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Effects of Gender Difference on Cardiac Myocyte Dysfunction in Streptozotocin-Induced Diabetic Rats

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The main characteristics of type 1 diabetic cardiomyopathy include depressed contractility and altered electrophysiological properties in ventricular myocytes. The goal of the present study was to determine the potential influence of gender in the diabetes-induced pathogenesis of ventricular myocyte function. Diabetes in both male and female rats was induced by a single intravenous injection of streptozotocin (STZ). Diabetic rats exhibited hyperglycemia and reduced body weight gain in both male and female groups. Neither contractile profiles nor activity of three types of K^+ channels of ventricular myocytes was significantly different between nondiabetic male and female rats. Ventricular myocytes isolated from diabetic rats exhibited significant depression in cell contraction and relaxation, which was associated with depression of intracellular Ca^{2+} ($[Ca^{2+}]_i$) transient. The degrees of contractile depression were comparable in ventricular myocytes obtained from both male and female diabetic rats. Similarly, diabetes depressed three types of outward K^+ currents (Ito, Ik, and Iss) to the same extent in both gender myocytes. These data demonstrate that in this animal model of diabetes, gender difference in cardiac myocyte functions was eliminated.

Key Words: Diabetes; gender; cardiac myocyte; cell shortening; Ca^{2+} transient; potassium channels.

Introduction

Cardiac complications remain the leading cause of morbidity and mortality in diabetic patients. Sustained diabetes leads to deterioration of heart function known as diabetic cardiomyopathy that is independent of coronary artery complications *(1,2)*. One of the common complications in diabetic cardiomyopathy is ventricular contractile dysfunction. Both systolic and diastolic dysfunctions have been identified in whole heart and cardiac tissue with diminished peak tension or pressure, elevated end-diastolic pressure, and reduced values for stroke volume, cardiac work, and cardiac output *(1–7)*. In individual ventricular myocytes isolated from the experimental diabetic animals, contractile dysfunction was characterized as reduced peak shortening and slowed shortening and relaxation kinetics *(8,9)*. Another common complication of diabetic cardiomyopathy is that diabetic patients have a higher incidence of cardiac arrhythmias, ventricular fibrillation, and sudden death *(10)*. These incidences of diabetic cardiomyopathy are associated with electrophysiological alterations at the cellular level. Myocytes isolated from diabetic animals exhibited a prolonged action potential duration that is correlated with reduced repolarizing K^+ currents including the transient outward current Ito, the delayed rectifier current Ik, and the sustained current Iss *(11–14)*.

Although many characteristics of cardiovascular disease are similar between male and female, clinical and experimental evidence has convincingly suggested that there is a gender difference, especially in the progression of heart failure. Premenopausal women usually have a lower risk of cardiovascular diseases than age-matched men and postmenopausal women *(15)*. However, this female protective advantage in cardiovascular function seems not to exist in clinical observations with diabetes mellitus. The incidence of heart failure is doubled in diabetic male patients and five times higher in diabetic female patients when compared with nondiabetic men and women *(16)*. In addition, young girls with type-1 diabetes may have more pronounced signs of diabetic cardiomyopathy *(17)*.

Nevertheless, most studies on diabetic cardiomyopathy only included male animals as the experimental subjects. Thus, our understanding of potential gender-specific difference in diabetic cardiomyopathy is largely limited. In the present study, we examined the possible influence by gender on single ventricular myocyte contractility, intracellular Ca^{2+} homeostasis, and electrophysiological properties in diabetic rats induced with streptozotocin. Our results demonstrate that while there is some difference in cardiac myocyte function between healthy male and female rats, ventricular myo-

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Table 1
Body Weight and Blood Glucose in Different Groups of Rats

		Body weight (g)		Blood glucose (mg/dL)	
Animal group	*n*	Initial	8 wk	3 d	8 wk
Male control	12	260.5 ± 11.2	470.0 ± 7.8	131.8 ± 6.8	153.2 ± 10.3
Male diabetic	9	243.6 ± 15.4	276.7 ± 14.8*	417.2 ± 45.2*	572.8 ± 12.4*
Female control	6	200.7 ± 9.3^{+}	305.8 ± 10.3^{+}	151.5 ± 6.5	153.7 ± 4.2
Female diabetic	6	208.7 ± 6.2^{+}	218.3 ± 14.3*$^{+}$	464.0 ± 36.1*	535.0 ± 19.5*

Data are mean ± SE. *n* represents the numbers of animals used in each group. * represents significantly different when compared with the age-matched control animals in the same gender. $^{+}$ represents significantly different when compared with the age-matched male animals.

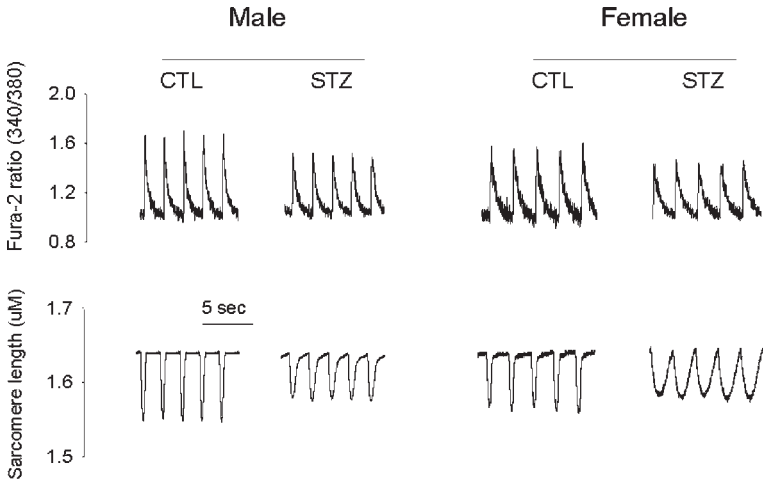

Fig. 1. Representative recordings of myocyte shortening and intracellular Ca^{2+} transient. Sarcomere lengths and intracellular fura-2 ratios were recorded simultaneously in ventricular myocytes isolated from both gender control (CTL) and streptozotocin-induced diabetic (STZ) rats. Myocyte contraction was initiated by field stimulation at 0.5 Hz.

cyte contractility and K^{+} channel activities were depressed at the same level in both male and female diabetic rats.

Results

General Features of Normal and Diabetic Male and Female Animals

The impact of gender on body weight and blood glucose in both diabetic and control rats is shown in Table 1. Age-matched control animals showed a significant gain of body weight and a stable level of blood glucose throughout the experiment. Body weight of the female animals was lower than male rats both before and after induction of diabetes. Blood glucose levels were not significantly different between normal male and female rats. Rats injected with STZ exhibited the characteristic symptoms of uncontrolled diabetes including hyperglycemia, polyuria, decreased body weight gain, polydipsia, and polyphagia when compared to age-matched controls. The blood glucose levels in both male and female diabetic rats were not significantly differ. These data demonstrate that injection of STZ induced diabetes in rats, which was not influenced by gender difference.

Influence of Gender on Myocyte Mechanical and Intracellular Ca^{2+} Transients

To assess the possible difference of gender in the diabetes-induced myocyte contractile dysfunction, we compared myocyte contractility in both genders of diabetic rats. Myocytes were stimulated at 0.5 Hz and data were collected at 3 min after stimulation to ensure a steady-state contraction in all groups of myocytes. Figure 1 shows the representative traces of sarcomere shortening and fura-2 ratio simultaneously recorded from ventricular myocytes isolated from an age-matched male, a diabetic male, an age-matched female, and a diabetic female animal. The resting sarcomere lengths of myocytes are comparable among myocytes obtained from all four groups of animals (Table 2). Sarcomere shortening of ventricular myocytes obtained from female control rats was relatively lower but not significantly different from that of age-matched control male counterpart (Table 2). The maximal rates of cell shortening and re-lengthening were significantly lower in female myocytes. Induction of diabetes severely depressed myocyte contractility in both gender animals as indicated by the reduced fractional sarcomere shortening and the maximal rate of shortening

Table 2
Myocyte Contraction and $[Ca^{2+}]_i$ in Different Groups of Rats

	Male		Female	
Parameters	Control (n = 12)	Diabetic (n = 9)	Control (n = 6)	Diabetic (n = 6)
Resting sarcomere length (μm)	1.67 ± 0.01	1.68 ± 0.04	1.64 ± 0.02	1.65 ± 0.01
Sarcomere shortening (%)	7.22 ± 0.53	3.75 ± 0.33*	6.82 ± 0.63	3.18 ± 0.26*
+dL/dt (μm/ms)	2.06 ± 0.17	1.34 ± 0.11*	1.23 ± 0.16$^+$	0.78 ± 0.09*$^+$
–dL/dt (μm/ms)	1.08 ± 0.11	0.57 ± 0.10*	0.68 ± 0.12$^+$	0.42 ± 0.04*$^+$
TL90 (s)	0.42 ± 0.02	0.56 ± 0.02*	0.60 ± 0.02$^+$	0.72 ± 0.03*$^+$
Resting fura-2 ratio	1.17 ± 0.04	1.17 ± 1.14	1.15 ± 0.05	1.16 ± 0.03
Peak fura-2 ratio	1.75 ± 0.05	1.53 ± 0.03*	1.74 ± 0.06	1.56 ± 0.06*
+dR/dt (unit/ms)	46.7 ± 2.4	31.0 ± 1.8*	45.5 ± 3.6	36.8 ± 3.4
–dR/dt (unit/ms)	3.02 ± 0.14	2.10 ± 0.10*	3.09 ± 0.22	2.35 ± 0.16*
TR90 (s)	0.27 ± 0.01	0.38 ± 0.01*	0.29 ± 0.01	0.40 ± 0.01*

Data are mean ± SE. ±dL/dt, maximal rates of sarcomere shortening and re-lengthening. TL90, time to 90% sarcomere re-lengthening. ±dR/dt, maximal rates of fura-2 ratio rise and fall. TR90, time to 90% fura-2 ratio fall. *n* represents the numbers of animals tested in each group. * represents significantly different when compared with the age-matched control animals in the same gender. $^+$represents significantly different when compared with the age-matched male animals.

(Table 2). The degree of the depressed cell contraction was similar in both male and female diabetic rats. Fractional sarcomere shortening was reduced 48% in male diabetic myocytes, and 52% in female diabetic myocytes, when compared to the same gender control myocytes. Similarly, the maximal rates of sarcomere shortening were depressed by 35% and 37%, respectively, in male and female diabetic myocytes. In addition to the depressed systolic function, diastolic function was also altered in both male and female diabetic myocytes. Maximal rates of sarcomere re-lengthening were significantly reduced, and times to 50% re-lengthening were significantly prolonged in the similar degree in myocytes from both male and female diabetic rats when compared to same gender control rats (Table 2). Again, the extent of reduction in these parameters is comparable between both genders.

Associated with depressed cell contraction, intracellular Ca^{2+} ($[Ca^{2+}]_i$) transients in myocytes isolated from both gender diabetic rats also were depressed at the similar level. Resting fura-2 ratios in diabetic groups were not significantly different from the controls (Table 2). However, fura-2 ratio development and the maximal rates of ratio rise and fall were all significantly lower, and the time to 50% ratio decline was significantly longer in myocytes from both male and female diabetic rats (Table 2). There is no significant difference in these parameters between male and female control myocytes. Peak fura-2 ratio was reduced by 38% in male diabetic myocytes, and 34% in female diabetic myocytes when compared to the same gender control myocytes. These results demonstrate that depressed cell shortening was closely associated with the reduced $[Ca^{2+}]_i$ in diabetic myocytes and reduction of $[Ca^{2+}]_i$ in diabetic myocytes was not affected by gender difference.

Influence of Gender on K^+ Channel Activities in Diabetic Cardiac Myocyte

Previous studies have shown a significant reduction in three different K^+ currents, Ito, Ik, and Iss, in myocytes from male diabetic rat hearts *(13,19,20)*. We compared the possible influence of gender on the diabetes-induced alteration in K^+ channel activity. Averaged Ito densities in male and female control myocytes were comparable and not significantly different when recorded at all the test potentials. Induction of diabetes depressed Ito densities at the test potentials between 0 and +50 mV (Fig. 2), and the fractional reduction of Ito densities was similar in both male and female diabetic myocytes. Ito density was attenuated by 30% and 32%, respectively, in male and female diabetic myocytes when measured at +50 mV. Similar to Ito, Ik and Iss densities were not significantly different in both male and female control myocytes at all the test potentials recorded, while these values were depressed in diabetic myocytes. There is no gender difference in the diabetes-induced attenuation of Ik and Ito. Ik densities were reduced by 19% and 20% (Fig. 3), respectively, and Iss densities were reduced by 42% and 50% (Fig. 4), respectively, in myocytes isolated from male and female diabetic rats when compared to same gender control myocytes at the test potential of +50 mV.

Discussion

The primary observation from the present study is that there is no gender difference in diabetes-induced cardiac myocyte dysfunction in STZ-induced type-1 diabetic rats. Myocytes isolated from female control rats exhibited slower rates of cell shortening and re-lengthening along with a prolonged time for 90% cell re-lengthening. On the other hand,

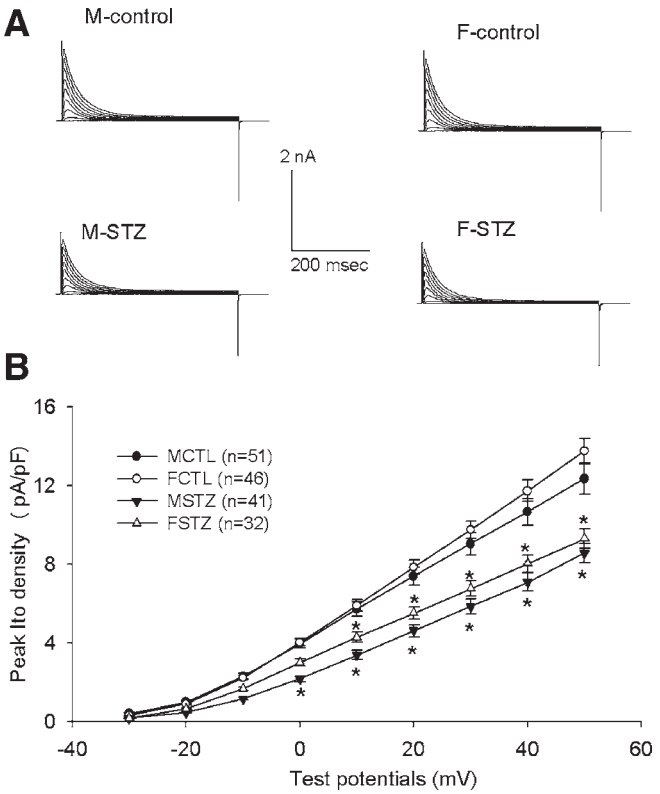

Fig. 2. Effects of gender on the transient outward potassium current (Ito) in control and diabetic myocytes. (**A**) Representative traces of Ito recorded in the ventricular myocytes from each groups of animals at different test potentials. (**B**) Averaged peak Ito current–voltage relationships in myocytes from different groups of animals. MCTL: male control; MSTZ: male diabetic; FCTL: female control; FSTZ: female diabetic. Currents were recorded 3 min after whole-cell voltage clamp configuration. Currents were elicited by the test potentials ranging from −30 to +50 mV from a holding potential of −60 mV in the presence of TEA. Test potentials were changed with 10 mV increment at 6-s intervals. Each value represents means ± SE, and *n* represents number of myocytes tested. Three to six cells from each animal were tested. *Significantly different from the same gender control ($p < 0.05$).

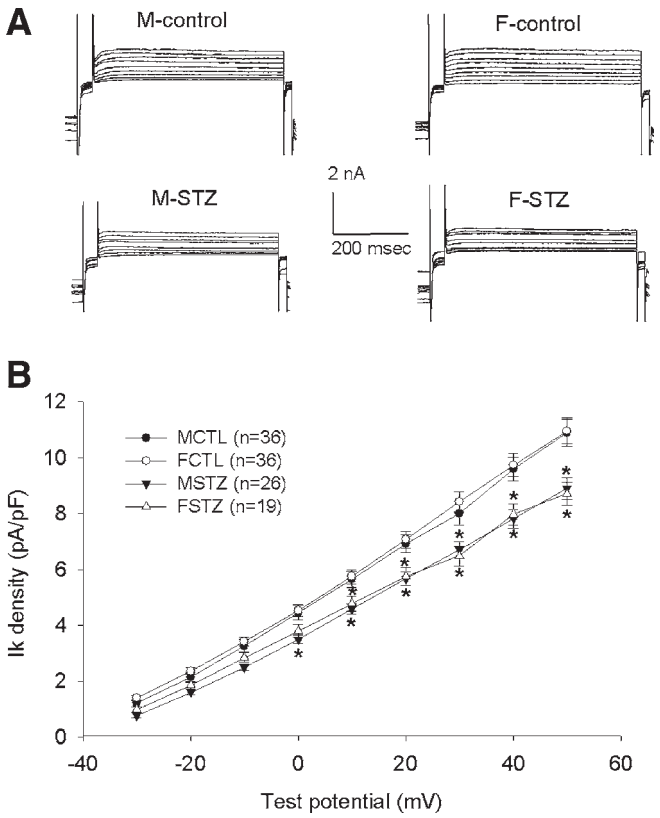

Fig. 3. Effects of gender on the delayed rectifier potassium current (Ik) in control and diabetic myocytes. (**A**) Representative traces of Ik recorded in the ventricular myocytes from each groups of animals at different test potentials. (**B**) Averaged Ik current–voltage relationships in myocytes from different groups of animals. MCTL: male control; MSTZ: male diabetic; FCTL: female control; FSTZ: female diabetic. Currents were recorded 3 min after whole-cell voltage clamp configuration. Resting membrane potential was held at −120 mV. Currents were elicited by the test potentials ranging from −30 to +50 mV, with a short prepulse to −40 mV to inactivate Na^+ currents, in the presence of 4-AP. Test potentials were changed with 10 mV increment at 6-s intervals. Each value represents means ± SE, and *n* represents number of myocytes tested. Three to sex cells from each animal were tested. *Significantly different from the same gender control ($p < 0.05$).

fractional cell shortening, intracellular Ca^{2+} homeostasis, and three types of outward K^+ channel activities are similar between both control genders. Induction of diabetes significantly depressed myocyte contraction and relaxation, systolic intracellular Ca^{2+} transient, and outward K^+ channel activities in both gender rats. The extent of the depression in all the parameters tested was comparable between male and female diabetic counterparts. Our results are consistent with the clinical observation that female advantage in other cardiovascular complications was canceled out in the diabetes-induced cardiac dysfunction.

Gender-related differences in myocardial contraction have been reported, but remain controversial. Schaible and Scheuer *(21)* reported that the isolated working heart performance is moderately greater in male rats than female rats. Capasso et al. *(22)* reported that isolated papillary muscles from female rats had greater contractile performance than those from male rats. A recent study by Brown et al. *(23)* demonstrated a similar contractile performance in isolated papillary muscles obtained from both male and female rat hearts. Leblanc et al. *(24)* demonstrated that the contractile profile of isolated papillary muscles was similar to the age-matched male counterpart in young female rats (2–4 mo of age), but was weaker in female rats older than 6 mo. On the other hand, there were no age- or gender-dependent differences in action potentials, outward rectifier K^+ currents, and L-type Ca^{2+} currents *(24)*. Results from our present study are consistent with those of Brown et al. *(23)* and Leblanc et al. *(24)*. All the rats in our experiments were sacrificed at 4 mo of age. Although female control myocytes exhibited slower rates of cell shortening and re-lengthening and prolonged time to re-lengthening, the fractional cell shorten-

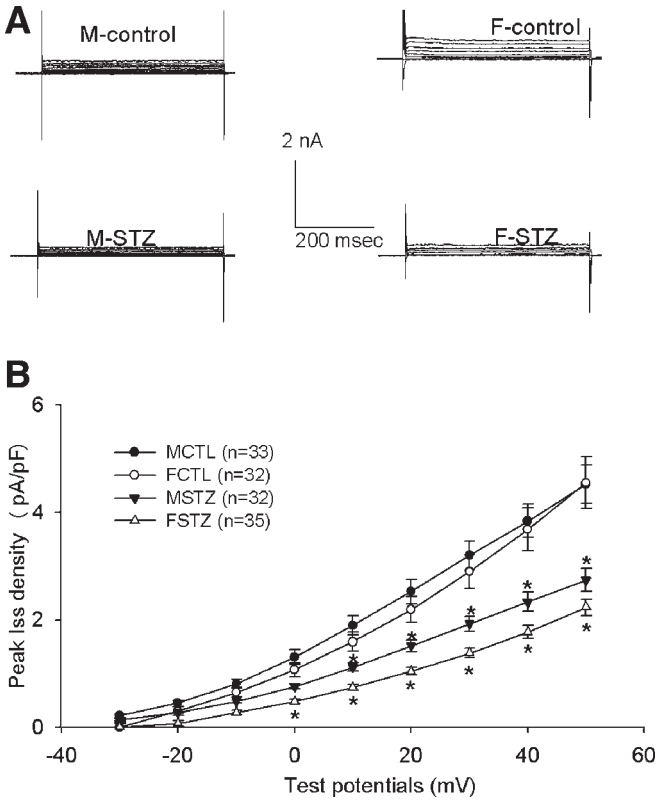

Fig. 4. Effects of gender on the sustained potassium current (Iss) in control and diabetic myocytes. (**A**) Representative traces of Iss recorded in the ventricular myocytes from each groups of animals at different test potentials. (**B**) Averaged Iss current–voltage relationships in myocytes from different groups of animals. MCTL: male control; MSTZ: male diabetic; FCTL: female control; FSTZ: female diabetic. Currents were recorded 3 min after whole-cell voltage clamp configuration. Resting membrane potential was held at –20 mV to inactivate both Ito and Ik. Currents were elicited by the test potentials ranging from –30 to +50 mV. Test potentials were changed with 10 mV increment at 6-s intervals. Each value represents means ± SE, and *n* represents number of myocytes tested. Three to six cells from each animal were tested. *Significantly different from the same gender control ($p < 0.05$).

ing was similar to that in male control counterpart. In addition, the resting and peak systolic $[Ca^{2+}]_i$ and outward rectifier K^+ currents in female control myocytes were similar to that in age-matched male control myocytes. These data suggest that although there may be a gender difference in cardiac contractility when animals reached older age, myocardial contractile profiles are comparable between both genders at younger age.

Clinical evidence has shown a female advantage in cardiovascular protection with a lower incidence and better survival rate of heart failure *(25–27)*. This lower risk of cardiovascular diseases in women has been linked to the female sex hormone estrogen and its actions on the vascular system and heart itself *(15,27–31)*. However, this female advantage in the cardiovascular protection seems to disappear during onset of diabetes. Premenopausal diabetic women display a comparable incidence of cardiovascular complications compared to age-matched male patients *(32)*. Among young diabetic patients, girls exhibit more pronounced signs of diabetic cardiomyopathy *(17)*. However, only limited information is available for the gender-specific difference in the diabetic cardiomyopathy, as very few studies of diabetic cardiomyopathy included female subjects. Brown et al. *(23)* demonstrated that the duration and maximal velocity of contraction and relaxation (TPT/RT90, and ±VT) of left ventricular papillary muscles were similar between normal male and female rats. Streptozotocin-induced diabetes prolonged TPT and RT90 in both genders, and reduced ±VT in males but not females. In addition, diabetes-induced vascular contractile dysfunction was comparable between both genders *(23)*. Using a calmodulin-overexpression-induced diabetic transgenic mice model, Ren and his colleagues reported that ventricular myocytes obtained from both gender diabetic mice exhibited an impaired contractility *(33,34)*. However, a female advantage may still exist as the younger female diabetic mice had better preserved mechanical function *(33,34)*. Results from our present study showed that ventricular myocyte contraction and intracellular Ca^{2+} homeostasis was severely impaired in both male and female rats. Importantly, the degree of myocyte contractile dysfunction induced by diabetes was comparable between male and female. In addition, there is no significant difference in the depressed systolic Ca^{2+} transient as well as the rates of Ca^{2+} transient rise and fall between the myocytes from both gender diabetic rats. The rats used in our experiments are at premenopausal age (approx 4 mo). Thus, our data strongly suggest that the "female advantage" in the cardiovascular protection is significantly dampened with diabetes even at young age. A possible explanation for the discrepancy between our results and Ren *(33,34)* may be due to the difference of diabetic models and animal species.

Abnormal electrophysiological properties are another hallmark of diabetes-induced cardiac complications *(14)*. Experimental studies have shown that the depressed outward K^+ channel activity was responsible for the prolonged action potential duration in ventricular myocytes obtained from diabetic subjects *(11–14)*. Our present study was focused on the gender influence on the diabetes-induced cardiac complications. Our results demonstrated that normalized current densities of Ito, Ik, and Iss were similar in myocytes from age-matched male and female control rats. This is consistent with the report from Leblanc et al. *(24)*. Our results also show that diabetes significantly depressed all three types of outward K^+ currents in ventricular myocytes, and the extent of diabetes-induced K^+ current reductions is comparable between both genders. Our data are somewhat different from that of Shimoni and Liu *(35,36)*. In their studies Shimoni and Liu observed that both the transient (Ipeak) and the sustained (Isus) outward potassium currents were significantly attenuated in myocytes obtained from female control rats when compared to male counterpart.

Diabetes significantly depressed both Ipeak and Isus in male myocytes. Only Isus was reduced in female diabetic myocytes, but the extent of Isus reduction was smaller than males. Interestingly, the values of Ipeak and Isus were not significantly different between myocytes obtained from both male and female diabetic rats. In addition, Ipeak in female nondiabetic myocytes was comparable to those in myocytes from both gender diabetic rats. Thus, the outward K^+ channel activity in female control myocytes is similar to that in male ventricular myocytes elicited by diabetes *(35,37)*. One possible explanation for this discrepancy is the duration of onset of diabetes. In our study the animals were sacrificed 8 wk after STZ-injection, while in their study myocytes were obtained 1–2 wk post-STZ-injection.

In summary, results from our present study suggest that both male and female are equally accessible to diabetes-induced cardiac dysfunction. Our results are consistent with the clinical observation that female advantage in other cardiovascular complications was canceled out in the diabetes-induced cardiac dysfunction.

Material and Methods

Animal Treatment

Male and female Wistar rats (8 wk old) were injected intravenously with a single dose of freshly prepared streptozotocin (STZ, 50 mg/kg; 0.05 *M* citrate buffer; pH 4.5) or the same volume of citrate buffer only (control) via the tail vein. The blood glucose concentration was determined using an Accu-Chek glucometer (Roche, Indianapolis, IN) at 72 h and 8-wk post-injection. Body weight was recorded daily. STZ and all the chemicals were purchased from Sigma (St. Louis, MO). All animal handling procedures were approved by Auburn University Institutional Animal Care and Use Committee (AU-IACUC).

Ventricular Myocyte Isolation

Viable left ventricular myocytes were isolated from rats using previously described methods with minor modifications *(18)*. Briefly, rats were killed by decapitation under deep pentobarbital sodium anesthesia. Heart was rapidly removed by thoracotomy and perfused via an aortic cannula in a retrograde fashion with oxygenated Ca^{2+}-free Tyrode's solution for 5 min, followed with a Tyrode's solution containing 0.4 mg/mL collagenase (Worthington; Type II, 371 U/mg) and 30 μ*M* $CaCl_2$ for 8–9 min. Left ventricle was then isolated and mechanically dispersed in Kraftbrühe (KB) solution. Isolated myocytes were centrifuged and resuspended in KB solution with increasing millimolar concentrations of Ca^{2+} to yield Ca^{2+}-tolerant cells. Myocytes were kept in KB solution with 1.0 m*M* Ca^{2+} for 1 h at room temperature before the experiment and used within 8 h.

Tyrode's solution was composed of (in mmol/L): 137 NaCl, 5.4 KCl, 4.4 $NaHCO_3$, 1.5 KH_2PO_4, 1.0 $MgCl_2$, 1.8 $CaCl_2$, 10 HEPES, and 10 glucose (pH 7.4). KB solution was composed of (in mmol/L): 25 KCl, 70 L-glutamic acid, 1.0 $MgCl_2$, 10 KH_2PO_4, 1.0 EGTA, 10 HEPES, 10 glucose, 10 taurine, 10 DL-β-hydroxybutyric acid, and 1 mg/mL albumin (pH 7.4).

Measurement of Myocyte Contraction and Intracellular Ca^{2+} Concentration

Cardiac myocyte contraction and intracellular Ca^{2+} transient ($[Ca^{2+}]_i$) were recorded simultaneously *(18)* using fura-2 fluorescence and edge detection system (IonOptix, MA). Briefly, myocytes were loaded with fura-2/AM (2.5 μ*M*) at room temperature (approx 22°C) for 20 min, washed twice with Tyrode's solution, and then stored in the Tyrode's solution for 30 min before use. Fura-2 loaded myocytes were placed in a cell perfusion chamber mounted on an inverted microscope (Nikon TE 2000, Japan) and perfused with normal Tyrode's solution by gravity (approx 2 mL/min) at room temperature. Myocyte contraction was elicited by field stimulation with two platinum electrodes on both sides of the cell perfusion chamber. Myocyte contraction was determined by measuring the changes in sarcomere length and the rates of sarcomere shortening and re-lengthening. Intracellular fura-2 fluorescence was excited with a collimated light beam from a 150-W xenon arc lamp. An adjustable rectangular diaphragm restricted recording to one cell in the field of view. The emitted fluorescence was passed to a spectrophotometer and converted to voltage. The signals were then digitized via an analog-to-digital converter for data storage and subsequent analysis.

Measurement of Outward K^+ Currents

Outward rectified K^+ currents were recorded in ventricular myocytes using the whole-cell patch-clamp technique. Patch pipets were prepared from borosilicate glass with a PE-830 pipet puller (Japan) and polished with a MF-830 microforge (Japan). The pipet resistance was 2–4 MΩ when filled with the appropriate pipet solution. After the whole-cell configuration, capacity transients and series resistance were measured by a 20-mV hyperpolarizing potential and partially compensated. A period of 3–5 min following break-in was utilized to attain cell dialysis before recording. Membrane currents of myocytes were measured via an Axopatch 200B patch-clamp amplifier (Axon, CA). Voltage clamp protocols were applied to the cells using the data acquisition package pClamp 9 (Axon, CA) and filtered at 5 kHz. All the experiments were performed at room temperature (25 ± 1°C).

Ito was recorded in the presence of external tetra-ethyl-ammonium chloride (TEA-Cl, 50 m*M*). The resting membrane potential was held at −60 mV. Current was elicited by test pulses between −30 and +50 mV applied in 10 mV increments at a frequency of 0.1 Hz. Values were corrected by subtracting the TEA-resistant Iss.

Ik was recorded in the presence of 4-aminopyridine (4-AP, 5 m*M*). The holding potential was set at −120 mV. A pre-

pulse to –40 mV (15 ms) was applied to inactivate I_{Na}, followed by test pulses between –30 and +50 mV (10 mV increment) at a frequency of 0.05 Hz.

Iss was recorded in the absence of TEA and 4-AP. The holding potential was set at –20 mV to inactivate I_{Na}, Ito, and Ik. The test pulses between –30 to +50 mV were applied at a frequency of 0.1 Hz.

The bath solution for K^+ current recordings was composed of (in mmol/L): 137 NaCl, 4 KCl, 1 $MgCl_2$, 10 HEPES, 0.5 $CaCl_2$, 10 glucose, pH adjusted to 7.4 with NaOH. When 4-AP is added to the solution, pH is readjusted to 7.4 with HCl. When TEA-Cl was added to the solution, NaCl was equimolarly substituted by TEA-Cl. The pipet solution was (in mmol/L): 80 L-aspartic acid, 50 KCl, 10 KH_2PO_4, 1 $MgSO_4$, 5 HEPES, 3 ATP_{Na}, 10 EGTA, pH adjusted to 7.4 with KOH.

Statistical Analysis

Experiments were performed on five to seven myocytes from the same animal for each protocol and data averaged to represent that animal. Values were reported as mean ± SE and *n* as the number of animals studied except noted in the figure legend. Data from different groups of cells were compared using two-tailed unpaired and paired Student's *t* test, and two-way ANOVA with a Student–Newman–Kuels post test, whenever appropriate. p value < 0.05 was considered significantly different.

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