Potential Therapeutic Value of Antioxidants for Abnormal Prolongation of QT Interval and the Associated Arrhythmias in a Rabbit Model of Diabetes

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Key Words

Vitamin E • Diabetic complications • QT prolongation • Rapid delayed rectifier K⁺ current I_{Kr} • HERG • Reactive oxygen species

Abstract

Abnormal QT prolongation is the major cardiac electrical disorder and a predictor of mortality in diabetic patients. Our previous studies suggest that dysfunction of delayed rectifier K⁺ current (I_{Kr}) is the main cause for the problem. Here we report the potential therapeutic role and mechanisms of vitamin E in the rabbit model of diabetes. The QT interval and action potential duration were considerably prolonged with frequent occurrence of ventricular tachyarrhythmias in diabetic rabbits. Administration of vitamin E corrected the abnormal QT prolongation and abolished the arrhythmic incidence. I_{Kr} was found markedly reduced resulting in slowing of cardiac repolarization thereby QT prolongation in diabetic hearts. The diabetic depression of I_{Kr} is primarily ascribed to oxidative damages to the cardiac membrane and proteins, as indicated by the overproduction of reactive oxygen species leading to severe lipid peroxidation and protein oxidation.

Moreover, I_{Kr} depression is most likely due to the dysfunction of HERG K⁺ channel, the major subunit underlying native I_{Kr}, in response to oxidative stress, for peroxide anion-generating system produced similar depression of HERG channels. Vitamin E restored the depressed I_{Kr} and HERG by its antioxidant actions which likely underlie its beneficial effects on diabetic QT prolongation and the associated arrhythmias. The data indicate that an antioxidant is sufficient for reversing the I_{Kr}/I_{HERG} dysfunction and the consequent electrical disorders in diabetic hearts. Our study also conceptually simplifies the complex nature of diabetic electrical disorders to primarily oxidative stress, and should stimulate interest in antioxidants as a therapeutic strategy for diabetic QT prolongation.

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Introduction

The abnormal QT prolongation is the most frequently identified cardiac electrical disorders in patients with diabetes mellitus (DM) and the prevalence is as high as some 25% in both type I insulin-dependent (IDDM) and

type II insulin-independent (NIDDM) diabetic patients. The QT prolongation has been suggested as the predictor of mortality in both IDDM and NIDDM [1-5] because it can be the cause of the higher incidence of ventricular early afterdepolarizations, the marked increase in complex arrhythmias and the higher incidence of sudden death that have been demonstrated in DM patients. Development of efficient therapeutic approaches is therefore absolutely necessary and timely. Studies aiming to understand the mechanisms of diabetic QT prolongation demonstrated that multiple ion currents are depressed in the hearts of animals of experimental diabetes [6, 7]. Our recent study further revealed that among the various ion currents, the rapid delayed rectifier K^+ current (I_{Kr}) is the major determinant of diabetic QT prolongation. In diabetic hearts, I_{Kr} is decreased by ~80% leading to ~30% prolongation of action potential duration (APD), in agreement with the clinical diabetic QT prolongation. Depression of I_{Kr} is likely a combined result of functional impairment by metabolic perturbations [7] in diabetic hearts and expression down-regulation of the human *ether-a-go-go* related gene (HERG) encoding the pore-forming α -subunit of I_{Kr} . Particularly striking is our finding that oxidative stress due to increased intracellular reactive oxygen species (ROS) is the common pathway for I_{Kr} /HERG dysfunction caused by diabetic metabolic perturbations such as hyperglycemia, overproduction of tumor necrosis factor- α (TNF- α) and ceramide, etc [8-10]. This raises a possibility of a novel therapeutic approach using I_{Kr} /HERG as a target and antioxidants to treat the diabetic QT prolongation. The present study is designed to test this notion.

Materials and Methods

Preparation of rabbit model of IDDM

Male New Zealand white rabbits weighing 1.6–2.0 Kg (Charles River Canada Inc) were housed individually in stainless steel wire-bottomed cages in a room with a 12:12-hrs light-dark cycle with standard laboratory rabbit chow and drinking water *ad libitum*. The animals were randomly assigned to control, IDDM, and IDDM/vitamin E (VitE) groups. To establish diabetes, a single injection of 140 mg/kg (body weight) of pre-warmed (37°C) alloxan monohydrate (Sigma-Aldrich) was administered via marginal ear vein under local anesthesia. To establish diabetes, a single injection of pre-warmed (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. For IDDM/VitE group, after stable diabetes had been established for 3 days, animals were administered with VitE acetate (100

mg/kg/day, i.m.). To prevent fatal hypoglycemia due to massive insulin release, 10% glucose solution (100 mg/kg sc) was administered 4 and 6 h after alloxan treatment. The control age-matched animals were sham-operated by injecting equal volumes of saline. 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 serum glucose concentrations ≥ 15 mM 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; Longisil, Vétoquinol) containing penicillin G benzathine (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. Surface ECG was also recorded in rabbits anesthetized with ketamine (65 mg/kg) and xylazine (13 mg/kg, i.m.). The QT measurements and simultaneously recorded RR intervals were used to derive heart rate corrected QT intervals using Carlsson's formula ($QT_c = QT - 0.175 (RR - 300)$) [11]. Incidences of ventricular tachycardia (VT) and ventricular fibrillation (VF) determined. An episode of VT was defined as a run of extra-excitations with at least three consecutive beats. All procedures are in accordance with the guidelines set by the Animal Ethics Committee of the Montreal Heart Institute.

Isolation of rabbit ventricular myocytes and cell culture

Ten weeks after alloxan treatment, myocytes were isolated from rabbit left ventricular endocardium via enzymatic digestion of the whole heart on a Langendorff apparatus with the procedures similar to previously described [12]. The freshly isolated myocytes were stored in either the extracellular solution for patch-clamp recordings or in standard culture medium (M199 with Earl's salts and L-glutamine; Gibco) for ROS measurement, supplemented with penicillin (50 U/ml; Sigma) and 2% FBS (Gibco). HEK293 cells stably-expressing HERG were maintained as described previously [10].

Whole-cell patch-clamp recording

Patch-clamp of I_{Kr} and HERG currents have been described in detail elsewhere [9, 10, 13]. Briefly, currents were recorded with whole-cell patch-clamp techniques in the voltage-clamp mode and single cell action potentials were recorded in the current-clamp mode, using an Axopatch-200B amplifier (Axon Instruments). The internal solution contained (mM): 130 KCl, 1 $MgCl_2$, 5 Mg -ATP, 10 EGTA (or 0.05 mM for AP recording), and 10 HEPES (pH adjusted to 7.3 with KOH). The external solution contained (mM): 149 N-methyl-D-glutamine, 5 $MgCl_2$, 0.9 $CaCl_2$, and 5 HEPES (pH adjusted to 7.4 with HCl). All experiments were conducted at $36 \pm 1^\circ C$.

Measurement of intracellular reactive oxygen species (ROS)

The same procedures have been described previously in detail [9, 10]. 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 from Cayman Chemical (Arbor, Michigan). Rabbit left ventricular preparation of 0.2 g was homogenized in HPLC grade H_2O 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.

Protein oxidation assay

Protein Carbonyl Assay kit from Cayman Chemical was used according to the manufacturer's protocols. Ventricular tissue (0.2 g) 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 x 340, Biotech Instrument, Inc.).

Data analysis

Group data are expressed as mean $\pm$ S.E. 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 square curve fitting was performed with CLAMPFIT in pCLAMP 8.0 or GraphPad Prism.

Fig. 1. Correction by vitamin E (VitE) of abnormal prolongation of QT interval and action potential duration (APD) in rabbits with insulin-dependent diabetes mellitus (IDDM). (A) Representative ECG recordings before alloxan injection for baseline data and 10 weeks after alloxan for endpoint data in anesthetized rabbits show the prolongation of QT interval. Ctl: control sham-treated and age-matched rabbits. Mean data are heart rate-corrected QT interval (QTc interval) * $p < 0.05$ vs. Ctl (endpoint); + $p < 0.05$ vs. IDDM (baseline); ^ $p < 0.05$ vs. IDDM (endpoint); $n = 8$ rabbits for Ctl, $n = 8$ for IDDM, and $n = 6$ for IDDM/VitE. Cellular and ionic mechanisms for the effectiveness of vitamin E in correcting diabetic QT prolongation. (B) Single cell action potentials in isolated left ventricular endocardial myocytes showing VitE reversal of abnormal lengthening of APD by IDDM. * $p < 0.05$ vs. Ctl; + $p < 0.05$ vs. IDDM alone; $n = 7$ batches of cells for each group.

Results and Discussion

Vitamin E corrects abnormal QTc and APD prolongation in diabetic rabbits

The heart rate-corrected QT interval (QTc interval) were consistently prolonged in rabbits treated with alloxan (188 ± 5 ms) for 6 weeks compared with the baseline values obtained before treatment (155 ± 2 ms, $p < 0.05$, $n = 19$), but not in healthy animals (159 ± 3 ms for baseline vs. 158 ± 4 ms for time-matched comparison). Strikingly, the IDDM rabbits treated with VitE (100 mg/kg/day, i.m.) failed to develop QT prolongation (QTc = 157 ± 5 ms for baseline and 159 ± 4 ms for after VitE) (Fig. 1) despite

Fig. 2. Recovery by vitamin E (VitE) of depressed I_{Kr} in cardiomyocytes of IDDM rabbits and impaired HERG by hyperglycemia in HEK293 cells. (A) Whole-cell patch-clamp recordings of I_{Kr} in isolated left ventricular endocardial myocytes showing VitE rescuing of depressed I_{Kr} by IDDM. $*p < 0.05$ vs. Ctl; $+p < 0.05$ vs. IDDM alone; $n = 6$ batches of cells for each group. I_{Kr} was elicited by the voltage protocols shown in the inset at an interpulse interval of 10 s. (B) Whole-cell patch-clamp recordings of I_{HERG} in HERG-expressing HEK293 cells showing VitE restoring of depressed I_{HERG} by simulated hyperglycemia. I_{HERG} was elicited by the voltage protocols shown in the inset at an interpulse interval of 10 s. G5: 5 mM external glucose concentration (normoglycemia), G20: 20 mM external glucose concentration (hyperglycemia). $*p < 0.05$ vs. Ctl (G5); $+p < 0.05$ vs. G20 alone; $n = 6$ batches of cells for each group.

that hyperglycemia was not corrected by VitE; the blood glucose concentration was 5.4 ± 0.7 mM for control, 22.6 ± 1.1 mM for IDDM, and 23.1 ± 2.3 mM for IDDM/VitE group. The QTc prolongation was accompanied by occurrence of ventricular arrhythmias, mainly the polymorphic VT which was otherwise absent in healthy animals. Out of eight rabbits of IDDM group, five developed VT (62%). The VT often predisposed to VF leading to sudden death; of the five rabbits with VT two (60%) died before completion of the experimental procedures. The total incidence of VT was 33 and VF 14 in the IDDM group. With VitE treatment, however, no arrhythmias were seen in five treated animals.

To investigate the cellular mechanisms, we went on to record the action potentials (AP) in single myocytes isolated from left ventricular endocardium. APD was significantly prolonged in IDDM myocytes relative to healthy cells. Consistently, the APD lengthening seen in single cells isolated from diabetic hearts was substantially smaller after the rabbits had been treated with VitE. These results are shown in Fig. 1B.

Ionic mechanisms underlying correction of diabetic QTc/APD abnormalities by vitamin E

We have previously found that the diabetic QTc/APD prolongation is primarily ascribed to dysfunction of delayed rectifier K^+ current (I_{Kr}) and its molecular counterpart HERG [7]. It is thus conceivable that VitE

corrects the diabetic QTc/APD abnormalities by protecting I_{Kr} /HERG function. To test this notion, we assessed the effects of VitE administration on I_{Kr} in diabetic rabbit hearts and on HERG current (I_{HERG}) in HERG-expressing HEK293 cells. As illustrated in Fig. 2, VitE efficiently prevented impairment of I_{Kr} in IDDM myocytes and hyperglycemia-induced depression of I_{HERG} in HEK293 cells. The results indicate that preservation of I_{Kr} /HERG function is the major ionic mechanisms underlying VitE's actions on diabetic QT/APD abnormalities and the associated arrhythmias.

Mechanisms underlying recovery of I_{Kr} /HERG function by vitamin E

Increasing evidence from both experimental and clinical studies suggests that oxidative stress plays a major role in the pathogenesis of both type I and type II diabetes mellitus. 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 lead to damages of cellular organelles and enzymes, increased lipid peroxidation, and development of insulin resistance. Indeed, TNF- α that is also known to elevate in its concentration and activity in diabetic myocardium suppresses I_{HERG} [9]. Intriguingly, we found that the common mechanism for HERG

Fig. 3. Prevention by vitamin E of oxidative damages to myocardium and to I_{Kr} /HERG function. (A) Lipid hydroperoxidation in myocardium from healthy (Ctl), IDDM, or IDDM/VitE rabbits. Shown are averaged data obtained from experiments performed in duplicate from three hearts for each group. * $p < 0.05$ vs. Ctl; + $p < 0.05$ vs. IDDM alone. (B) Protein carbonyl oxidation in myocardium from healthy (Ctl), IDDM, or IDDM/VitE rabbits. Shown are averaged data obtained from experiments performed in triplicate from three hearts for each group. * $p < 0.05$ vs. Ctl; + $p < 0.05$ vs. IDDM alone. (C) Overproduction of intracellular reactive oxygen species (ROS) in left ventricular endocardial myocytes isolated from healthy (Ctl), IDDM, or IDDM/VitE rabbit hearts. Example images are shown and the green fluorescence indicates ROS staining. (D) Effects of antioxidants VitE (100 μ M) on I_{HERG} impairment induced by the superoxide anion-generating system xanthine/xanthine oxidase (X/XO) on I_{HERG} in HERG-expressing HEK293 cells. X: 500 μ M and XO: 5mU/ml, $n=5$ for each group. * $p < 0.05$ vs. Ctl; + $p < 0.05$ vs. X/XO alone; $n=5$ batches of cells for each group.

dysfunction induced by ceramide and TNF- α is overproduction of reactive oxygen species (ROS), especially superoxide anion (O_2^-). These data suggest that oxidative stress as a result of metabolic perturbations is the major cause for I_{Kr} /HERG dysfunction and the consequent QT prolongation in diabetes. It is well established that hyperglycemia itself is an important stimulant of ROS generation [10]. In addition, alloxan, in the presence of intracellular thiols, generates ROS in a cyclic reaction with its reduction product, dialuric acid. ROS generation destruct pancreatic β -cells, which is the main mechanism for alloxan to induce diabetes.

To this end, we revealed 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. As depicted in Fig. 3A, the degree of lipid peroxidation was increased by ~45% in diabetic heart relative to control heart. This increase was ~50% abrogated by VitE (100 mg/kg). Similarly, the

protein oxidation, determined by carbonyl assay, was also significantly increased in IDDM rabbits relative to healthy animals (Fig. 3B) and the effect was abrogated by VitE. Consistently, the myocytes isolated from IDDM rabbits had considerably higher percentage of positive ROS staining and strikingly stronger staining, indicating an elevated level or over-production of ROS in the cell, compared to the cells isolated from healthy rabbits. Strikingly, in the myocytes from IDDM rabbits treated with VitE, the ROS level was restored to nearly identical to the healthy animals (Fig. 3C). Furthermore, I_{HERG} was critically diminished when the HERG-expressing HEK293 cells had been treated with the superoxide anion generating system xanthine/xanthine oxidase (X/XO) and this functional depression was restored by co-treatment with VitE (100 nM) (Fig. 3D). It is therefore likely that vitamin E preserves I_{Kr} /HERG function by protecting the membrane lipids and proteins from oxidative damages which manifests in diabetic conditions.

The novel finding of this study is the efficacy of antioxidant vitamin E in correcting diabetic QT prolongation and the associated arrhythmias via rescuing depressed I_{Kr} /HERG function and the resultant APD lengthening. This finding suggests a potential alternative approach for the therapy of diabetic electrical disturbances. Antioxidants, albeit with minimal therapeutic value for diabetes *per se*, can be used for correcting the abnormal APD/QT prolongation in diabetic complications. This is particularly important considering that I_{Kr} /HERG enhancer is currently unavailable. These points imply that “metabolic correction” may be considered a rational therapeutic approach for QT prolongation and the associated arrhythmias in diabetic patients. However, it should be noted that the results from a rabbit model of diabetes using alloxan to create diabetes is not a resemblance of natural etiology of diabetes; thus our finding should not be directly extrapolated to humans.

In conclusion, the present study shows that an antioxidant is sufficient for reversing the I_{Kr}/I_{HERG} dysfunction and the consequent cardiac electrical disorders in a rabbit model of diabetes. Our data also have the potential to conceptually simplify the complex nature of diabetic electrical disorders to primarily oxidative stress, and to stimulate the interest in translational studies

and clinical trials for the potential use of antioxidants as a therapeutic strategy for abnormal diabetic QT prolongation.

Abbreviations

APD (Action potential duration); APD₅₀ and APD₉₀ (Action potential duration at 50% and 90% repolarization, respectively); HERG (Human *ether-a-go-go* related gene); IDDM (Induced insulin-dependent diabetes mellitus); I_{Kr} (Rapid delayed rectifier K⁺ current); VitE (Vitamin E).

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References

- Christensen PK, Gall MA, Major-Pedersen A, Sato A, Rossing P, Breum L, Pietersen A, Kastrup J, Parving HH: QTc interval length and QT dispersion as predictor of mortality in patients with non-insulin-dependent diabetes. *Scand J Clin Lab Invest* 2000;60:323–332.
- Okin PM, Devereux RB, Lee ET, Galloway JM, Howard BV: Strong Heart Study: Electrocardiographic repolarization complexity and abnormality predict all-cause and cardiovascular mortality in diabetes: the strong heart study. *Diabetes* 2004;53:434–440.
- Rossing P, Breum L, Major-Petersen A, Sato A, Winding H, Pietersen A, Kastrup J, Parving HH: Prolonged QTc interval predicts mortality in patients with type 1 diabetes mellitus. *Diabet Med* 2001;18:199–205.
- Veglio M, Chinaglia A, Cavallo-Perin P: QT interval, cardiovascular risk factors and risk of death in diabetes. *J Endocrinol Invest* 2004;27:175–181.
- Veglio M, Sivieri R, Chinaglia A, Scaglione L, Cavallo-Perin P: QT interval prolongation and mortality in type 1 diabetic patients: a 5-year cohort prospective study. *Neuropathy Study Group of the Italian Society of the Study of Diabetes, Piemonte Affiliate. Diabetes Care* 2000;23:1381–1383.
- Wang DW, Kiyosue T, Shigematsu S, Arita M: Abnormalities of K⁺ and Ca²⁺ currents in ventricular myocytes from rats with chronic diabetes. *Am J Physiol* 1995;1269: H1288–H296.
- Zhang Y, Wang J, Wang Z: Ionic mechanisms underlying the diabetic cardiocomplications. *Biophys J* 2005;88:472a.
- Chapman H, Ramström C, Korhonen L, Laine M, Wann KT, Lindholm D, Pasternack M, Törnquist K: Downregulation of the HERG (KCNH2) K⁺ channel by ceramide: evidence for ubiquitin-mediated lysosomal degradation. *J Cell Sci* 2005;118:5325–5334.
- Wang J, Wang H, Zhang Y, Gao H, Nattel S, Wang Z: Impairment of HERG K⁺ channel function by tumor necrosis factor- α : role of reactive oxygen species as a mediator. *J Biol Chem* 2004;279:13289–13292.
- Zhang Y, Han H, Wang J, Wang H, Yang B, Wang Z: Impairment of HERG (Human ether-a-go-go Related Gene) K⁺ channel function by hypoglycemia and hyperglycemia: similar phenotypes but different mechanisms. *J Biol Chem* 2003;278:10417–10426.
- Carlsson L, Abrahamsson C, Andersson B, Duker G, Schiller-Linhardt G: Proarrhythmic effects of the class III agent almokalant: importance of infusion rate, QT dispersion, and early afterdepolarizations. *Cardiovasc Res* 1993;27:2186–2193.
- Shi H, Wang H, Wang Z: Extracellular barium blocks the cardiac transient outward potassium current. *Am J Physiol* 2000;278:H295–H299.
- Wang Z, Fermini B, Nattel S: Rapid and slow components of delayed rectifier current in human atrial myocytes. *Cardiovasc Res* 1994;28:1540–1546.