Sphingolipid Metabolite Ceramide Causes Metabolic Perturbation Contributing to HERG K⁺ Channel Dysfunction

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Key Words

Ceramide • HERG • Reactive oxygen species • Vitamin E • MnTBAP • QT prolongation

Abstract

Ceramide, a sphingolipid metabolite, has emerged as a key second messenger molecule that mediates multiple cellular functions. Its *de nova* synthesis and accumulation in ischemic myocardium, congestive heart failure and diabetic cardiomyopathy is associated with the abnormalities such as abnormal QT prolongation and increased risk of arrhythmias. To investigate how ceramide is involved in modulating cardiac repolarization, we performed whole-cell patch-clamp studies on HERG current (I_{HERG}), a critical determinant of cardiac repolarization, expressed in HEK293 cells. Acute application (superfusion for 25min) of membrane permeable ceramide (C2, 5 μ M) did not alter I_{HERG} . Prolonged incubation with C2 for 10hrs caused pronounced I_{HERG} inhibition in a concentration-dependent and voltage-independent

fashion and positive shift of voltage-dependent HERG activation. The IC_{50} for I_{HERG} suppression was 19.5 μ M. C2 did not affect the inactivation property and time-dependent kinetics of I_{HERG} . Similar effects were observed with production of endogenous ceramide catalyzed by sphingomyelinase. Tyrosine kinase inhibitors failed to reverse C2-induced suppression of HERG function, and PKA and PKC inhibitors only slightly reversed the I_{HERG} depression. Western blotting and immunocytochemical analyses indicate that C2 does not alter HERG protein expression on the cytoplasmic membrane. The inhibitory effect of C2 on I_{HERG} was reversed by antioxidants vitamin E or MnTBAP. C2 caused considerable production of intracellular reactive oxygen species (ROS), which was prevented by vitamin E or MnTBAP. We conclude that ceramide depresses I_{HERG} mainly via ROS overproduction and ceramide-induced I_{HERG} impairment may contribute to QT prolongation in prolonged myocardial ischemia, heart failure and diabetic cardiomyopathy.

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Introduction

The rate of cardiac repolarization is important in maintaining the normal cardiac electrophysiology and in determining the propensity of arrhythmias. Ischemic myocardium demonstrates characteristic sequential alterations in electrophysiology with an initial shortening of action potential duration (APD) and QT interval during the early phase (<15min) of acute ischemia and subsequent lengthening of APD/QT after a prolonged ischemic period and chronic myocardial ischemia [1, 2]. While these alterations may be adaptive to the altered metabolic status, they occur at the cost of arrhythmogenesis. Ischemic arrhythmias are generally categorized into three phases based on the time when they appear: phase I arrhythmias occurring during the first 30min of ischemia, phase II arrhythmias between 5 to 72 hours and phase III arrhythmias at a chronic stage after an infarct. The electrophysiological remodeling during chronic myocardial ischemia has been revealed to manifest as APD/QT prolongation which provides the substrate for reentry and EAD-dependent triggered activity. Our current understanding of the ionic mechanisms for the repolarization slowing in ischemic myocardium has been incomplete. The rate of repolarization is determined by several ion currents, of which the rapid delayed rectifier K⁺ current (I_{Kr}) has a crucial role, particularly to the plateau phase of action potentials [3-5]. The major molecular component of native I_{Kr} has been identified as the human *ether-a-go-go*-related gene (*HERG*) which when expressed in heterologous systems generates I_{Kr}-like current [5]. Impairment of I_{Kr}/HERG can cause excessive prolongation of APD and QT interval, which is commonly believed to be a mechanism for both genetic and drug-induced long QT syndromes.

Ceramide, a long-chain sphingolipid generated intracellularly on hydrolysis of sphingomyelin catalyzed by sphingomyelinase (SMase), has emerged as an important second messenger molecule that is capable of mediating multiple physiological effects including regulation of cellular differentiation, proliferation, and apoptosis by activating a variety of signaling cascades, including those triggered by cytokines, growth factors, and stress [6-8]. Ceramide generation can be triggered by diverse stimuli and *de novo* ceramide synthesis and accumulation take place in the myocardium under various pathological conditions such as myocardial ischemia (MI) or hypoxia, congestive heart failure (CHF), and diabetic cardiomyopathy (DCM) [9-16]. In these situations,

ceramide is involved in triggering of cardiomyocyte apoptosis, cardiac pump failure and cardiac arrhythmias. In particular, prolongation of action potential duration (APD) and QT interval is a common feature with regard to cardiac electrical disturbances in chronic MI [1, 2], CHF [17, 18] and DCM [19, 20]. HERG function (or I_{Kr}) has been shown to be impaired under these conditions [21-28].

These notions prompted us to investigate whether ceramide can modulate HERG K⁺ channels, in order to have better understanding of the metabolic and ionic mechanisms for the APD/QT prolongation in these diseased settings. To this end, we performed studies to investigate the effects of ceramide on HERG K⁺ channel function and to elucidate the potential signaling mechanisms. Our data identified HERG as a target for ceramide action and intracellular reactive oxygen species (ROS) as a mediator for ceramide-induced impairment of HERG function.

Materials and Methods

Cell Culture

HEK293 cells stably expressing HERG (a kind gift from Drs. Zhou and January) [29] were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 200 μM G418, 100 units/ml penicillin, and 100 μg/ml streptomycin, as previously described in detail [30, 31].

Whole-Cell Patch-Clamp Recording

Patch clamp recording of HERG K⁺ current (I_{HERG}) has been described in detail elsewhere [30, 31]. Currents were recorded in the whole-cell voltage-clamp mode with an Axopatch-200B amplifier (Axon Instruments). Borosilicate glass electrodes had tip resistances of 1–3 MΩ when filled with the internal solution containing (mM) 130 KCl, 1 MgCl₂, 5 Mg-ATP, 10 EGTA, and 10 HEPES (pH 7.3). The extracellular (Tyrode) solution contained (mM) 136 NaCl, 5.4 KCl, 1 CaCl₂, 1 MgCl₂, 10 glucose, and 10 HEPES (pH 7.4). Experiments were conducted at 36 ± 1°C. Junction potentials were zeroed before formation of the membrane-pipette seal. Series resistance and capacitance were compensated, and leak currents were subtracted.

Drugs and Treatment

N-acetyl-D-sphingosine, synthetic membrane permeable ceramide (C2), sphingomyelinase (SMase, the neutral form with long-lasting activity), okadaic acid (OA), bisindolylmaleimide (Bis; PKC inhibitor), H89, and vitamin E (VitE) were purchased from Sigma (Oakville, ON). Dihydro-, N-acetyl-D-erythro-sphingosine (dihydro-C2, an inactive analogue of C2), herbimycin A (HA) and genistein (Gen) were purchased from Calbiochem (La Jolla, CA). PDD, Bis, bisindolylmaleimide, C2,

Fig. 1. Analog data showing the effects of membrane permeable ceramide (C2) on HERG current (I_{HERG}) expressed in HEK293 cells. I_{HERG} was elicited by 2.5-s depolarizing pulses to voltages ranging from -60 to +40 mV to record the step current, followed by 2.5-s repolarization step to -50 mV to record the tail current, as shown with the voltage protocol shown in the inset.

dihydro-C2, HA, LA, Gen, and FSK were dissolved in DMSO. OA was dissolved in phosphate-buffered saline (PBS). SMase was prepared in a working solution of 50% glycerol containing 50 mM Tris-HCl. All agents were prepared as 1000 x stock solutions and stored at -20°C.

For acute exposure, drugs were added to the bathing solution 10min after baseline recording and the current amplitudes before and after drugs were compared. For prolonged exposure, cells were incubated in normal culture medium containing agents at the desired final concentrations for 10hrs prior to patch-clamp recordings and group comparison between treated and untreated cells (treated with the same vehicles as for drug treatment groups) was made. Step I_{HERG} was defined as the current amplitude at the end of the 2.5-sec depolarizing voltage steps and tail I_{HERG} was measured as the peak value of the decaying tail currents upon repolarization to -50 mV. To have more rational comparisons between groups, I_{HERG} density was calculated by dividing the current amplitude by the capacitance of the same cell. For experiments involving protein kinase inhibitors, cells were pre-incubated with the drugs for 2hrs and then incubated with C2 for another 10hrs in the continuous presence of the inhibitors.

Western Blot

The procedures for measuring HERG protein levels were essentially the same as described previously [27, 28]. The primary antibody, polyclonal anti-HERG raised in rabbit against highly purified peptide (CY)EEL PAGAPELPQD GPT, corresponding to residues 1118-1133 of human HERG was purchased from Alomone Labs (Jerusalem, Israel). Bound antibodies were detected using the chemiluminescent substrate (Western Blot Chemiluminescence Reagent Plus, NEN Life Science Products, Boston, USA) and quantified using a Phosphorimager (Bio-Rad). The presence of HERG channel proteins was verified by the presence of a prominent band with expected molecular mass in the range of previous reports and by elimination of the band in preparations preincubated with the antigenic peptide. Coomassie staining was performed to verify equal protein loading for each sample.

Immunocytochemistry

The procedure was the same as previously described in detail [32]. The anti HERG antibody was the same as used for Western blot analysis. The cells were examined under a Laser scanning confocal microscope (Zeiss LSM 510).

Intracellular Reactive Oxygen Species (ROS) Measurement

The procedures have been described in detail previously [33]. CM-H2DFDA from Molecular Probes was used to detect oxidative activity in living cells. Cells were divided into 5 groups: control group, C2 10-hrs group, C2 20-min group, C2+VitE, and C2+MnTBAP group. For experiments involving VitE or MnTBAP, the cells were pretreated with VitE (100 μM) or MnTBAP (5 μM) for 2hrs and then incubated in the culture medium containing C2 in the continual presence of VitE or MnTBAP. The percentage of positively stained cells and the fluorescence intensity of staining were determined by densitometric scanning with LSM software (Zeiss).

Data Analysis

Group data are expressed as mean $\pm$ s.e.m. Comparisons among groups were made by analysis of variance (*F*-test) and Bonferroni-adjusted *t*-tests were used for multiple group comparisons and paired or unpaired *t*-test was used, as appropriate, for single comparisons. 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.

Results

Effects of Membrane Permeable Ceramide on I_{HERG} Expressed in HEK293 Cells

Depolarizing voltage steps from -60 mV to +40 mV elicited delayed rectifier type of outward currents with characteristic inward rectification that manifests at

Fig. 2. Characterization of I_{HERG} with prolonged exposure to ceramide (C2). (A) Current density-voltage relationships. The number of cells studied is indicated in the brackets. (B) Steady-state voltage-dependent activation curves. The activation curves were constructed by plotting the conductance G as a function of depolarizing potentials. G was calculated by normalizing the tail currents at -50 mV by dividing the amplitude of the tail currents measured at various antecedent depolarizing potentials by that of the tail current at +40 mV. Symbols are mean of experimental data and lines represent the Boltzmann fit: $G/G_{\text{max}} = 1 / \{1 + \exp[(V_{1/2} - V)/k]\}$, where G_{max} represents the maximal conductance at +40 mV, $V_{1/2}$ is a half-maximal activation voltage, and k is a slope factor. Note that C2 produces positive shift of HERG activation. (C) Concentration-dependent block of I_{HERG} by C2. Symbols are experimental data at a test potential of 0 mV and the curve represents the fit to the Hill equation: $Y = B + (T - B) / (1 + 10^{-(\log \text{IC}_{50} - [\text{C2}])})$, where Y is the percentage of I_{HERG} inhibition, B is the minimum inhibition and T the maximum inhibition observed, and IC_{50} represents the concentration of C2 [C2] for half-maximal inhibition of I_{HERG} . (D) Percent decrease in I_{HERG} produced by various concentrations of C2. * $P < 0.05$ vs. Ctl.

stronger depolarization. Upon repolarization back to -50 mV, slowly decaying tail currents are recorded. Application of the membrane permeable ceramide (C2) to the superfusate at a concentration of 25 μM for up to 20 min did not significantly alter the HERG currents (Fig. 1A), suggesting that ceramide does not directly act on the HERG channels.

In contrast to the acute exposure, prolonged incubation of cells with C2 (1-50 μM) in the culture medium for 10 hrs caused consistent changes of I_{HERG} in two different ways. First, I_{HERG} density was decreased by C2 at a concentration-dependent (Fig. 1B and Fig. 2) and voltage-independent (Fig. 2D) manner. The IC_{50} of C2 for I_{HERG} suppression was calculated by the Hill equation to be 19.5 μM . Second, the steady-state voltage-dependent activation of HERG channels was appreciably shifted by C2 towards depolarizing voltages (Fig. 2B). For example, the voltage for half maximum activation ($V_{1/2}$) was changed from -27.0 ± 0.5 mV ($n=27$) to -24.2 ± 1.0 mV ($n=13$, $p < 0.05$ vs. control) and -22.0 ± 1.1 mV ($n=17$, $p < 0.05$) by 1 and 5 μM C2, respectively, with no changes in the slope factor (k). Intriguingly, the IC_{50} for the $V_{1/2}$ shift was around 1.1 μM , some 20-fold lower than the effect on the current density. Higher concentrations of C2 (10, 25 and 50 μM) did not produce

further shift of I_{HERG} activation. The inactive analog of C2, dihydro-C2 (25 μM), failed to cause any appreciable alterations of I_{HERG} (Fig. 2A). No effects of C2 on HERG kinetics and inactivation properties were observed (data not shown).

Effects of Endogenous Ceramide Generated by Sphingomyelinase on I_{HERG}

To verify the modulation of I_{HERG} by ceramide and the potential pathophysiological implication, we studied the effects of sphingomyelinase (SMase) that catalyzes production of endogenous ceramide on I_{HERG} . Application of SMase is known to lead to ceramide generation in the cell and the endogenous ceramide is expected to produce similar effects on I_{HERG} as exogenous ceramide did. Indeed, qualitatively the same effects were observed with SMase as with exogenously applied C2. SMase significantly decreased I_{HERG} in a concentration-dependent (reduction ranging from 18% to 65%) but voltage-independent manner (Fig. 3). SMase also produced positive shifts of I_{HERG} activation voltage; $V_{1/2}$ was changed from -30.6 ± 0.6 mV ($n=25$) for control to -27.3 ± 0.8 mV ($n=20$, $p < 0.05$) and -24.4 ± 0.6 mV ($n=13$, $p < 0.05$) in the presence of 0.2 U/ml and 0.6 U/ml SMase, respectively. SMase did not affect the activation and

Fig. 3. Characterization of I_{HERG} depression caused by sphingomyelinase (SMase). (A) Current density-voltage relationships. The number of cells studied is indicated in the brackets. (B) Steady-state voltage-dependent activation curves. Note that similar to C2, SMase also produces positive shifts of HERG activation. (C) Concentration-dependent block of I_{HERG} by SMase. Symbols are experimental data and the curve represents the fit to the Hill equation. (D) Percent decrease in I_{HERG} produced by various concentrations of SMase. * $P < 0.05$ vs. Ctl.

deactivation kinetics and inactivation properties of HERG channels (data not shown).

Effects of Inhibitors to PTK, PKA or PKC on I_{HERG} Modulation by Ceramide

It has been shown that ceramide inhibits T lymphocyte voltage-gated potassium channel via tyrosine phosphorylation of the channel proteins [34]. On the contrary, the I_{HERG} -like current has been found to be enhanced by protein tyrosine kinase (PTK) activation in MLS-9 rat microglia cell line [35]. To investigate whether this mechanism also applies to HERG modulation by ceramide, we evaluated the effects of two PTK inhibitors herbimycin A (HA) and genistein (Gen). As illustrated in Fig. 4A, preincubation of cells with HA (5 μM) alone for 2hrs remarkably suppressed I_{HERG} (~66% at 0 mV) to a similar extent as the I_{HERG} inhibition caused by 25 μM C2 (~58% at 0 mV). Co-incubation of cells with HA and C2 caused a slightly further decrease in I_{HERG} (by ~77%). Similarly, Gen alone also reduced I_{HERG} albeit to a less extent compared with C2. Pretreatment with Gen (80 μM) did not alter the effects of C2 on I_{HERG} density (Fig. 4B).

Ceramide is characterized by its ability to simulate activation of the atypical protein kinase C (PKC ξ) [36]. PKC ξ can be inhibited by a phospholipase C inhibitor okadaic acid (OA). Our experiments demonstrated that OA (1 μM) alone significantly diminished I_{HERG} and

pretreatment of cells with OA failed to prevent I_{HERG} reduction induced by C2; instead, further decreases in I_{HERG} were found in the presence of both OA and C2 (Fig. 4C).

PKA phosphorylation of HERG channels was shown to cause reduction of current amplitude due to positive shift of HERG activation voltages [37, 38]. To investigate the potential involvement of PKA pathway in mediating ceramide-induced I_{HERG} depression, we assessed the effects of a PKA inhibitor H89. As illustrated in Fig. 5A, incubation of cells with H89 (1 μM) alone for 10hrs produced a marked shift of the HERG I-V relationship and activation curve towards more negative potentials. The maximum step I_{HERG} was unaltered despite that because of the positive shift I_{HERG} was increased at potentials negative to -10 mV and decreased thereafter. The maximum tail I_{HERG} , however, was reduced by approximately 11%. Pretreatment with H89 only slightly reduced the degree of C2-induced I_{HERG} diminishment (Fig. 5A).

PKC is another target of ceramide action. To investigate the possible involvement of PKC in mediating I_{HERG} modulation by ceramide, we evaluated the effects of a PKC inhibitor bisindolylmaleimide (Bis, 0.1 μM) on C2-induced I_{HERG} depression. Cells treated with Bis alone for 10 hrs had significantly smaller I_{HERG} density (30.5 ± 2.2 pA/pF, $p < 0.05$) compared with control untreated cells (39.8 ± 2.8 pA/pF) (Fig. 5B). The I_{HERG} activation property

Fig. 4. Effects of inhibitors of tyrosine protein kinases (TPKs) and atypical protein kinase C (PKC β) on I_{HERG} modulation by ceramide (C2). Left panels: current density-voltage relationships; right panels: steady-state voltage-dependent activation curves. PTK inhibitors herbimycin A (HA, 5 μM) and genestein (Gen, 80 μM); PLC/PKC β inhibitor okadaic acid (OA, 1 μM). Comparisons were made among 4 groups: Ctl-control untreated cells (Ctl), C2-cells treated with C2 alone, cells treated with an inhibitor alone, and cells co-incubated with an inhibitor and C2 for 10hrs after 2-hrs preincubation with the inhibitor alone. * $P < 0.05$ vs. Ctl.

was not different between Bis and control groups. Bis pretreatment partially reduced C2-induced I_{HERG} depression (Fig. 6A). Similarly, pretreatment with another PKC inhibitor cherylethrine (1 μM) partly restored C2-induced I_{HERG} reduction and cherylethrine alone also significantly decreased I_{HERG} (data not shown).

Lack of Effects of Ceramide on HERG Protein Expression Level

There is a possibility that the decrease of I_{HERG} in presence of ceramide was due to down-regulation of HERG channel protein expression. To test this notion, we carried out Western blot analysis with membrane protein samples extracted from HERG-expressing HEK293 cells to compare the HERG protein levels between control cells and C2-treated cells. As shown in Fig. 6A, anti-HERG antibody recognized a band of ~135 kDa, consistent with the molecular mass of HERG protein [29, 32], and the band disappeared if the antibody had been pretreated with its antigenic peptide. HERG protein levels were comparable between the C2-treated cells and control untreated cells.

This result was further reinforced by our immunocytochemical analysis demonstrating similar pattern and intensity of HERG staining on the surface membrane between C2-treated cells and control untreated cells (Fig. 6B).

Role of Reactive Oxygen Species (ROS) in I_{HERG} Modulation by Ceramide

It has been well established by numerous studies that ceramide can act on mitochondria and stimulate production of ROS, particularly superoxide anion ($\text{O}_2^{\cdot -}$) [7, 39-40]. We have previously demonstrated that $\text{O}_2^{\cdot -}$ causes impairment of HERG K^+ channel function [33]. It is quite plausible that C2-induced depression of I_{HERG} is related to increased ROS generation. We tested this notion by preincubating the cells with vitamin E (VitE, 100 μM) alone for 2hrs and then incubating the cells with C2 (25 μM) in the continual presence of VitE for another 10hrs. As shown in Fig. 7A, VitE substantially weakened the depressing effects of C2, but VitE alone did not produce any appreciable effects, on I_{HERG} . For example, I_{HERG} density was ~50% smaller in control than in the cells pretreated with C2 alone, but it was only ~24% smaller in the cells pretreated with both VitE and C2. Or in other words, I_{HERG} density in the VitE+C2 group was approximately 52% greater than in the group with C2 alone (48.2 ± 3.2 pA/pF, $n=19$, vs. 31.7 ± 2.0 pA/pF, $n=16$, $p < 0.05$), at 0 mV (Fig. 7A). Furthermore, Mn(III) tetrakis(4-benzoic acid) porphyrin chloride (MnTBAP), a superoxide dismutase (SOD) mimic, resembled the effects of VitE; it reversed C2-induced I_{HERG} reduction (Fig. 7B).

To confirm that ROS production was indeed

Fig. 5. Effects of inhibitors of protein kinase A (PKA) or protein kinase C (PKC) on I_{HERG} modulation by ceramide (C2). (A) Effects of PKA inhibitor H89 (1 μM) on C2-induced I_{HERG} depression. Left panels: current density-voltage relationships; right panels: steady-state voltage-dependent activation curves. * $P < 0.05$ vs. Ctl. (B) Effects of PKC inhibitor bisindolylmaleimide (Bis) on C2-induced I_{HERG} depression. For experiments involving co-application of H89 or Bis and C2, the cells were preincubated with H89 or Bis for 2hrs and then incubated with C2 (5 μM) in the presence of H89 or Bis for 10hrs before patch-clamp recordings. * $P < 0.05$ vs. Ctl.

increased by C2 and the C2-induced ROS was mainly of O_2^- , we proceeded to measure the intracellular ROS levels using CM-H2DCFDA fluorescence dye. The ROS level was measured in cells incubated with the culture medium containing 25 μM C2 for 10hrs. The cells showing fluorescence intensity ≥ 5 times the background were defined as positive staining, and the number of cells with positive staining was pooled from 5 fields for each batch of cells (a total of 4 independent batches of cells for each group were studied). The intensity of staining by the fluorescent probe for ROS was analyzed by densitometric scanning using the LSM program, and cells with positive staining were taken for analysis, and the data were normalized to the control values. Under control conditions, cells with positive staining were sparse. Yet in the cells treated with C2, the number of the cells with positive staining, as well as the intensity of staining, was consistently higher. If pretreated with VitE or MnTBAP prior to exposure to C2, the cells had significantly lower ROS levels, as indicated by the smaller number of positively stained cells and the weaker intensity of staining (Fig. 8A and 8B). Since 20min superfusion of cells with C2 failed to affect I_{HERG} , we also conducted CM-H2DCFDA experiments with cells treated with C2 for

only 20min. In sharp contrast with 10-hrs exposure, 20min treatment did not alter the level of intracellular ROS.

Discussion

The present study identifies HERG K^+ channel as a new target for ceramide action. The major findings of the study are: (1) prolonged exposure (chronic incubation), but not brief exposure (acute superfusion), of cells to C2 significantly impairs HERG K^+ channel function and (2) stimulation of intracellular reactive oxygen species (ROS) by C2 mediates the depressing effects of C2 on HERG K^+ channel function. In view of the critical role of HERG K^+ channel in regulating cardiac repolarization and the fact that ceramide is overproduced in MI, CHF or DCM, it is possible that HERG impairment by ceramide contributes to the electrical disturbances in these pathological conditions.

It has been reported that the HERG-equivalent K^+ current in rat microglia MLS-9 cell [35] is significantly reduced by inhibitors of protein tyrosine kinases (PTK), indicating the role of PTK in maintaining HERG function. Consistent with these findings, our data demonstrated that

Fig. 6. Expression level of HERG protein determined by immunoblotting with membrane protein preparations extracted from HERG-expressing HEK293 cells. (A) Upper panel: examples of immunoblotting bands of ~135 kDa, corresponding to the molecular mass of HERG protein, from C2 treated (C2) and untreated cells (Ctl). Cells were incubated with C2 (25 μ M) for 10hrs in the culture medium prior to the procedures for membrane protein extraction. Lower panel: mean band density from 3 independent protein samples. AP-, without antigenic peptide pretreatment for anti-HERG antibody; AP+, with antigenic peptide pretreatment for anti-HERG antibody. (B) Upper panel: examples of immunocytochemical staining of HERG proteins on the cytoplasmic membrane. Lower panel: averaged data from four independent experiments, showing % cells with positive staining with anti-HERG antibody and intensity of staining.

PTK inhibitors herbimycin A and genestein alone both markedly depressed I_{HERG} amplitude, indicating a role of basal PTK activity in maintaining HERG function. Co-application of these inhibitors with ceramide did not alter ceramide-induced I_{HERG} depression, or if anything, caused further reduction of I_{HERG} . The latter suggests that ceramide has the ability to enhance HERG function and partially offsetting its inhibitory effect by stimulating PTK activities, but failed to do so in the presence of PTK inhibitors. Thus, PTKs do not account for ceramide-induced I_{HERG} depression. Involvement of PKA or PKC in ceramide-induced I_{HERG} depression appears to be minimal, if any. This conclusion was drawn on the basis that neither PKA inhibitor H89 nor PKC inhibitor Bis significantly reversed the effects of C2, despite that these compounds themselves when applied in the absence of C2 produced direct effects on I_{HERG} . Obviously, these direct effects of H89 and Bis are unlikely related to ceramide action on I_{HERG} , but the positive shift of HERG activation may be caused by PKA because H89 completely abolished ceramide-induced shift.

A growing body of evidence is emerging indicating that oxidative stress and ceramide generation are intimately connected in cell death signaling. The major source for ROS in most cell types is probably the electron leakage from the mitochondrial electron transport chain, which results in the formation of superoxide anion ($\text{O}_2^{\cdot-}$). Experimental evidence indicates that cell-permeable ceramide analogs elicit a direct effect on mitochondria, ranging from inhibition of the respiratory chain and ROS overproduction to induction of mitochondrial permeability transition in intact cells and cytoplasts [41, 42]. Several studies have identified the mitochondrial ubiquinone pool of complex III (a protein complex that links proton translocation to electron transfer from ubiquinone to cytochrome c) as a ceramide target to produce ROS. Indeed, it has been reported that MnTBAP, an SOD mimic, inhibits ceramide-induced apoptosis in neuronal cells [43]. The present study confirmed that C2 stimulates intracellular production of ROS as indicated by the increased ROS level and the increase was attenuated by VitE. Furthermore, our data suggest that C2-induced

Fig. 7. Role of reactive oxygen species (ROS) on I_{HERG} modulation by ceramide (C2). (A) Effects of vitamin E (VitE) on C2-induced I_{HERG} depression. (B) Effects of MnTBAP, a superoxide dismutase mimic, on C2-induced I_{HERG} depression. Left panels: current density-voltage relationships; right panels: steady-state voltage-dependent activation curves. The cells were preincubated with VitE (100 μM) or MnTBAP (5 μM) for 2hrs and then incubated with C2 (25 μM , $n=16$ cells) in the presence of VitE ($n=16$ cells) or MnTBAP ($n=16$ cells) for 10hrs before patch-clamp recordings. * $P<0.05$ vs. Ctl ($n=14$ cells).

generation of ROS was mainly of $\text{O}_2^{\cdot -}$ because MnTBAP nearly abolished the ROS increases. These data are consistent with the electrophysiological results that VitE or MnTBAP reversed the C2-induced reduction of I_{HERG} amplitude. The fact that 20min superfusion of the cells with C2 failed to affect I_{HERG} in our experiments (Fig. 1A) may well be due to failure of C2 to increase ROS within such a short time frame and this notion is indeed supported by the data shown in Fig. 8A. In other words, the reason why prolonged exposure (>2.5 hrs) was required for C2 to produce effects on I_{HERG} is that sufficient level of ROS needs to be raised with time in the presence of C2 to affect I_{HERG} . The ability of ceramide to induce ROS production in cultured neonatal rat ventricular myocytes has indeed been recently confirmed by Suematsu *et al* [44]. Noticeably, neither VitE nor MnTBAP altered C2-induced positive shift of HERG activation, indicating that ROS is not responsible for ceramide-induced shift.

The present study has several important pathophysiological implications. First, it is known that ischemic myocardium demonstrates characteristic bi-phase changes of electrophysiology with APD shortening during early phase of acute ischemia and subsequent APD lengthening after prolonged ischemic period, which are associated with different types of arrhythmias [1, 2].

However, the ionic mechanisms underlying these sequential alterations of APD remained unresolved. This study together with our previous work seems to provide an explanation. It is well recognized that altered lipid metabolism is an important deleterious factor in ischemic myocardial injury; some lipid metabolites such as lysophosphatidylcholines (LPCs) are overproduced rapidly within the first 10min of acute myocardial ischemia, while others like ceramide accumulate progressively in the late phase (30min after) of ischemia [6-8, 12]. We have recently reported that LPCs produce pronounced enhancement of HERG function [30, 31]. This finding may explain in part the APD shortening and the associated arrhythmias occurring in the early phase of ischemia. On the other hand, *in vitro* ischemia leads to a progressive accumulation of ceramide in cardiomyocytes. The content of ceramide in ischemic area was found to be elevated to 155% baseline levels after 30min, and to 330% after 210min, of ischemia. Ischemia (30min) followed by reperfusion (180min) increased the ceramide level to 250% in the ischemic area [9]. In another study, the total basal ceramide concentration in the myocardium was 135 nM/g tissue, and it was increased by 14.1% and 48.4% in 30min global ischemia and 30min ischemia/30min reperfusion groups, respectively [10]. Cordis *et al* [11] showed a 50% decrease of sphingomyelin both during

Fig. 8. Effects of vitamin E (VitE) or MnTBAP (an SOD mimic) on intracellular levels of ROS measured by CM-H2DCFDA fluorescence dye. (A) and (B) Upper panels: laser scanning confocal microscopic images of CM-H2DCFDA staining reflecting the intracellular ROS levels. Lower panels: percentage of positively stained cells (mean $\pm$ S.E.), obtained from 5 fields of 4 experiments by counting the cells with staining intensity ≥ 5 times the background and averaged intensity of CMH-2DCFDA fluorescence measured from the positively stained cells. Data were obtained from control untreated cells (Ctl), cells treated with C2 (25 μ M for 20min or 10hrs), and cells pretreated with VitE (100 μ M) or MnTBAP (5 μ M). * P <0.05 vs. Ctl; + P <0.05 vs. C2.

ischemia and subsequent reperfusion with a corresponding increase in ceramide. Another study suggests that a longer time period (>30min) may be needed to alter ceramide mass in tissues in response to ischemic insult [12]. Intriguingly, the cardiac AP is abnormally lengthened after prolonged ischemia [1, 2]. In lieu of the critical role of HERG K^+ channel in cardiac repolarization and the ability of ceramide to inhibit I_{HERG} , it is tempting to speculate that I_{HERG} depression produced by ceramide that accumulates during the late phase of myocardial ischemia might contribute to the observed prolongation of APD and the associated arrhythmias.

The second implication of our study is related to arrhythmias in CHF. One characteristic electrophysiological alteration in CHF is abnormally prolonged APD at the cellular level and QT interval as reflected in electrocardiogram [18, 45]. This prolongation

provides an electrophysiological substrate for early afterdepolarizations (EADs) to occur, which can result in ventricular tachycardias that often predispose to ventricular fibrillation leading to sudden cardiac death [17]. Polymorphic ventricular tachycardias, likely related to arrhythmogenic EADs, are common in CHF [17]. The ionic mechanisms underlying APD prolongation in CHF have not been precisely defined. Intriguingly, *de novo* ceramide synthesis has been found to be increased in heart failure [13]. Increased ceramide level in cardiomyopathy can cause impairment of HERG function and result in excessive APD prolongation. More importantly, our data demonstrate that ROS mediates the inhibitory effect of ceramide on I_{HERG} and antioxidants can prevent the I_{HERG} depression. This explains at least partly the benefits of antioxidants in preventing and suppressing arrhythmias in ischemic myocardium and

failing hearts [46, 47]. Indeed, Tsuji *et al.* [25] showed I_{Kr} , measured as E-4031-sensitive tail current, to be ~36% smaller in rabbits with ventricular tachypacing-induced CHF than in healthy rabbits. Lodge and Normand [24] demonstrated earlier that I_{Kr} , measured as dofetilide-sensitive tail current, reduced by ~45% in the BIO TO-2 strain of cardiomyopathic hamster of 10 months old, derived from the BIO 53.58 animals and providing a model of dilated low output heart failure, compared with the 10-month-old control (BIO F1B) hamsters. Moreover, simulations of cellular electrophysiology predict I_{Kr} inhibition to cause EADs in failing, but not nonfailing, myocytes [48].

Finally, the results of this study also have implications in diabetic cardiomyopathy (DCM). DCM is characterized by electrical remodeling with aberrant electrophysiology, metabolic remodeling with malignant biochemical processes and anatomical remodeling with progressive loss of cardiomyocytes, which result in impaired cardiac contractile and increased risk of lethal arrhythmias. QT prolongation and QT dispersion have been suggested as the predictor of mortality in both type I and type II [19, 20, 49]. It has been documented that *de novo* synthesis and accumulation of ceramide in diabetic

myocardium contributes to the development of DCM [13, 15, 16]. In addition, oxidative stress is also known to be a critical deleterious factor for DCM [49-51]. Moreover, we have recently found that I_{Kr} /HERG function is impaired in the rabbit model of DCM [27, 28]. Taken together, it seems plausible that ceramide-induced HERG depression may be one of the multiple determinants for diabetic QT prolongation.

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