Defective calcium inactivation causes long QT in obese insulin-resistant rat

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OBESITY IS DIRECTLY LINKED to type 2 diabetes, which has become an increasingly public health problem. In 2004, heart disease was noted on 65–68% of diabetes-related death among people aged 65 years and older (statistics from American Diabetes Association and American Heart Association). Obesity is also a significant contributor to insulin resistance, which is the main feature that occurs early and contributes to the development of diabetes. Cardiac ventricular dysfunction, concomitant with ventricular hypertrophy, is manifested in obese diabetics. Hypertrophied cardiomyocytes are prone to arrhythmias. It may be not coincidental that the major heart diseases associated with diabetic patients are cardiac arrhythmias (26, 39), leading to ventricular dysfunction. Abnormal diastolic left ventricular function in the diabetic heart has been recognized for many years without understanding of the molecular mechanism (22). Recently, evidence for impaired calcium homeostasis in the diabetic heart provides an intriguing mechanism that can directly cause ventricular dysfunction (23).

Ca2+ entry is mediated by L-type calcium channels (LTCC) in the ventricular myocytes. In an insulin-deficient mouse model, L-type calcium current (ICaL) has been shown to be reduced in the ventricular myocytes (25). The suppressed phosphatidylinositol 3-kinase (PI3K) signaling was proposed to mediate the reduced cell surface expression of LTCC, providing an explanation for the weakened cardiac contractility in this mouse model (25). Additionally, the expression levels of key calcium-handling proteins have been shown to be either reduced (sodium/calcium exchanger, ryanodine receptor, sarco/endoplasmic reticulum Ca2+-ATPase) or increased (phospholamban) (23). In contrast, there is a paucity of research in cardiac electrophysiology remodeling under insulin-resistant conditions.

Obesity creates a condition for a worsening state, diabetes. One major feature in obesity is the insulin resistance that must result from the defective insulin signaling. Insulin resistance is a multifaceted syndrome that causes not only an impaired glucose regulation but also a number of other diseases at different organs, including dysfunctional cardiac ventricular contraction. Using 16- to 17-week-old obese Zucker rats (OZR) as an animal model, the purpose of this work was to begin understanding of cardiac arrhythmogenesis directly linked to the sudden cardiac arrest in obese diabetic patients.

MATERIALS AND METHODS

Electrocardiogram recording. Isoflurane (2%) mixed with oxygen at the flow rate of 2 l/min inhaled by OZR and lean Zucker rats (LZR) was used for anesthetization in accordance with Animal Care and Use Committee guidelines for the measurement of electrocardiogram (ECG). ECG was recorded by using a two leads vector. Values for ECG measures are derived by 1-KHz sampling rate. All results are repeated eight times.

Cardiac tissue and myocyte preparation. LZR and OZR at 16–17 wk of age were killed by intraperitoneal injection of pentobarbital in accordance with the Institutional Animal Care and Use Committee protocols. The heart was excised, and ventricles were cut out and prepared for protein chemistry experiments. The ventricular myocytes were prepared using a collagenase dissociation protocol as described previously (19). Briefly, the aorta was cannulated, and the heart was perfused with oxygenated Ca2+-free Tyrode solution at 37°C for 5 min, followed by oxygenated Ca2+-free Tyrode solution containing
0.6 mg/ml collagenase II (Worthington) for 20–30 min. The ventricles were minced into small pieces in Krafe-Brühe (KB) solution, dispersed, and filtrated through a 200-µm mesh. The isolated myocytes were stored in KB solution at room temperature for 1 h before patch-clamp experiments. The animal protocols in this study were reviewed and approved by the Institutional Animal Care and Use Committee at West Virginia University and the National Institute for Occupational Safety and Health at Morgantown, WV.

Whole cell patch-clamp studies of action potential and ICaL. Whole cell patch-clamp studies were carried out for action potential and ICaL properties in ventricular myocytes isolated from OZR and LZR at the temperature of 35–37°C. The bath chamber temperature was controlled by a temperature controller (Cell MicroControls). Action potential was recorded at current-clamp mode with normal Tyrode and pipette solutions. The Tyrode solution contains (in mM): 143 NaCl, 5.4 KCl, 1.8 CaCl2, 0.5 MgCl2, 0.25 NaH2PO4, and 5 HEPES; pH was adjusted to 7.4 by NaOH. The pipette solution contains (in mM): 120 KCl, 1 CaCl2, 5 MgCl2, 5 Na2ATP, 11 EGTA, 10 HEPES, and 11 glucose; pH was adjusted to 7.3 by KOH. The current stimulus was 1 nA for 2–5 ms.

I CaL currents (Ba2+ were used as the charge carrier) and I Na currents (Ba2+ were used as the charge carrier) were recorded in the solutions similar to that of a previous report with no Na+ and K+ in both pipette and bath solutions (24). For ICaL recordings, the pipettes had a resistance of 2–5 MΩ when filled (in mM): 108 CsCl, 4 MgCl2, 9 EGTA, and 9 HEPES. The bath solution contained (in mM): 2 CaCl2, 1 MgCl2, 10 HEPEs, 40 TEA, 10 glucose, and 65 CsCl. For INaL recordings, 2 mM BaCl2 was used to replace CaCl2. Both pipette and bath solutions were adjusted by CsOH to pH 7.2. P/4 protocols were repeated for each group, whereas at least five myocytes were repeated in INaL recordings. The capacitive currents were minimized with 75–85% series resistance compensation.

Data were collected by Clampex (8.0) using an Axon 200B amplifier. Data analysis was performed using Clampfit (8.0). The statistical analysis was performed by Student’s t-test. The averaged data were presented as means ± SE.

Total and membrane protein preparation, Western blots, and immunoprecipitation. The ventricular tissue removed from OZR and LZR hearts was pulverized under liquid nitrogen and homogenized in buffer containing 20 mM Tris, 150 mM NaCl, 10 mM EGTA, and 10 mM EDTA (pH 7.4), supplemented with protease inhibitors. After centrifuge at 10,000 g (4°C) for 1 h, the supernatant was subjected to ultracentrifugation at 100,000 g (4°C) for 1 h. Membrane proteins were collected as pellets after ultracentrifugation and solubilized in homogenization buffer plus 1% digitonin for 30 min at 4°C. Protein concentration was determined using BCA or Bradford assay. For immunoprecipitation experiments, equal amounts of total protein (0.5–1 mg) were incubated with a specific antibody (2–4 µg) for 1 h at 4°C, and 30–50 µl protein A/G PLUS-agarose (Santa Cruz) was then added and incubated overnight with gentle rocking. The beads were washed extensively with cold PBS buffer and resuspended in 2× sample buffer. The immune complexes were separated by SDS-PAGE and analyzed by Western blot using the specific antibody of interest. The molecular weight of total protein (5–20 µg/sample) was subjected to SDS-PAGE using 4–12% gradient gels (Invitrogen) and analyzed by Western blot using the specific antibody of interest. The molecular weight of total protein (5–20 µg/sample) was subjected to SDS-PAGE using 4–12% gradient gels (Invitrogen) and analyzed by Western blot using the specific antibody of interest. The molecular weight of total protein (5–20 µg/sample) was subjected to SDS-PAGE using 4–12% gradient gels (Invitrogen) and analyzed by Western blot using the specific antibody of interest.

RESULTS

ECG and increased size of ventricular myocytes in OZR. Figure 1A shows the representative lead II ECG recordings from a 16-wk-old LZR and an age-matched LZR. The enlarged ECGs depicting the ECG characteristics are shown in Fig. 1A, right. Compared with LZR, the averaged corrected QT interval (QTc) is prolonged by 22% (LZR: 123 ± 3 ms, n = 8; OZR: 153 ± 9 ms, n = 8), shown in Fig. 1B. Additionally, the T wave of OZR is enhanced, and the descending limb of the T wave is increased. We used the QTc-Fridericia approach for QTc measurement, which has been widely used at fast heart rates and produces more consistent results (16).

To assess the potential hypertrophied heart that associates with the altered ECG, we studied cell capacitance (Cm) of isolated single ventricular myocytes. Cell capacitance directly reflects the cell volume. Figure 1C shows that the isolated ventricular myocytes were 37.9% larger in OZR than in LZR (CmLZR: 94.5 ± 4.3 pF, CmOZR: 130.7 ± 5.7 pF, P < 0.0001). Action potentials of LZR and OZR ventricular myocytes. Figure 2 shows the representative recordings of action potentials in ventricular myocytes isolated from 16-wk-old LZR (Fig. 2A) and OZR (Fig. 2B) as well as Sprague Dawley (SD) rat (Fig. 2C) hearts. LZR action potential is close to that of SD rat (43). OZR action potential, however, exhibited a remarkable plateau phase similar to those in canine (42) and human (18, 35) ventricles. On average of 10 myocytes for LZR and OZR, respectively, the duration of APD90 (measured at the 90% of action potential amplitude from the peak potential) is increased by over twofold in OZR compared with LZR (LZR: 91.5 ± 4.6 ms, n = 10; OZR: 199.8 ± 11.2 ms, n = 10) (Fig. 2D). ICaL in isolated ventricular myocytes. Prolonged QT interval results from either an increased inward ionic flow, a decreased outward current, or a combination of both. We focused on the LTCCs because of its primary role in Ca2+ influx. Figure 3A shows the representative currents of ICaL recorded at +10 mV in ventricular myocytes isolated from a 16-wk-old OZR compared with an age-matched LZR heart. ICaL inactivation was apparently disrupted in OZR compared with LZR. The ICaL currents from both LZR and OZR myocytes were confirmed by blockade of 10 µM verapamil. Because of the disrupted inactivation of ICaL, the Ca2+ influx at 0 mV, calculated from the area enclosed by activation and inactivation of ICaL, as well as the zero current line, was increased by 43 ± 12% in OZR compared with LZR (P = 0.002, n = 8) (Fig. 3B).

Figure 4 shows the representative currents illustrating the disrupted ICaL inactivation in OZR compared with LZR. At the potentials of −40 and −30 mV, ICaL has little inactivation in OZR (Fig. 4B). At −20 mV, the OZR ICaL does inactivate, but not as complete as LZR ICaL (Fig. 4A). The fraction of residual currents remaining at the end of 400 ms to the step (R400) was plotted against the test potentials and shown in Fig. 4C. Ca2+ dependence of ICaL inactivation is dramatically attenuated in OZR compared with LZR. The voltage-dependent inactivation kinetics were shown in Fig. 4D. The inactivation was best fit by a two-component exponential function (Fig. 4D). The kinetics
of the fast component of inactivation in OZR are not significantly different from LZR at negative voltages but significantly slower at positive voltages near the plateau of action potential compared with LZR. The kinetics of the slow component of inactivation, however, are much slower in OZR than in LZR.

Figure 5 shows that the current-voltage relationship of $I_{\text{CaL}}$ activation is unaltered in OZR (Fig. 5A). However, the $I_{\text{CaL}}$ current density was decreased in OZR myocytes compared with LZR (at $+10\,\text{mV}$, LZR: $10.2 \pm 0.4\,\text{pA/pF}$; OZR: $8.4 \pm 0.5\,\text{pA/pF}$; $P < 0.05$, $n = 8$ for each group). Figure 5B shows the voltage-dependent activation curves derived from the current-voltage relationships of Fig. 5A. Boltzmann fit revealed the midpoint voltage of activation of $-25.8 \pm 1.2\,\text{mV}$ ($n = 8$) for LZR and $-31.9 \pm 7.4\,\text{mV}$ ($n = 8$) for OZR. The difference is statistically significant ($P > 0.05$). Figure 5C shows that, if the altered $I_{\text{CaL}}$ results from the impaired insulin regulation, the LTCC and the insulin signaling molecules should be close beneath the plasma membrane. Figure 7 shows that the insulin receptor and the pore-forming subunit of LTCC (Cav1.2) are indeed associated with each other in the rat ventricle. Using an antibody against the β-subunit of insulin receptor (IR-β antibody, generously provided by Dr. Martin Myers from the University of Michigan), we first verified the presence of the insulin receptor expression in SD rat ventricles (Fig. 7A). We then precipitated the sample using a Cav1.2 antibody and detected insulin receptor signals using the IR-β antibody (Fig. 7B). If we precipitated the sample using the IR-β antibody, the Cav1.2 signals were also detected using the Cav1.2 antibody (Fig. 7C). The double bands of Cav1.2 are presumably due to the glycosylation of the channel proteins in the heart, and observed by others using the same Cav1.2 antibody (13). Absence of IR immunoblots after immunoprecipitation with a hyperpolarization-activated cation channel (HCN) 3 antibody to identify the insulin signaling molecules beneath the plasma membrane. Figure 7 shows that the insulin receptor and the pore-forming subunit of LTCC (Cav1.2) are indeed associated with each other in the rat ventricle. Using an antibody against the β-subunit of insulin receptor (IR-β antibody, generously provided by Dr. Martin Myers from the University of Michigan), we first verified the presence of the insulin receptor expression in SD rat ventricles (Fig. 7A). We then precipitated the sample using a Cav1.2 antibody and detected insulin receptor signals using the IR-β antibody (Fig. 7B). If we precipitated the sample using the IR-β antibody, the Cav1.2 signals were also detected using the Cav1.2 antibody (Fig. 7C). The double bands of Cav1.2 are presumably due to the glycosylation of the channel proteins in the heart, and observed by others using the same Cav1.2 antibody (13). Absence of IR immunoblots after immunoprecipitation with a hyperpolarization-activated cation channel (HCN) 3 antibody was used as a charge carrier, disrupted inactivation is not significantly altered ($V_{1/2}_{\text{LZR}}: -19.6 \pm 3\,\text{mV}$, $V_{1/2}_{\text{OZR}}: -17.1 \pm 5\,\text{mV}$, $n = 5$, $P > 0.05$).

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changed. Figure 8 shows that the association is weakened in OZR compared with LZR (Fig. 8B), although the expression levels of insulin receptors are unaltered in OZR (Fig. 8A). The similar results were repeated in an additional four pairs of rat hearts. The quantitative analysis revealed a 43% weaker IRβ signal in OZR compared with LZR (Fig. 8C). These results highly suggested an altered protein expression in the Cav1.2 complex in OZR ventricles. Multiple bands of the β-subunit of insulin receptor have also been observed by others (26).

Reduced expression of Cav1.2 and calmodulin in OZR. Figure 9 shows that indeed the total Cav1.2 protein expression was reduced in OZR ventricles (Fig. 9A). On average, the total Cav1.2 protein levels (260 ± 95 kDa) were decreased by 47 ± 14% compared with LZR (Fig. 9C). Negative and positive controls are shown in Fig. 9, B and D, respectively. The total membrane protein expression levels of Cav1.2 were also decreased in OZR compared with LZR, shown in Fig. 10A. Averaging over six repeated experiments, the OZR total membrane protein levels of Cav1.2 (260 ± 95 kDa) were reduced by 55 ± 7% compared with LZR (Fig. 10B). Furthermore, the ratio of signals at 260 kDa over that at 95 kDa was significantly increased in OZR (r_LZR: 0.45 ± 0.07, r_OZR: 0.82 ± 0.04, n = 6, P < 0.05) (Fig. 10C).

Calmodulin and the regulatory Cavβ2 subunit have been demonstrated to be key molecules that determine 1) inactivation (10), 2) cell surface expression (10), and 3) protein stability of Cav1.2 (4). Thus, we also examined the protein expression of these two molecules in OZR. Figure 11A shows the protein expression levels of Cavβ2 and calmodulin in OZR and LZR. On average of five hearts for each group, Cavβ2 protein expression levels were barely changed in OZR, but the calmodulin protein levels were significantly decreased in OZR (27 ± 8%, n = 5, P < 0.05) compared with LZR ventricles (Fig. 11B). Consistently, application of a specific calmodulin blocker, W-7 (15 µM), for 15–30 min significantly slowed inactivation and inhibited the current amplitude of LZR $I_{CaL}$ (Fig. 11C), which mimicked the abnormal $I_{CaL}$ in OZR (Fig. 3A).

DISCUSSION

In this study, we focused on the impaired gating properties of $I_{CaL}$ by the pathological state, collectively called metabolic syndrome, that is induced by obesity, in the 16- to 17-wk-old OZR (17). Enlarged myocytes (Fig. 1C) indicated a hypertrophied heart at this age, in response to increased arterial pressure and total peripheral resistance (29).

We detected a prolonged QTc interval in OZR compared with the age-matched LZR (Fig. 1). Not surprisingly, we found a significantly increased duration of action potential in OZR myocytes compared with LZR myocytes (Fig. 2). What is surprising is the apparent plateau phase that has never been observed in rat ventricles. The underlying ionic mechanisms of the plateau phase are complex and well understood (30). It is unlikely that the dramatic change in OZR action potential compared with LZR is caused by alteration of one channel or exchanger protein. Increased $I_{CaL}$ is one of the underlying ionic...
mechanisms for long QT (15); we thus focused on the potential alteration of \( I_{\text{CaL}} \) and its underlying mechanisms.

\( I_{\text{CaL}} \) in OZR ventricular myocyte. We found that \( I_{\text{CaL}} \) current density is reduced in OZR compared with LZR (Fig. 5A). On the other hand, the voltage dependence of \( I_{\text{CaL}} \) is not altered (Fig. 5A and B). More intriguingly, we found a disrupted \( I_{\text{CaL}} \) inactivation in OZR ventricular myocytes (Fig. 3A). \( I_{\text{CaL}} \) in OZR barely inactivates at negative potentials such as \(-30\) mV (Fig. 4B). The slow-component inactivation is much slower, probably reflecting a disrupted calcium-dependent inactivation.

Fig. 4. Disrupted \( I_{\text{CaL}} \) inactivation in OZR compared with LZR. Representative current traces recorded at indicated voltages in myocytes isolated from LZR (A) and OZR (B) ventricles. The depolarizing pulse duration was 400 ms. C: voltage dependence of the fraction of remaining current at the end of 400 ms into the step (\( R_{400} \)) for LZR and OZR. D: voltage-dependent inactivation kinetics for LZR and OZR, including the fast component (left) and the slow component (right).

\( I_{\text{CaL}} \) gating properties. A: current-voltage (I-V) relationship of \( I_{\text{CaL}} \) in OZR and LZR myocytes. B: voltage-dependent activation curves (\( G/G_{\text{max}} \)), derived from I-V relationship curves (A). Conductance (\( G \)) was calculated from the equation, \( G = I/(V_m - V_{\text{rev}}) \), where \( I \) is the peak current of \( I_{\text{CaL}} \), \( V_m \) is the test voltage, and \( V_{\text{rev}} \) is the reversal potential. The values of \( V_{\text{rev}} \) were obtained by extrapolating the I-V curves. \( V_{\text{CaL}} \) was \(-69 \pm 6\) mV for LZR (\( n = 8 \)) and \(-74 \pm 5\) mV for OZR (\( n = 8 \)), respectively. \( G_{\text{max}} \) is the maximal conductance. C: steady-state inactivation curves of \( I_{\text{CaL}} \) were obtained using a two-pulse protocol. The conditioning pulses of 500 ms were from \(-70\) to \(+20\) mV in 10-mV increments. After a brief pulse of 5 ms, the test pulse was applied to 10 mV for 300 ms. The holding potential was \(-70\) mV. The data were best fit by a Boltzmann equation to obtain \( V_{1/2} \) for the steady-state inactivation curve.

Fig. 5. \( I_{\text{CaL}} \) gating properties. A: current-voltage (I-V) relationship of \( I_{\text{CaL}} \) in OZR and LZR myocytes. B: voltage-dependent activation curves (\( G/G_{\text{max}} \)), derived from I-V relationship curves (A). Conductance (\( G \)) was calculated from the equation, \( G = I/(V_m - V_{\text{rev}}) \), where \( I \) is the peak current of \( I_{\text{CaL}} \), \( V_m \) is the test voltage, and \( V_{\text{rev}} \) is the reversal potential. The values of \( V_{\text{rev}} \) were obtained by extrapolating the I-V curves. \( V_{\text{CaL}} \) was \(-69 \pm 6\) mV for LZR (\( n = 8 \)) and \(-74 \pm 5\) mV for OZR (\( n = 8 \)), respectively. \( G_{\text{max}} \) is the maximal conductance. C: steady-state inactivation curves of \( I_{\text{CaL}} \) were obtained using a two-pulse protocol. The conditioning pulses of 500 ms were from \(-70\) to \(+20\) mV in 10-mV increments. After a brief pulse of 5 ms, the test pulse was applied to 10 mV for 300 ms. The holding potential was \(-70\) mV. The data were best fit by a Boltzmann equation to obtain \( V_{1/2} \) for the steady-state inactivation curve.
in OZR (Fig. 4D), whereas the fast-component inactivation was slower in OZR at the potentials near the action potential plateau phase, probably indicating the decreased voltage-dependent inactivation in OZR (Fig. 4D) (11). Lack of efficient inactivation caused a larger Ca²⁺ influx (Fig. 3B), consistent with a much less voltage-dependent change in $R_{400}$ (Fig. 4C). A larger Ca²⁺ influx, especially at potentials near 0 mV, can contribute directly to prolonged duration of action potential (Fig. 2D) and the QTc interval in OZR (Fig. 1B).

The voltage-dependent inactivation of LTCC was also changed (Fig. 6). In addition to the shifted current-voltage relationship for OZR, $I_{\text{Cat}}$ also exhibited a depolarized shift of activation, consistent with the shift in the current-voltage relationship. These altered properties were not observed for $I_{\text{Cat}}$ in OZR. However, like $I_{\text{Cat}}$, the steady-state inactivation of $I_{\text{Cat}}$ was not altered in OZR compared with LZR, indicating the differential voltage- and calcium-dependent regulation in LTCC. The altered LTCC voltage- and calcium-dependent properties can result from a disruptive modulation of the channel expressed at the plasma membrane, an altered protein expression of the channel complex, or both.

**Protein expression of Cav1.2, Cavβ2, and calmodulin.** Cardiac LTCC is constituted of the pore-forming subunit, Cav1.2 (α₁C), β subunit (mainly β₂), and α₂δ (5, 36). Cav1.2 has four repeats of the six-transmembrane domains with its NH₂- and COOH-termini located inside the cell. The α-interaction domain in the I-II linker is associated with the β-subunit. A disulfide bond links two extracellular α-subunits and the δ-subunit that has one transmembrane domain. Both β- and α₂δ-subunits enhance the membrane trafficking of Cav1.2 and $I_{\text{Cat}}$ current density and moderate $I_{\text{Cat}}$ inactivation (1, 8, 10). In the COOH-terminal region, critical amino acid residues have been identified for association with calmodulin to form the molecul-

Fig. 6. L-type Ba²⁺ current ($I_{\text{Hlat}}$) gating properties. A: $I_{\text{Hlat}}$ traces in response to a depolarizing pulse of 450 ms to +10 mV from the holding potential of −70 mV in a LZR (black) and in an OZR (gray) myocyte, respectively. Both currents were blocked by 10 μM verapamil (broken line). B: $I-V$ relationship curves of $I_{\text{Hlat}}$ for LZR (black) and OZR (gray). C: activation curves of $I_{\text{Hlat}}$ for LZR (black) and OZR (gray). D: steady-state inactivation (SSINA) curves of $I_{\text{Hlat}}$ for LZR (black) and OZR (gray). The results were averaged from 5 myocytes for each group.

Fig. 7. Association of insulin receptor (IR) with α₁C in SD rat ventricles (RV). A: IR expression in SD heart. IB, immunoblot. B: IR β-subunit signals were readily detected in three separate samples with the IR β-subunit antibody (Ab) after the samples were first immunoprecipitated using an antibody against α₁C. HCN, hyperpolarization-activated cation channel. C: α₁C signals were detected in RV samples with the α₁C antibody (Ab) after the samples were first immunoprecipitated using the IR β-subunit antibody. The amount of IR β-subunit was increased 5-fold (lane 3) and 10-fold (lane 4) compared with that in lane 2 to confirm the appearance of α₁C signals.
ular inactivation machinery, including at least the EF hand, peptide A, C, and the IQ motif (3, 6, 27).

A significant decrease in Cav1.2 protein expression in OZR compared with LZR (Figs. 9A and 10A) is consistent with the reduced $I_{Ca,L}$ current density (Fig. 5A). The antibody we used can detect two bands of intact Cav1.2 protein near its calculated molecular weight of 260 kDa (Figs. 9A and 10A). They represent possibly the mature form (posttranslational modification) and immature form (no posttranslational modification) (Alomone). Because of the high proteolytic activity in cardiac (and skeletal) muscle, strong bands at low molecular weight near 95 kDa were detected (Figs. 9A and 10A). It is noticed that Cav1.2 in both total protein and membrane protein levels was significantly decreased in OZR compared with LZR. It is also found that Cav1.2 has less degradation in OZR than in LZR ventricles as indicated by weaker bands at 95 kDa (Figs. 9A and 10A). It is worth emphasizing that not all channels expressed at the plasma membrane function at the same time. While the functional channels are better characterized by patch-clamp studies, membrane protein expression provides an overall structural basis for the altered function of LTCC induced by disease.

We have found no changes in protein expression levels of CavH9252 in OZR compared with LZR. Currently, it is unknown whether the phosphorylation state of CavH9252 is altered in OZR compared with LZR, since the protein kinase B (Akt)-depender-
dent phosphorylation of Cavβ2 increases the protein stability of Cav1.2 (4). The double band of Cavβ2 (Fig. 11A) may be explained by five isoforms in the Cavβ2 family resulting from alternative splicing (12). The difference of these isoforms is located in the NH2-terminus. The predicted molecular weights of these isoforms vary from each other slightly. Therefore, the double band is likely the result of detecting more than one isoform of Cavβ2 (7).

Intriguingly, the levels of calmodulin protein expression are significantly decreased in OZR (Fig. 11A). A decreased calmodulin protein expression explains the impaired voltage- and calcium-dependent inactivations when expressed in Chinese hamster ovary cells and in Xenopus oocytes, respectively. The Ca2+

Fig. 10. Cav1.2 membrane protein expression. A: membrane protein expression of Cav1.2 in OZR and LZR ventricles. Three repeated data are shown. The α-actin bands (bottom) were used as loading controls. M, marker. B: average changes in total membrane protein levels of Cav1.2 in OZR (gray) compared with LZR (dark), n = 6. The expression levels were normalized to LZR protein level. C: average changes in ratio of Cav1.2 signals at 260 kDa (intact) over that at 95 kDa (proteolyzed) in OZR (gray bar) and LZR (dark bar). *Statistical significance (P < 0.05) compared with LZR.

Association of insulin receptor and Cav1.2. The common underlying mechanism in type 1 and type 2 diabetes is the impaired insulin signaling, caused by either insufficient release of insulin (type 1) or reduced insulin sensitivity to insulin receptor signaling (type 2). Although OZR is frequently used as an animal model of metabolic syndrome, it is also a good model for studying cardiac insulin resistance (28, 44). As an early event, insulin resistance occurs as early as 7 wk in OZR (9). Even in the late state of metabolic syndrome, insulin resistance remains as a main feature.

Binding of insulin to its receptor activates the receptor’s tyrosine kinase activity, leading to the activation of insulin receptor substrate, which, in turn, activates the PI3K/Akt pathway (2). Our coimmunoprecipitation experiments have shown that the insulin receptor is associated with Cav1.2 in the ventricle, and this association is weakened in the OZR compared with LZR (Fig. 8B). Because the levels of insulin receptor are unaltered (Fig. 8A), the weakened association is likely due to a decreased level of total and membrane protein expression of Cav1.2 (Figs. 9A and 10).

Massive effort has been invested in the pathophysiology of diabetes caused by obesity. In the meantime, there is a paucity of research for the altered cardiac electrophysiology that occurs as an early marker associated with insulin resistance. Given the recent realization for a significantly higher risk of sudden cardiac arrest in diabetes, it is essential to understand the underlying mechanisms that predispose the heart to cardiac arrhythmias in obese patients. One of the early and most consistent observations in the heart of diabetics is the prolongation of QTc interval (23). Disrupted ICaL inactivation may cause a long QTc interval, a precursor for sudden cardiac arrest that occurred in patients with type 1 and type 2 diabetes (14, 21), under obese conditions. Prolongation of QTc interval has also been demonstrated as an independent risk factor in type 1 and type 2 diabetes (34, 37, 38), as well as a significant predictor of cardiac death (31, 34). Our work has provided one ionic mechanism: defective ICaL inactivation contributed to the prolonged QTc interval in the OZR heart.

Defective inactivation of ICaL was also found to cause prolonged QT (LQTS) in Timothy Syndrome (33). A mutation was identified at residue 406 with glycine replaced by arginine, creating a missense mutant, G406R, located in the intracellular loop linking domain I and domain II of Cav1.2. G406R exhibited a significant decrease in both calcium- and voltage-dependent inactivations when expressed in Chinese hamster ovary cells and in Xenopus oocytes, respectively. The Ca2+

Fig. 11. Protein expression of Cavβ2 and calmodulin (CaM) in OZR and LZR ventricles. Three repeated data are shown in A. The middle lane in OZR was ignored due to the distorted signal. The α-actin bands were used as loading controls. B: average total protein levels of Cavβ2 and CaM in LZR (dark bar) and OZR (gray bar). Signals were normalized to α-actin. *Statistical significance (P < 0.05) compared with LZR. C: ICaL at +10 mV in a LZR myocyte (dark line) and the effects of W-7 (gray line). LZR ICaL was verified by 10 μM verapamil (broken line). The holding potential = −50 mV.
current-voltage relationship and the voltage-dependent activation are not significantly altered. Computer simulation predicted a 17% prolongation of ventricular action potential duration, which causes the prolonged QT leading to the lethal arrhythmia, the ultimate trigger of death in this disorder (33). Compared with the wild-type I_CaL, the alterations induced by G406R are similar to our results, but there are apparent differences. The defective inactivation of Ca^{2+} currents, unchanged current-voltage relationship, and voltage-dependent activation are similar to ours (Figs. 3–5). However, we found that the action potential duration is nearly doubled in OZR myocytes (Fig. 2), suggesting altered ionic mechanisms in addition to I_CaL. Furthermore, the underlying mechanisms for the defective inactivation of I_CaL may be different. It is unknown whether mutation in Cav1.2 may occur in OZR. It is known, however, that OZR at 16 wk old is obese and fully developed insulin resistance (17). Our findings of the weakened association of insulin receptor with Cav1.2 and the reduced protein expression of calmodulin provided novel mechanisms for defective inactivation of I_CaL. Mechanistic understanding of altered cardiac electrophysiology associated with insulin resistance is also important for potential therapeutic treatment of heart disease in diabetes patients that are overweight. For instance, a cyclin-dependent kinase inhibitor, roscovitine, has recently been found to enhance the voltage-dependent inactivation of Cav1.2 (40), which may be useful to correct the disrupted I_CaL inactivation.

While the present work provided mechanistic rationale for some calcium channel blocker to limit the excessive Ca^{2+} influx in the prevention of cardiac remodeling in type 2 diabetes (20), our results also raised many intriguing questions. First, does insulin modulate I_CaL gating directly through association of insulin receptor and Cav1.2? Second, are reduced calmodulin protein levels (which can explain the disrupted I_CaL inactivation) caused by insulin resistance? Third, will Cavβ2 phosphorylation be affected by impaired insulin signaling? Fourth, is the protein expression of δ-subunit (another integrated regulatory subunit of Cav1.2) altered in OZR? Fifth, is the downstream signaling pathway such as PI3K/Akt involved in the disruptive regulation of I_CaL gating in the pathological conditions of OZR? Answers to these questions will provide us with mechanistic insights into cardiac arrhythmias associated with diabetes patients that are obese, which should have a significant impact in the prevention of sudden cardiac arrest in the increasing diabetes population.

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DISCLOSURES

The authors declare that they have no conflict of interest. The findings and conclusions in this report are those of the authors and do not necessary represent the views of the National Institute for Occupational Safety and Health.


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