Restoring depressed HERG K⁺ channel function as a mechanism for insulin treatment of abnormal QT prolongation and associated arrhythmias in diabetic rabbits

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diabetes; cardiovascular disease; insulin-dependent diabetes mellitus; action potential duration

DIABETIC MELLITUS (DM) is one of the most prevalent chronic conditions associated with significant morbidity and mortality from cardiovascular diseases. An impaired cardiac function independent of vascular and other diseases suggests a primary myocardial defect in DM. Diabetic cardiomyopathy is characterized by electrical remodeling, metabolic remodeling with malignant biochemical processes, and anatomic remodeling with progressive loss of cardiomyocytes, which result in impaired cardiac contractility and increased risk of lethal arrhythmias. The abnormal prolongation of the QT interval is the most prominent electrical remodeling in diabetic hearts; clinically, its prevalence is as high as ~25% in DM patients, including type 1 insulin-dependent (IDDM) and type 2 non-IDDM populations (6, 19, 27). QT prolongation is a significant predictor of mortality in IDDM and non-IDDM (4, 27), because it is associated with an increased risk of sudden cardiac death in DM patients consequent to lethal ventricular arrhythmias known as torsades de pointes. Correction of QT prolongation, therefore, is an important step toward reducing cardiac death of DM patients. To achieve this goal, sufficient knowledge about the ionic mechanisms underlying diabetic QT potential is essential.

We recently identified depression of multiple ion currents in diabetic rabbits (40), including transient outward K⁺ current (I_o), L-type Ca⁺² current (I_CaL), rapid delayed rectifier K⁺ current (I_Kr), and slow delayed rectifier K⁺ current (I_Ks). Our data on I_o and I_CaL are consistent with the results from earlier studies carried out in rats and mice (10, 15, 21–23, 26, 28). However, our finding that I_Kr is the major ionic determinant for, whereas other ion currents play a minor role in, diabetic QT prolongation has significant implications for therapeutic interventions (40). Moreover, our previous studies revealed that human ether-a-go-go-related gene (HERG), the pore-forming α-subunit of the native I_Kr, is negatively modulated by hyperglycemia, tumor necrosis factor-α (TNF-α), ceramide, and reactive oxygen species (ROS) (29–31, 38), the cellular metabolites accumulating in diabetic tissues. Also pertinent to I_Kr in the diabetic QT prolongation is our finding that basal activities of PKB (or Akt), a downstream mediator of the insulin signaling pathway, are crucial for maintaining the normal function of HERG K⁺ channels (39). Our understanding of diabetic QT prolongation, although still incomplete, should be adequate to allow us to develop rational therapeutic approaches. The present study was designed to shed light on this issue, evaluating the potential of I_Kr/HERG as a therapeutic target and the efficacy of insulin in protecting I_Kr/HERG function and, thereby, preventing action potential (AP) duration (APD)/QT prolongation in the rabbit model of IDDM.

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**METHODS**

Preparation of a rabbit model of IDDM. Male New Zealand White rabbits (1.6–2.0 kg body wt; Charles River Canada) were housed individually in stainless steel wire-bottomed cages in a room with a 12:12-h light-dark cycle with standard laboratory rabbit chow and drinking water ad libitum. The animals were randomly assigned to groups as follows: control, IDDM, and IDDM with insulin treatment (IDDM/INS). To establish diabetes, a single injection of prewarmed (37°C) alloxan monohydrate (140 mg/kg body wt; Sigma-Aldrich), freshly dissolved in saline at a concentration of 100 mg/ml, was administered via marginal ear vein under local anesthesia. To prevent fatal hypoglycemia from massive insulin release, 10% glucose solution (100 mg/kg sc) was administered 4 and 6 h after alloxan treatment. After stable diabetes had been established for 3 days in the IDDM/INS group, the animals were treated with diluted insulin zinc (7–10 IU/kg sc, followed by 5–7 IU/kg every 2–3 days) to lower the plasma glucose level. The blood was collected via marginal ear vein after local anesthesia for determination of the plasma level of glucose with a glucometer (TheraSense), and the blood glucose level was monitored weekly thereafter until week 10. Only those animals with ≥15 mM serum glucose were considered diabetic and were used for further studies. The protocol for animal use was approved by the Animal Ethics Committee of the Montreal Heart Institute.

Implantation of telemeters and ECG recording in conscious rabbits. The rabbits were anesthetized with an injection (1.2 ml/3 kg im) of a mixture (7:1) of ketamine (Vetalar, BioNiche Animal Health Canada, Belleville, ON, Canada) and xylazine (Rompun, Bayer, Toronto, ON, Canada). Abdominal hair was shaved, and skin was cleaned and sterilized with antiseptic. A small incision was made on the skin for subcutaneous implantation of an ECG telemeter (EMKA Technologie, Paris, France), and the probes of the telemeter were fixed to the right and left underarm positions. Antibiotic cream (Polytopic, Sabex, Boucherville, QC, Canada) was applied to the closed skin wounds, which were covered with adherent surgical dressing. Bandages were used to protect the wounds. Antibiotic solution (0.5 ml; Longsil, Vetosquil) containing penicillin G benazine (150,000 IU/ml) and penicillin G procaine (150,000 IU/ml) was administered by intramuscular injection daily for 5 days after the surgery. At 7 days after implantation, the transducer was activated to record the real-time ECG as the basal measurement in conscious rabbits before induction of diabetes as the basal measurement. The ECG signal was acquired and analyzed by EMKA Technologie IOX acquisition software and ECG-Auto, respectively. ECG was monitored continuously for 24 h immediately after treatment with alloxan; 2 days later, ECG was recorded for 20 min at 3-h intervals. ECG recorded in this way is equivalent to the standard lead II ECG.

Surface ECG recording in anesthetized rabbits. Standard lead II ECG was recorded before and after diabetes was established in rabbits. Sedation and induction of anesthesia were accomplished with intramuscular injection of ketamine (65 mg/kg) and xylazine (13 mg/kg). Three-lead surface ECG was recorded with silver electrodes placed under the skin at optimized positions to obtain maximal-amplitude recordings, enabling accurate measurements of QT intervals. The QT measurements and simultaneously recorded R-R intervals were used to derive heart rate-corrected QT (QTc) intervals using Carlsson’s formula: QTc = QT - 0.175(RR)^1/2, where RR is R-R interval (s).

**Isolation of rabbit ventricular myocytes.** Myocytes were isolated from rabbit left ventricular endocardium of the apical region via enzymatic digestion. The rabbits were anesthetized with pentobarbital sodium (60 mg/kg iv). The hearts were rapidly excised and mounted on a Langendorff apparatus and perfused retrogradely with 1 mM Ca^2+ -Tyrode solution (2 min), Ca^2+ -free-Tyrode solution (3–5 min), and Ca^2+ -free-Tyrode solution containing collagenase (Worthington type II) in a sequential order for 25–35 min. The endocardial layer was shaved from the left ventricular wall, and the samples were minced in the storage solution and filtered. The freshly isolated myocytes were gently centrifuged and resuspended in the storage solution for patch-clamp studies. The solution for cell storage contained 20 mM KCl, 10 mM KH2PO4, 25 mM glucose, 70 mM potassium glutamate, 5 mM β-hydroxybutyric acid, 20 mM taurine, 10 EGTA, 40 mannitol, and 0.1% albumin (pH 7.4).

**HEK-293 cell culture.** HEK-293 cells stably expressing HERG were a kind gift from Drs. Zhou and January (41). Cell culture and handling procedures have been described previously (38, 39).

Whole cell patch-clamp recording. Patch-clamp techniques have been described in detail elsewhere (29, 32). Currents were recorded in the whole cell voltage-clamp mode, and APs were recorded in the current-clamp mode with an Axopatch 200B amplifier (Axon Instruments). Borosilicate glass electrodes had tip resistances of 1–3 MΩ when filled with the internal pipette solution. The pipette solution for K+ current recording contained (mM) 130 KCl, 1 MgCl2, 5 Mg2+-ATP, 10 EGTA, and 10 HEPES, with pH adjusted to 7.25 with KOH. The internal pipette solution for AP recording was the same as that for K+ current recording, except EGTA concentration was 0.05 mM. The normal Tyrode solution used as the extracellular superfusate for AP recordings (in ventricular myocytes and fat cells; HERG-expressing) recordings in HERG-expressing HEK-293 cells contained (mM) 136 NaCl, 5.4 KCl, 1 CaCl2, 1 MgCl2, 5 glucose, and 10 HEPES, with pH adjusted to 7.4 with NaOH. For Ikr recordings, the superfusate was changed to a N-methyl-D-glucamine solution composed of (in mM) 149 N-methyl-D-glucamine, 2 MgCl2, 1 CaCl2, and 5 HEPES, with pH adjusted to 7.4 with HCl. The Na+ current was inactivated by holding the membrane at −50 mV, and Icalc was blocked by 200 μM CdCl2 in the bathing solution. 4-Aminopyridine (1 mM) was used to inhibit Ito, and external glyburide (10 μM) + internal Mg2+-ATP (5 mM) was used to prevent ATP-sensitive K+ current. HMR-1556 (1 μM; Avanti Polar Lipid, Alabaster, AL) was used to block Iks. Experiments were conducted at 36 ± 1°C. Junction potentials, in the range −5.2 to −10.4 mV (−7.4 ± 1.1 mV, n = 35 cells), were zeroed before formation of the membrane-pipette seal and were not corrected for our data analyses. Series resistance and capacitance were compensated, and leak currents were subtracted.

Because our study was designed for group comparisons of the experimental results, all currents were recorded immediately after membrane rupture and series resistance compensation to minimize the possible time-dependent rundown, run-up, or negative shift of currents. Individual currents were normalized to the membrane capacity to control for differences in cell size and expressed as current density (pA/pF). Ikr was expressed as dofetilide-sensitive currents by subtraction of the currents recorded 10 min after administration of 1 μM dofilte from the baseline currents before dofetide administration. The amplitude of Ikr was measured from both step currents at various test potentials (the difference between the current level at the end of the pulse and zero level) and tail currents (the difference between the peak tail current and zero level) at a repolarizing potential of −40 mV.

Western blot. The membrane protein samples were extracted from rabbit ventricles for immunoblotting analysis of the HERG channel protein essentially as described in detail elsewhere (27). The protein content was determined with a protein assay kit (Bio-Rad, Mississauga, ON, Canada), with bovine serum albumin as the standard.

The membrane protein sample (~150 μg) was fractionated by SDS-PAGE (7.5–10% polyacrylamide gels) and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The sample was incubated overnight at 4°C with the primary antibodies diluted 1:50–1:200. Affinity-purified polyclonal primary antibodies against the COOH terminus of HERG raised in goat was used. HERG is used for rabbit ether-a-go-go (ERG) for simplicity, inasmuch as the rabbit ERG channel sequence (GenBank accession no. U87513) is 93% and 96% homologous to HERG at the nucleotide and amino acid levels, respectively (18, 35, 42). Inhibitory peptide for each antibody was used to test antibody specificity. On the next day, the membrane was washed three times (10 min each) in TBS + Tween 20 and incubated for 2 h with the horseradish peroxidase-conjugated donkey
anti-goat IgG (1:600 dilution) in the blocking buffer. Primary and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Bound antibodies were detected using the chemiluminescent substrate (Western Blot Chemiluminescence Reagent Plus, NEN Life Science Products, Boston, MA). GAPDH was used as an internal control for equal input of protein samples with use of anti-GAPDH antibody (RDI, Flanders, NJ). The membrane was also stained with Coomassie blue to verify the size of the sample. Western blot bands were quantified using QuantityOne software by measurement of the band intensity (area × optical density) for each group and normalization to GAPDH. The final results are expressed as fold changes by normalization of the data to the control values.

Pulse-chase metabolic labeling of HERG proteins. The method for pulse-chase metabolic labeling of HERG proteins was similar to that described by Akhavan et al. (2). HEK-293 cells were divided into three groups, control, hyperglycemia, and hyperglycemia/INS, and incubated with 5 mM unlabeled methionine/cysteine in the presence of 0.25% bovine serum albumin. Then the cells were metabolically labeled in 5 ml of methionine-free DMEM containing 10% FCS and 200 cells were incubated with 0.25% paraformaldehyde (pH 7.35 in PBS) at room temperature for 30 min, and then washed three times in PBS. Triton X-100 (1% in PBS) was used to permeabilize the cell membrane by incubation at room temperature for 5 min followed by blocking with 1% normal donkey serum for 1.5 h at room temperature. Goat polyclonal primary antibody against HERG (and rHERG; Santa Cruz Biotechnology) was diluted 1:50 in antibody dilution buffer (containing 1% normal donkey serum in PBS) and reacted with cells on coverslips at 4°C overnight. Specificity of the antibody was verified using antigenic blocking peptides. Alexa Fluor 594-conjugated donkey anti-goat IgG was used as secondary antibody (1:600 dilution). After the blocking procedure with 1% BSA, the cell membrane was stained with Alexa Fluor 488-conjugated wheat germ agglutinin (10 µg/ml; Molecular Probes) for 30 min. The coverslips were then mounted onto slides with antifading medium, and the sample was examined by confocal microscopy. The images were deconvolved to minimize the background noise.

Measurement of intracellular ROS. Detailed procedures for measurement of intracellular ROS have been described previously (29, 38). Briefly, 5-(and 6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (Molecular Probes) was used to detect oxidative activity in living cells, according to the manufacturer’s protocols. The cells were examined under a laser scanning confocal microscope (model LSM 510, Zeiss), with an excitation wavelength of 480 nm and an emission wavelength at 505–530 nm. The percentage of positively stained cells and the fluorescence intensity of staining were determined by densitometric scanning with LSM software (Zeiss).

Lipid peroxidation assay. Lipid peroxidation was measured using a lipid hydroperoxide (LPO) assay kit (Cayman Chemical, Ann Arbor, MI). A 0.2-g rabbit left ventricular preparation was homogenized in HPLC-grade H2O on ice, and LPO was immediately extracted from the sample into chloroform and assayed in a 96-well plate in triplicate according to the manufacturer’s protocols. The standard curve was generated with the materials provided with the kit using a microplate reader (Power Wave X340, Biotec Instrument) by measurement of the absorbance at 500 nm for calculation of LPO. LPO was expressed as hydroperoxide in nanomoles per gram heart tissue.

Protein oxidation assay. A protein carbonyl assay kit (Cayman Chemical) was used for quantification of protein oxidation in the samples extracted from the hearts of IDDM and age-matched healthy rabbits, according to the manufacturer’s protocols. Briefly, 0.2 g of ventricular tissue was homogenized, and total proteins were extracted. The reactions were carried out in a 96-well plate, and the absorbance was measured at a wavelength of 370 nm in duplicate using a microplate reader (Power Wave X340). The relative optical density values were calculated for comparison, and protein oxidation was expressed as protein carbonyl in nanomoles per milligram total protein.

Total endogenous antioxidant assay. Blood was collected from the marginal ear vein in citrate-containing tubes. Plasma was obtained by centrifugation of blood at 1,000 × g for 20 min at 4°C. Plasma total antioxidant status was measured using an antioxidant status assay kit (Cayman Chemical), according to the manufacturer’s instructions. The reactions were read at 750 nm in duplicate.

Data analysis. Group data are expressed as means ± SE. Statistical comparisons (performed using ANOVA followed by Dunnett’s method) were carried out using Microsoft Excel. A two-tailed P < 0.05 was taken to indicate a statistically significant difference. Nonlinear least-squares curve fitting was performed with CLAMPFIT 8.0 or GraphPad Prism.
RESULTS

Insulin corrects diabetic QT prolongation and suppresses ventricular arrhythmias. The average nonfasting blood glucose level measured 6 wk after alloxan treatment was elevated to 21.8 ± 1.6 mM \((n = 10)\) in the IDDM group and partially restored to 11.9 ± 1.5 mM \((n = 5)\) in the IDDM/INS group compared with 5.4 ± 0.7 mM \((n = 8)\) in the age-matched healthy animals. The QTc interval was markedly prolonged 6 wk after alloxan treatment \((187 ± 4\) ms, \(P < 0.05\) vs. control) in the IDDM group compared with age-matched healthy animals \((156 ± 2\) ms), and this prolongation was largely prevented by insulin administration; the QTc interval of the IDDM/INS group was shortened to 164 ± 7 ms \((P < 0.05\) vs. IDDM, \(n = 5\); Fig. 1A). Most profoundly, the spontaneous ventricular tachycardias, which occurred frequently in IDDM rabbits \((\text{total incidence} = 35)\), were nearly abolished by insulin \((\text{incidence} = 2\) in IDDM/INS group), consistent with correction of the QTc interval. Furthermore, in some IDDM animals, the ventricular tachycardia predisposed to ventricular fibrillation, leading to sudden death, but this was not seen in the IDDM/INS group (Fig. 1B).

Insulin prevents IDDM-induced APD prolongation and rescues depressed \(I_{Kr}\) function. At the cellular level, APDs at 50% and 90% repolarization were significantly lengthened by ~36% and 28%, respectively, in the left ventricular endocardial myocytes isolated from the IDDM rabbits relative to those from healthy animals. This prolongation was nearly abolished with insulin treatment (Fig. 2A).

Consistent with the APD/QTc prolongation, \(I_{Kr}\) density was markedly diminished by ~50–80%, depending on membrane potentials, in IDDM myocytes. As expected, insulin completely rescued \(I_{Kr}\), preventing its IDDM-induced depression (Fig. 2B).

Metabolic mechanisms by which insulin maintains \(I_{Kr}/HERG\) function. Diabetes is a pathological process caused by, and resulting in, metabolic disorders in the cell, e.g., diminished glucose metabolism, impaired insulin signaling, and increased oxidative stress. We previously demonstrated that high glucose suppresses HERG channel activity (38), which is likely a cause of \(I_{Kr}\) dysfunction in IDDM hearts. Thus it was expected that reduction of the blood glucose level with insulin should restore \(I_{Kr}/HERG\) function. However, insulin completely restored \(I_{Kr}\) function, despite only partial normalization of blood glucose by insulin at the concentration examined; the level in IDDM rabbits was still twice that in healthy rabbits.

This finding suggests that, in addition to hyperglycemia, some other factors also contribute to diabetic \(I_{Kr}/HERG\) dysfunction. Indeed, our data in Fig. 3 further demonstrate that insulin prevents lengthening of APD and functional impairment of \(I_{Kr}\).

Fig. 1. Electrical disorders in a rabbit model of alloxan-induced insulin-dependent diabetes mellitus (IDDM). A: abnormal prolongation of QT interval in IDDM rabbits. Representative ECG recordings were obtained before alloxan injection for baseline data and 6 wk after alloxan treatment for end-point measurements in anesthetized rabbits. QTc, heart rate-corrected QT interval; Ctl, control sham-treated and age-matched rabbits; INS, insulin. Values are means ± SE \((n = 8\) for Ctl, \(n = 10\) for IDDM, and \(n = 5\) for IDDM/INS). \(^*P < 0.05\) vs. Ctl (end point). \(^{++}P < 0.05\) vs. IDDM (baseline). \(^{++}P < 0.05\) vs. IDDM (end point). B: ventricular arrhythmias in IDDM rabbits. Top trace and bottom left traces: ECG telemetric recordings of polymorphic ventricular tachycardias (VT) in an IDDM rabbit and ventricular fibrillation (VF) 2 min before sudden death of an IDDM rabbit. Bottom right: incidence of VT and VF. Duration of sustained VT is >30 s; nonsustained VT persists for >3 successive beats, but its duration is <30 s. Data from 3 rabbits in each group represent 20 min of telemetric ECG recording at 3-h intervals for 24 h at 2 wk after induction of IDDM or from age-matched healthy rabbits.
in healthy rabbit ventricular myocytes and of \( I_{\text{HERG}} \) in HERG-expressing HEK-293 cells in the continuous presence of 20 mM glucose, which is equivalent to the blood glucose concentration in the IDDM rabbit. Under normoglycemic conditions, insulin slightly shortened APD and increased \( I_{\text{Kr}} \) and \( I_{\text{HERG}} \) (Fig. 3).

One plausible explanation for these findings is that insulin acts to maintain \( I_{\text{Kr}} \) function by counteracting the actions of glucose, but not simply by lowering the glucose concentration in the blood. Hyperglycemia may mediate its damaging effects through a series of secondary transducers, and a common element linking hyperglycemia-induced damage is overproduction of ROS, particularly superoxide, by the mitochondrial electron-transport chain (16). It is likely that high glucose impairs \( I_{\text{Kr}}/\text{HERG} \) through increasing intracellular ROS. Indeed, oxidative stress as a result of metabolic perturbation is a pivotal deleterious factor for diabetic cardiomyopathy. Moreover,
we previously identified the HERG channel as a target for the action of ROS; specifically, superoxide anion ($O_2^-$) depresses $I_{HERG}$ in the heterologous expression system (29–31, 38), and the depression can be reversed by antioxidants, i.e., vitamin E and manganese (III)tetrakis(4-benzoic acid) porphyrin (29, 38). Also, insulin has been shown to possess antioxidant effects (1, 11, 34). However, whether these results can be applied to in vivo diabetic conditions remained to be established. To this end, we first confirmed the presence of oxidative damage to myocardium in our rabbit IDDM model by measuring the degree of lipid peroxidation and protein oxidation of the myocardium and then evaluated the antioxidant effect of insulin.

Lipid peroxidation was increased by ~45% in the diabetic hearts relative to the control hearts (Fig. 4A). This increase was nearly abolished by 100 nM insulin. Protein carbonyls are a covalent modification of a protein induced by reactive oxygen intermediates or by-products of oxidative stress, such as xanthine oxidase, $O_2^-$, and lipid peroxide adducts. Carbonyls can result in protein aggregation and are often associated with dysfunction but may require more stringent oxidative conditions. The protein oxidation, determined by carbonyl assay, was significantly increased in IDDM rabbits relative to the healthy animals, and this increase disappeared with insulin treatment (Fig. 4B).

Fig. 4. Role of oxidative damage in diabetic $I_{Kr}$ impairment and APD prolongation and insulin treatment. A: lipid hydroperoxidation in myocardium from healthy (Ctl), IDDM, and IDDM/INS rabbits. Values represent averaged data obtained from experiments performed in duplicate from 3 hearts for each group. *$P < 0.05$ vs. Ctl. **$P < 0.05$ vs. IDDM. B: protein carbonyl oxidation in myocardium from healthy (Ctl), IDDM, and IDDM/INS rabbits. Values represent averaged data obtained from experiments performed in triplicate from 3 hearts for each group. *$P < 0.05$ vs. Ctl. **$P < 0.05$ vs. IDDM. C: total endogenous antioxidant level in plasma. Values represent averaged data obtained from experiments performed in duplicate from 4 hearts for each group. *$P < 0.05$ vs. G5. **$P < 0.05$ vs. G20. D–G: overproduction of intracellular reactive oxygen species (ROS) induced by high (20 mM) glucose (G20) in myocytes isolated from left ventricular endocardium of healthy rabbit hearts (D and F) or in HERG-expressing HEK-293 cells (E and G). D and E: examples of green fluorescence, indicating ROS staining. F and G: mean data from 24–26 cardiomyocytes and from 85–126 HEK-293 cells, respectively. *$P < 0.05$ vs. G5. **$P < 0.05$ vs. G20.
The oxidative damage might be caused by decreased endogenous antioxidant reserve and/or increased ROS production. To clarify this issue, we went on to quantify the total antioxidant capacity and intracellular ROS concentration. At 6 wk, antioxidant concentration in IDDM rabbits was diminished to \(\sim 45\%\) of the level in age-matched healthy rabbits (Fig. 4C). This reduction was likely due to depletion of endogenous antioxidant molecules by intracellular ROS, because pretreatment with insulin partially restored the endogenous antioxidant level; antioxidant concentrations were 75% of the control value with insulin. More direct evidence came from the experiments with intracellular ROS staining shown in Fig. 4D. In rabbit myocytes treated with 20 mM glucose for 40 min, the percentage of ROS-positive staining was considerably higher and staining was strikingly stronger, indicating an elevated level or overproduction of ROS compared with untreated cells. In the myocytes pretreated with insulin and then treated with 20 mM glucose, the ROS level was as low as the control level in the cells under normoglycemic conditions. In HERG-expressing HEK-293 cells treated with 20 mM glucose, levels of intracellular ROS were significantly higher and were diminished by preincubation with insulin (Fig. 4, E–G).

**Molecular mechanisms by which insulin maintains \(I_{\text{K,HERG}}\) function.** There is a possibility that the diabetic conditions can result in downregulation of HERG expression, contributing to the HERG dysfunction and, thereby, QT prolongation in diabetic hearts. This was indeed supported by our Western blotting analysis with membrane protein samples extracted from the hearts of healthy or diabetic rabbits. The anti-HERG antibody recognized two separate bands corresponding to the sizes of HERG proteins: 135 kDa, representing the immature protein, and 155 kDa, for the mature \(N\)-glycosylated form of HERG. When normalized to the internal control with GAPDH for protein sample input, the density of the 155-kDa band was \(\sim 40\%\) smaller and that of the 135-kDa band was 65% smaller in IDDM rabbits than in healthy animals. Remarkably, in IDDM/INS rabbits, the HERG protein levels were not diminished; instead, they were robustly increased (Fig. 5A), overshooting the control levels.

To delineate whether the reduced HERG protein level in IDDM was due to a decrease in \(I_{\text{K,HERG}}\) expression, real-time RT-PCR experiments were conducted to compare the quantities of HERG transcripts in IDDM and healthy hearts. HERG mRNA concentration was unaltered in IDDM samples relative to that in healthy hearts (\(P < 0.05, n = 5\) for each group; Fig. 5B). However, insulin significantly increased the concentration of HERG transcripts in IDDM rabbits, by \(\sim 40\%\), which may be responsible for at least part of the insulin-induced increase in the HERG protein level shown above.

Real-time RT-PCR experiments did not explain how the HERG protein level was reduced in IDDM hearts. To shed light on this issue, we used the pulse-chase method with the radiolabeled proteins in HERG-expressing HEK-293 cells (Fig. 5C) to compare the relative stability of HERG in diabetic conditions with that in normal conditions. The half-life of mature HERG proteins was shortened from \(\sim 15.4\) h for control to 4.9 h with hyperglycemia, and by 24 h only 25% of mature HERG proteins were retained. Insulin treatment stabilized HERG proteins by extending the glucose-shortened half-life to 13.2 h. HERG proteins remained 71% and 56% of the initial levels.

**Fig. 5. Alterations of expression levels of HERG \(K^+\) channels.** A: HERG protein level assessed by Western blot analysis with membrane samples extracted from rabbit hearts. Top: Western blot bands with anti-HERG and anti-GAPDH antibodies. Bottom: densitometric analysis of bands corresponding to non-\(N\)-glycosylated (135-kDa) and \(N\)-glycosylated (155-kDa) forms of HERG proteins. Data were normalized to GAPDH and expressed as fold changes over control 155-kDa band. Values are means ± SE (\(n = 6\) hearts for Ctl, \(n = 7\) for IDDM, and \(n = 4\) for IDDM/INS). *\(P < 0.05\) vs. Ctl. **\(P < 0.05\) vs. IDDM. B: HERG mRNA concentration determined by real-time RT-PCR with RNA samples purified from rabbit hearts. Values are means ± SE (\(n = 6\) hearts for Ctl, \(n = 7\) for IDDM, and \(n = 4\) for IDDM + INS) relative to control. *\(P < 0.05\) vs. Ctl. **\(P < 0.05\) vs. IDDM. C: results from pulse-chase experiments for assessment of HERG stability in HEK-293 cells. HEK-293 cells expressing HERG plasmid were labeled for 1 h with radioactive methionine and cysteine and chased for 0–24 h. Lysates were subjected to immunoprecipitation followed by SDS-PAGE and fluorography. Density of immature (135-kDa) band was calculated for cells treated with 5 mM (G5) and 20 mM (G20) glucose, respectively, at the indicated chase intervals. Mean data were obtained from 4 independent experiments.
values at 18 and 24 h, respectively, in the presence of insulin, compared with 33% and 25% at the same time points without insulin. This pulse-chase analysis indicates that hyperglycemia results in a decrease in stability of the HERG channel.

Decrease in the mature N-glycosylated form of the HERG channel protein level suggests that the density of HERG channel proteins in the cytoplasmic membrane must decrease in IDDM myocytes. To clarify this issue, cellular localization of HERG channel proteins was analyzed first by immunohistochemistry with double staining for HERG (red) and endoplasmic reticulum (green) using confocal microscopy. The HERG proteins were stained along the cytoplasmic membrane, with stronger staining at the intercalated disks, and staining for ER was scattered in the cytosol, appearing as rod-shaped objects or punctations (Fig. 6A). Clearly, the HERG staining was less prominent overall in the diabetic heart than in the healthy heart, particularly at the intercalated disks. This was further confirmed by our immunocytochemical analysis (Fig. 6B), which showed less fluorescent intensity of HERG staining and fewer cells stained positively for HERG among IDDM than among normal control myocytes. HERG staining regained its intensity and the number of cells was restored nearly to the control level among myocytes from IDDM/INS rabbits.

DISCUSSION

Here we demonstrate that insulin is highly effective in correcting the abnormal QT prolongation and the associated arrhythmias in the rabbit model of IDDM and that dysfunction of I$_{Kr}$/HERG is the ionic mechanism, or at least one of the ionic mechanisms, for the effectiveness of insulin in our model. The present study indicates that I$_{Kr}$/HERG is a valid target for treatment of this cardiac disorder in diabetic patients.

Accumulating data from experimental, pathological, epidemiological, and clinical studies show that DM can result in cardiac functional and structural changes, independent of hypertension, coronary artery disease, autonomic neuropathy, or any other known cardiac disease, that support the existence of diabetic cardiomyopathy. The ECG of diabetic patients shows a variety of alterations with respect to healthy individuals. Among these alterations, the most frequent are those related to cardiac repolarization. These alterations can be the cause of the higher incidence of ventricular afterpotentials, the marked increase in complex arrhythmias, and the higher incidence of sudden death, which have been demonstrated in patients with DM (17, 19). Therefore, understanding the exact ionic mechanisms and identifying the therapeutic targets for diabetic QT prolongation are of pivotal importance to development of more rational approaches for prevention and treatment of the electrical disorders and sudden cardiac death. One of the major findings of the present study is that rescue of the depressed function of I$_{Kr}$/HERG channels is a valid approach for correction of the abnormal QT prolongation in diabetic hearts. We showed in our previous studies that diabetic QT prolongation is mainly a HERG channelopathy, although multiple ion channels are depressed in diabetic hearts (40), and this study provides further evidence in support of this notion.

DM is characterized by chronic hyperglycemia and alterations in carbohydrate, fat, and protein metabolism associated with total or partial deficiencies in insulin secretion or activity. In addition to elevated blood glucose, TNF-$\alpha$, ceramide, and
ROS have been shown to accumulate in the myocardium. We previously documented that hyperglycemia, TNF-α, ceramide, and ROS can depress HERG function and that the deleterious effects of hyperglycemia, TNF-α, and ceramide on HERG are primarily mediated by ROS (29–31, 38). A recent study on ceramide and HERG channels confirmed our earlier observations and conclusions (7). Activities of Akt, on the other hand, are diminished mainly as a result of insulin insufficiency, because Akt is a downstream component of the insulin signaling pathway, mediating metabolic activities and survival signals (1, 12). We previously demonstrated that Akt activity is essential in maintaining the normal function of HERG channels (39), and a decrease in Akt activities will result in a decrease in I_{Kr}/HERG function. Collectively, these metabolic perturbations characteristic of diabetes likely cause the diabetic I_{Kr}/HERG dysfunction, and ROS is the common pathway or are key elements linking metabolic perturbations with HERG dysfunction in diabetic myocardium.

In earlier studies, a Ca^{2+}-independent I_{kr} was proposed to be a target for treatment of APD prolongation in ventricular cells from streptozotocin-induced diabetic rats, because insulin reversed the attenuation of I_{kr} (22). However, whether the finding could be used to explain observations in the ECG of human diabetic patients remains uncertain because of the paradoxical function of I_{kr} in cardiac membrane repolarization; although inhibition of I_{kr} indeed can result in APD prolongation in species devoid of I_{kr}, such as rats and mice, it paradoxically shortens APD in species expressing I_{kr}, such as humans and rabbits (8, 24, 33). A recent elegant study with dynamic clamp techniques provides convincing experimental data and persuasive theoretical reasoning for APD shortening as a result of inhibition in I_{kr}/HERG function. Collectively, these metabolic perturbations characteristic of diabetes likely cause the diabetic I_{Kr}/HERG dysfunction, and ROS is the common pathway or are key elements linking metabolic perturbations with HERG dysfunction in diabetic myocardium.

One of the most significant findings of the present study is that I_{Kr}/HERG dysfunction and the resultant APD/QT prolongation and the associated arrhythmias in diabetic hearts are preventable and reversible, and insulin is highly effective in the treatment of these diabetic electrical problems. The mechanisms for the efficacy of insulin are likely multiple.

First, insulin corrects hyperglycemia by improving cellular metabolism. We show that insulin efficiently lowers blood glucose level in diabetic rabbits, although normalization was not complete at the dose used in our experiments. This partial normalization, however, may contribute significantly to amelioration of I_{Kr}/HERG function in light of the observation that hyperglycemia impairs the function of I_{Kr}/HERG (38).

Second, insulin has antioxidant properties: it reduces the intracellular ROS level and reverses the HERG depression induced by oxidative stress. Increasing evidence from experimental and clinical studies suggests that oxidative stress plays a major role in the pathogenesis of both types of DM (16). Free radicals are formed disproportionately in diabetes by glucose oxidation, nonenzymatic glycation of proteins, and the subsequent oxidative degradation of glycated proteins. Abnormally high levels of free radicals and the simultaneous decline of antioxidant defense mechanisms can damage cellular organelles and enzymes, increase lipid peroxidation, and lead to insulin resistance. Our study is the first to show that oxidative stress as a result of metabolic perturbations is the major cause of I_{kr}/HERG dysfunction and the consequent QT prolongation in diabetes. Insulin improves I_{kr}/HERG function at least partially via its antioxidative actions, and, indeed, the antioxidant property of insulin has been previously described in several studies (1, 11, 34). It has been shown that, within minutes of exposure to dihydroxyfumaric acid or xanthine/xanthine oxidase, both of which produce O_{2}^{-}, APD was prolonged in canine myocytes, papillary muscle, and small strips of right ventricular walls of guinea pig hearts, and this effect was followed by early afterdepolarization (3). The elevated oxidative stress in diabetes is also a deleterious factor for the function of I_{kr}. Incubation of diabetic rat cardiomyocytes with insulin or glutathione normalized I_{kr} density to the level in healthy myocytes as a result of increased glucose utilization and enhanced insulin signaling of reductive components in the redox system (36, 37). Before our studies, it was unknown whether other cardiac ion currents, such as I_{Kr} and I_{Kr}, are modulated by the overproduction of ROS in hyperglycemia and diabetes.

Third, insulin activates its downstream component Akt, which we have shown to be essential for maintaining normal HERG activity (1, 11, 34). Moreover, Akt mediates insulin’s effects on glucose transport and metabolism, which are also important in modulating I_{Kr}/HERG function. Supplementation with insulin is therefore expected to improve I_{Kr}/HERG function through promotion of Akt activity. The slight shortening of APD and increased I_{Kr}/HERG densities induced by insulin under normoglycemic conditions may be due to enhanced activities of Akt beyond the basal active Akt. Nonetheless, it was reported that the ability of insulin to improve the depressed I_{kr} in diabetic rats was not blocked by wortmannin, an inhibitor of phosphatidylinositol 3-kinase (37). In contrast, inhibition of the mitogen-activated protein kinase pathway by PD-98059 prevented restoration of I_{kr} (37). The authors of this study (37) suggested that insulin action on I_{kr} may involve changes in transcription or expression of channel proteins, rather than changes in cellular metabolism. Our study, however, demonstrates that insulin influences expression of HERG, HERG protein levels, and metabolism to maintain I_{kr}/HERG function. It is quite possible that insulin modulates different ion channels through different mechanisms.

There is disparity between the effects of insulin on I_{Kr} from in vivo experiments and effects from in vitro experiments: insulin completely abolished the depression of I_{Kr} in myocytes from the IDDM rabbits (Fig. 2B) but only partially reversed 20 mM glucose-induced depression of I_{Kr} in cells from the healthy animals (Fig. 3A). One possible explanation is that, with in vivo application, the duration of insulin action was sufficient to boost HERG protein level and reduce oxidative stress so as to enhance I_{Kr} amplitude, whereas with in vitro application, the duration of insulin action was adequate only for its antioxidative effects. Alternatively, it could be that the insulin concentration used for the in vitro experiments was actually lower than the blood level of insulin applied for the in vivo experiments and, therefore, insulin could not reach its maximum effect when applied to the isolated myocytes.

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