ to $I_{To slow}$ happens...

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<th>Table 1. Effect of diabetes and IGF-II treatment on body wt, ventricle wt, and serum glucose levels</th>
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|                      | Nondiabetic Control | DM | DM + IGF-
| Body wt, g           | 486 ± 30            | 379 ± 82* | 370 ± 51* |
| Ventricle wt, g      | 1.24 ± 0.13         | 1.13 ± 0.04 | 1.15 ± 0.13 |
| Ventricle wt/body wt | 2.55 ± 0.19         | 2.99 ± 0.14* | 3.12 ± 0.12* |
| Blood glucose, mg/dl | 146.8 ± 18.0        | 531.5 ± 111.3* | 539.0 ± 118.1* |

Values are means ± SD (n = 4 rats per group) for body weights (body wt), ventricle weights (ventricle wt), and serum glucose levels. Diabetes was induced by injection of streptozocin, and adult 12-wk-old diabetic (DM) and non-DM (control) rats were maintained for an additional 12 wk. DM rats were implanted subcutaneously with miniosmotic pumps that released either vehicle (DM rats) or 5 μg/day insulin-like growth factor-II (DM + IGF rats) during the last 6 wk of DM. Statistical significance was determined by Student’s t-test. *P < 0.01.
Abnormal Kv gene expression in diabetes. APD is prolonged in the STZ-induced diabetic rat ventricle (31, 38), and the attenuation of \( I_{TO} \) has been proposed as being responsible (48–50). Furthermore, an increased rate dependency (38) or prolongation of recovery kinetics of \( I_{TO} \) was reported (49, 50). When heterologously expressed, Kv1.4 and Kv4.2 produce rapidly activating and inactivating outward currents and are considered to underlying cardiac \( I_{TO} \) (9, 51, 55, 63). One of the greatest differences between these Kv channels is their recovery from inactivation. Kv1.4 recovers with a time constant of 3–8 s (2, 40, 41), whereas the Kv4.2 recovers much faster with a time constant of 200–1,000 ms (57, 58). Recent gene knockout and dominant negative approaches have confirmed that \( I_{TO\text{fast}} \) is encoded by Kv4.2, whereas \( I_{TO\text{slow}} \) is due to Kv1.4 (19, 62).

The data shown above indicate that Kv4.2 gene expression (Fig. 2B) and protein (Fig. 4, A and B, and Fig. 5) levels were reduced, whereas Kv1.4 gene expression (Fig. 2A) and protein levels (Fig. 4, G and H, and Fig. 5) were increased in diabetic heart ventricles. These changes are likely to represent the molecular mechanism responsible for the effects of diabetes on \( I_{TO} \) and the resulting increase in cardiac APD. Our findings support recently published work of Qin et al. (42), which shows that diabetes reduces ventricular Kv4.2 protein and mRNA levels. However, this study did not examine the levels of either Kv1.4 mRNA or protein. In contrast to our findings reported here, these investigators found Kv4.3 expression was also suppressed with diabetes.

Interestingly, Kv4.2 and Kv1.4 gene expression was changed without marked cardiac hypertrophy (Table 1, Fig. 1) (42), albeit MHC gene expression was abnormal. This shows that the alteration in Kv4.2 and Kv1.4 mRNA levels was not secondary to ventricular hypertrophy. \( K^+ \) channel expression in the heart is associated with cardiac hypertrophy (29), and it seems likely we would also have observed hypertrophy with longer duration or more deeply diabetic rats. The abnormal Kv1.4 and Kv4.2 gene expression in the ventricle was not observed in the brain (Fig. 3, A and B), showing that this abnormality could not be simply ascribed to hyperglycemia. Other possibilities include perhaps hemodynamic factors (12, 27, 56) or impaired autonomic neural control of the heart (17, 30, 53). In fact, the heart rate of the STZ-induced diabetic rats is reduced (12, 27, 56). It is interesting to note that, although both Kv4.2 and Kv4.3 contribute to cardiac \( I_{TO} \), only the Kv4.2 expression was altered.

Our present data are in contrast to several other studies examining gene expression in hypertensive hearts or after infarction. Takimoto et al. (51) showed that in renovascular hypertensive rats, both Kv4.2 and Kv4.3 expression were decreased, whereas Kv1.4 expression was unaltered. In one study examining altered \( I_{TO} \) expression after infarction both Kv4.2 and Kv4.3 were downregulated, whereas Kv1.4 expression increased (26).

Clearly, the cardiac \( K^+ \) gene expression responds differently to hypertension, infarction, and the diabetic state studied in our present work. IGF treatment of diabetic rats did not affect the changes in Kv channel expression in the ventricle or brain. This treatment also had no effect on these tissues in nondiabetic rats.

Low-dose IGF treatment protects against MHC isoform switching. \( \alpha \)-MHC mRNA was reduced 32% (Fig. 1A), whereas \( \beta \)-MHC was increased 259% (Fig. 1B) in diabetic ventricles. This isoform switching is observed in cardiac but not skeletal muscle (35). It is noteworthy that MHC isoform switching was prevented by low-dose (\( \sim 15 \mu g \cdot kg^{-1} \cdot day^{-1} \)) IGF-II treatment, despite continued hyperglycemia and body weight loss (Table 1). This suggests that isoform switching may not be a consequence of hyperglycemia per se but may result from reduced IGF gene expression in the heart (5, 28).

Likewise, low-dose IGF-I or IGF-II treatment can prevent diabetic neuropathy independently of hyperglycemia and weight loss (22, 64). Further study is needed to determine whether IGF protection results from indirect actions on autonomic function or direct actions on cardiac tissue. IGF treatment both prevents ultrastructural abnormalities in autonomic nerves (47) and attenuates myocardial injury and apoptosis after ischemia (7). Thus IGF-I improves cardiac output and decreases systemic vascular resistance after infarction (10). Both IGF-I and IGF-II act through the type I IGF receptor expressed on the surface of ventricular myocytes (18).

In summary, we have demonstrated that \( K^+ \) channel genes regulating the \( I_{TO} \) switch from Kv4.2 to Kv1.4 in diabetic ventricles. This change may well explain the reduced rate of repolarization and abnormal \( I_{TO} \) observed in the diabetic heart. The switch in Kv channel isoform expression preceded observable cardiac hypertrophy, albeit MHC isoform gene expression was altered. IGF treatment blunted the diabetes-induced changes in MHC isoform gene expression independently of continued hyperglycemia but had no effect on Kv channel expression. These findings suggest IGF treatment may ameliorate some but not all aspects of cardiac contractile dysfunction in diabetes.

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