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Involvement of Caveolin in Probucol-Induced Reduction in hERG Plasma-Membrane Expression

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ABSTRACT

The human *ether-à-go-go*-related gene (hERG) encodes the pore-forming subunit of the rapidly activating delayed rectifier K^+ current (I_{Kr}) important for cardiac repolarization. Dysfunction of the hERG channel causes long QT syndrome (LQTS). Although diverse compounds reduce the hERG current (I_{hERG}) by blocking the channel, probucol, a cholesterol-lowering drug that causes LQTS, reduces I_{hERG} by decreasing plasma-membrane hERG protein expression. Here, we investigated the mechanisms of probucol effects on hERG expression levels. Our data demonstrate that probucol accelerated the degradation of mature hERG channels, which associated with caveolin-1 (Cav1) in hERG-expressing HEK cells. In human embryonic kidney (HEK) cells without hERG expression, probucol promoted endogenous Cav1 degradation. In hERG-expressing HEK cells, overexpression of Cav1 enhanced, whereas knockdown of Cav1 impeded, probucol-induced reduction of mature hERG channels. Thus, probucol reduces hERG expression through accelerating Cav1 turnover. The effects of probucol on Cav1 and hERG result from probucol's cholesterol-disrupting action, because low-density lipoprotein (LDL), a potent cholesterol carrier, effectively prevented probucol-induced reduction of I_{hERG} in hERG-expressing HEK cells and of I_{Kr} in neonatal rat cardiomyocytes. Our data provide evidence that targeting hERG-interacting protein caveolin represents a novel mechanism for drugs to decrease hERG expression and cause LQTS.

Introduction

The human *ether-à-go-go*-related gene (hERG) encodes the pore-forming subunit of the rapidly activating delayed rectifier K^+ channel (I_{Kr}) (Sanguinetti et al., 1995; Trudeau et al., 1995), which is important for cardiac repolarization. The significant role of I_{Kr} in cardiac repolarization was unequivocally manifested by identifying inherited mutations in hERG as molecular mechanisms for long QT syndrome type-2 (Sanguinetti and Tristani-Firouzi, 2006). Mutations in hERG can reduce I_{Kr}, delay cardiac repolarization, and cause inherited LQTS, which can lead to ventricular arrhythmias and sudden cardiac death (Keating and Sanguinetti, 2001). In addition, a large number of medications can interfere with I_{Kr} function and cause LQTS, a phenomenon known as drug-induced LQTS. These drugs interfere with hERG channel function by either directly blocking the channel (Roy et al., 1996; Zhang et al., 2001) or disrupting hERG expression in the plasma membrane (Ficker et al., 2004; Kuryshev et al., 2005; Rajamani et al., 2006; Guo et al., 2007; Wang et al., 2007). We demonstrated previously that probucol [4,4′-(isopropylidenedithio)-bis-(2,6-di-t-butylphenol)], a cholesterol-lowering drug that causes LQTS, reduces cell surface expression levels of I_{Kr} in cardiomyocytes and hERG channels in a stable cell line (Guo et al., 2007). However, the mechanisms of probucol-induced reduction in hERG plasma-membrane expression remain unknown. In the present study, using electrophysiology, Western blot, and immunocytochemistry methods, we demonstrate that probucol reduces hERG membrane expression by promoting mature channel degradation through accelerated Cav1 turnover.

Materials and Methods

Molecular Biology. An HEK 293 cell line stably expressing hERG channels (Zhou et al., 1998) was a gift from Dr. Craig January (University of Wisconsin–Madison, Madison, WI). The cells were cultured in minimum essential medium supplemented with 10%

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ABBREVIATIONS: hERG, human *ether-à-go-go*-related gene; BFA, brefeldin A; Cav1, caveolin-1; CHX, cycloheximide; I_{hERG}, human *ether-a-go-go*-related gene current; LDL, low-density lipoprotein; LQTS, long QT syndrome; siRNA, small interfering RNA; HPLC, high-performance liquid chromatography; Cav3, caveolin-3; CoIP, coimmunoprecipitation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

fetal bovine serum and 0.2 mg/ml G418 to select for transfected cells. The human Cav1 cDNA was obtained from Dr. Eric Smart (University of Kentucky, Lexington, KY). Knockdown of the basal expression levels of Cav1 in hERG-HEK cells was performed using Cav1 siRNA (Santa Cruz Biotechnology, Santa Cruz, CA). Cells were grown in 35-mm dishes at 60 to 70% confluence, and 80 pmol of duplex siRNA was transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 24 to 48 h of transfection and subsequent treatment with probucol, cells were harvested for either Western blot or electrophysiological analysis. For electrophysiological studies, cells were harvested from the culture dish by trypsinization and stored in standard minimum essential medium at room temperature. Cells were studied within 8 h of harvest. Ventricular myocytes were isolated from 1- to 2-day-old Sprague-Dawley rats of either gender by enzymatic dissociation as described previously (Guo et al., 2007). Neonatal rat ventricular myocytes were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 medium (Invitrogen) with 10% fetal bovine serum. Cardiomyocytes were grown on glass coverslips for electrophysiological studies.

Patch-Clamp Recording Method. Whole-cell patch-clamp method was used (Hamill et al., 1981). The standard 135 mM K^+ pipette solution contained 135 mM KCl, 5 mM EGTA, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.2 with KOH. The standard 5 mM K^+ bath solution contained 135 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4 with NaOH. For I_{Kr} recordings in cultured neonatal rat ventricular myocytes, Cs^+-rich solutions were used to isolate I_{Kr} from the other potassium currents (Zhang, 2006). The pipette solution contained 135 mM CsCl, 10 mM EGTA, 5 mM MgATP, 10 mM HEPES, pH 7.2 with CsOH, and the bath solution contained 135 mM CsCl, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, and 0.01 mM nifedipine, pH 7.4 with CsOH. Procedures of whole-cell patch-clamp method and Cs^+-carried I_{Kr} recording were performed as described previously (Zhang, 2006). Patch-clamp experiments were performed at room temperature (22 ± 1°C).

Western Blot Analysis. Whole-cell lysates from hERG-expressing HEK 293 cells were used for analysis. For hERG detection, proteins were separated on 7.0% SDS polyacrylamide gels and electroblotted overnight at 4°C to polyvinylidene difluoride membrane. The membrane was then blocked using 5% nonfat milk and 0.1% Tween 20 in Tris-buffered saline and immunoblotted for 1 h with a rabbit anti-hERG primary antibody (anti-Kv11.1; Sigma-Aldrich, St. Louis, MO). hERG signals were detected using goat anti-rabbit horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence detection kit (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). For coimmunoprecipitation (CoIP), the total cell lysate was extracted using lysis buffer with protease inhibitor cocktail and phenylmethylsulfonyl fluoride (Sigma-Aldrich). Samples of 0.5 mg of protein were incubated with either anti-hERG (N20) or anti-Cav1 overnight at 4°C and then precipitated with protein A/G Plus-agarose beads for 4 h at 4°C. The samples were washed three times with lysis buffer. The beads were resuspended in 2× sample buffer and boiled to remove agarose beads. The immunoprecipitates were separated using SDS-polyacrylamide gel electrophoresis.

Immunofluorescence Microscopy. hERG-HEK cells grown on cover glasses were treated with probucol (100 μM) or vehicle (ethanol, 0.3%) as control for 48 h. Cells were washed and fixed with 4% ice-cold paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100, and blocked with 5% bovine serum albumin in phosphate-buffered saline solution. The hERG and Cav1 were labeled with appropriate primary and fluorescence-conjugated secondary antibodies. Nuclei were stained using Hoechst 33342 (0.2 μg/ml; Sigma-Aldrich). Images were acquired using a Leica TCS SP2 Multi Photon confocal microscope (Leica, Wetzlar, Germany).

Reagents. Probucol, brefeldin A, cycloheximide, digitoxin, glycerol, thapsigargin, anti-Kv11.1 antibody, anti-actin antibody, and chemicals for patch-clamp experiments were purchased from Sigma-Aldrich. Probucol was dissolved in ethanol to make a 30 mM stock solution. Digitoxin was dissolved in dimethyl sulfoxide to make a 0.1 mM stock solution. Anti-hERG (N20, for CoIP), anti-Cav1, anti-GAPDH antibodies, Protein A/G, Cav1 siRNA, and control siRNA were purchased from Santa Cruz Biotechnology. Low-density lipoprotein (LDL) was isolated from cholesterol-fed rabbits (Massaeli et al., 1999). Human LDL was purchased from Calbiochem (San Diego,

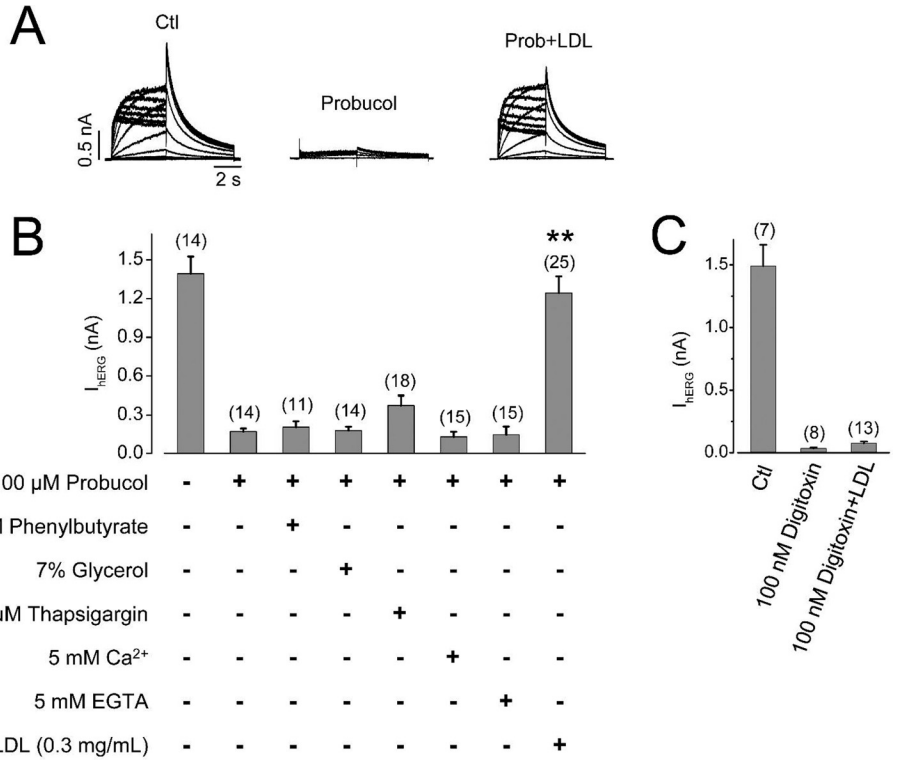

Fig. 1. Rescue of probucol-induced I_{hERG} reduction by LDL. A, families of hERG currents in control, after treatment with 100 μM probucol for 48 h, and with 100 μM probucol plus 0.3 mg/ml LDL for 48 h. I_{hERG} was elicited by depolarizing steps in 10-mV increments to voltages between −70 and +70 mV for 4 s. The holding potential was −80 mV. The tail currents recorded upon a repolarizing step to −50 mV for 5 s were used for analysis of the hERG current amplitude. B, summarized amplitudes of I_{hERG} in control conditions, in the presence of probucol alone, and probucol plus various agents. hERG-HEK cells were treated under each condition for 48 h. C, summarized amplitudes of I_{hERG} in control conditions, in the presence of digitoxin, or digitoxin plus LDL (0.3 mg/ml). The number above each column in B and C indicates the number of cells examined from at least three independent experiments. **, $P < 0.01$ versus probucol treatment.

CA). Sodium 4-phenylbutyrate was purchased from EMD Biosciences (San Diego, CA).

Data are expressed as the mean ± S.E. To test for statistical significance between control and test groups, one-way analysis of variance or two-tailed Student's *t* test was used. A *P* value of 0.05 or less was considered significant.

Results

LDL Effectively Rescues Probucol-Induced I_{hERG} Reduction. We demonstrated previously that probucol reduces hERG expression levels in the plasma membrane in a concentration-dependent manner within clinical relevant concentrations (Guo et al., 2007). Our data demonstrated that long-term probucol exposure reduces I_{hERG} in hERG-expressing HEK cells with an IC_{50} of 10.6 μM and native I_{Kr} in cultured neonatal rat cardiac myocytes with an IC_{50} of 20.6 μM (Guo et al., 2007). To investigate the mechanisms for probucol-induced hERG reduction, we used a concentration of 100 μM to maximize the effect of probucol. As shown in Fig. 1A, treatment of hERG-HEK cells with 100 μM probucol for 48 h essentially eliminated I_{hERG}. Several reagents have been shown to effectively rescue various trafficking-defective proteins. We examined whether these reagents can rescue probucol-induced I_{hERG} reduction. Sodium 4-phenylbutyrate is a protein-stabilizing chemical chaperone that improves ΔF508-CFTR trafficking in cystic fibrosis epithelial cells and in patients with ΔF508-homozygous cystic fibrosis (Rubenstein and Lyons, 2001). Glycerol rescues the defective ΔF508 CFTR channel trafficking (Zhang et al., 2003). As shown in Fig. 1B, neither phenylbutyrate nor glycerol had any effect on probucol-induced I_{hERG} reduction. On the other hand, thapsigargin (5 μM) slightly reversed the probucol-induced I_{hERG} reduction. Thapsigargin is a sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase inhibitor, which has been shown to rescue the trafficking-defective ΔF508 CFTR mutation (Egan et al., 2002). Sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase inhibitors such as thapsigargin deplete endoplasmic reticulum luminal $[Ca^{2+}]$ and are believed to alter Ca^{2+}-dependent molecular chaperone activity (Corbett and Michalak, 2000). Thapsigargin also rescues surface expression of trafficking-defective hERG mutants G601S and F805C (Delisle et al., 2003). Because thapsigargin may alter the intracellular Ca^{2+} homeostasis, we examined the potential role of Ca^{2+} in probucol-disrupted hERG expression levels. An increase in Ca^{2+} by adding 5 mM Ca^{2+} or decrease in Ca^{2+} by adding 5 mM EGTA, a Ca^{2+} chelator, to the cell culture medium had no effect on probucol-induced I_{hERG} reduction (Fig. 1B).

Probucol reduces plasma cholesterol levels by accelerating LDL removal (Naruszewicz et al., 1984). It also inhibits the ATP-binding cassette transporter 1-mediated cellular lipid metabolism in cultured J774 macrophages (Favari et al., 2004). To examine the role of cholesterol in probucol-disrupted hERG expression, we added LDL (0.3 mg/ml, from cholesterol-fed rabbits), a natural cholesterol carrier (Massaeli et al., 1999), to the probucol-containing cell culture medium for 48 h. The addition of LDL almost completely restored I_{hERG} (Fig. 1, A and B). Although less potent, human LDL also rescued the probucol-reduced I_{hERG} (data not shown). Probucol is a lipophilic compound. To exclude the possibility that LDL rescues the probucol effects on hERG via sequestering probucol in the cell culture medium, we tested the effects of LDL on digitoxin-induced I_{hERG} reduction. Digitoxin also reduces hERG expression levels in the plasma membrane (Wang et al., 2007) and is also lipophilic (Joubert, 1990; Belz et al., 2001). Incubation of hERG-HEK cells with 100 nM digitoxin for 48 h completely eliminated hERG current. However, LDL (0.3 mg/ml) had no effect on digitoxin-induced I_{hERG} reduction (Fig. 1C). Thus, LDL rescue is specific to probucol-induced I_{hERG} reduction. To further address whether LDL decreases probucol concentration, we compared probucol concentrations in supernatants isolated via centrifugation from cell culture medium containing probucol and from medium containing probucol plus LDL using C_{18} reversed-phase high-performance liquid chromatography (HPLC). We cultured hERG-HEK cells in the medium, medium with probucol (100 μM), or with probucol (100 μM) plus LDL (0.3 mg/ml) for 24 h. We then collected media from each culture condition and isolated supernatants using centrifugation (20,000*g*, 1 h). Supernatants (100 μl) from each medium were eluted at 1 ml/min with acetonitrile/water (80/20) mixture as the mobile phase in HPLC to detect probucol at a wavelength of 241 nm. Our data showed that probucol concentrations were comparable between samples from probucol-containing medium and probucol/LDL-containing medium (data not shown). Thus, LDL rescue of probucol-induced I_{hERG} reduction is not via decreasing probucol concentration in the cell culture medium.

To determine whether LDL also rescues probucol-induced I_{Kr} reduction in cardiomyocytes, we performed experiments on cultured neonatal rat ventricular myocytes using symmetric Cs^+ as charge-carrier for I_{Kr}. Figure 2 shows a family of Cs^+ currents obtained from a single cardiomyocyte. From a holding potential of −80 mV, depolarizations in 10-mV increments to voltages between −70 and +70 mV for 1 s were applied to evoke currents. Depolarizing steps to voltages more positive than 0 mV induced outward currents that inactivated in a voltage-dependent manner. Upon repolarization to −80 mV, the inward tail currents displayed an initial rising phase, which is described as a "hook," reflecting the

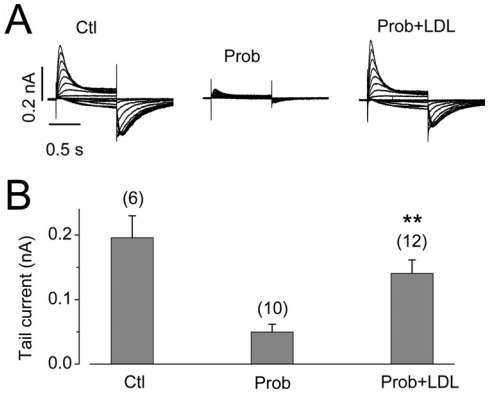

Fig. 2. LDL rescues probucol-induced I_{Kr} reduction in cultured neonatal rat cardiomyocytes. A, $I_{Kr\text{-}Cs}$ under control conditions, after 48 h treatment with 100 μM probucol, or 48 h treatment with 100 μM probucol plus 0.3 mg/ml LDL. Symmetric Cs^+ solutions were used to record $I_{Kr\text{-}Cs}$ elicited by depolarizing steps in 10-mV increments to voltages between −70 and +70 mV for 1 s. The tail currents upon repolarizing steps to holding potential of −80 mV were used for analyzing the current amplitudes. B, summarized $I_{Kr\text{-}Cs}$ amplitudes under control (Ctl), 100 μM probucol (Prob), or 100 μM probucol plus 0.3 mg/ml LDL (Prob + LDL). The number above each column indicates the number of cells examined from three independent treatments. **, $P <$ 0.01 versus probucol treatment.

rapid recovery of inactivated channels to the open state before deactivation, and is unique to I_{Kr}. Cs^+ is well known to block various types of K^+ channels (Zhang, 2006). By analyzing the current gating kinetics, especially inactivation and drug sensitivity of the Cs^+ current in cardiomyocytes and in hERG-expressing stable HEK cells, we have demonstrated previously that the Cs^+ current recorded in cardiac myocytes represents pure I_{Kr} (Zhang, 2006). Similar to expressed hERG channels, Cs^+-mediated I_{Kr} ($I_{Kr\text{-}Cs}$) in cardiomyocytes was essentially eliminated by the incubation of 100 μM probucol for 48 h. Also, the addition of LDL (0.3 mg/ml) effectively prevented probucol-induced I_{Kr} reduction (Fig. 2).

Probucol Reduces hERG Expression through a Cav1-Dependent Mechanism. The effects of probucol on hERG expression were examined using Western blot analysis. Total cell proteins were extracted from hERG-HEK cells cultured with 100 μM probucol and from cells cultured with 0.3% ethanol as a vehicle control for 48 h. The hERG protein from control cells displayed two bands with molecular masses of 135 and 155 kDa (Fig. 3). The 155-kDa band represents the mature, fully glycosylated form of functional hERG channels in the plasma membrane, and the 135-kDa band represents the immature, core-glycosylated form residing in the endoplasmic reticulum (Zhou et al., 1998; Guo et al., 2007, 2009). Inclusion of 100 μM probucol in the culture medium for 48 h eliminated the 155-kDa hERG band (Fig. 3). Interestingly, Cav1 expression level was also significantly reduced by probucol treatment. Furthermore, LDL effectively rescued both probucol-reduced 155-kDa hERG and Cav1 expression levels (Fig. 3).

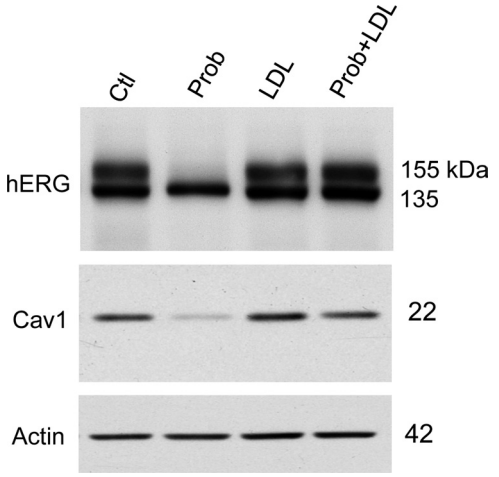

Fig. 3. Probucol-induced reduction in hERG expression is concomitant with a decrease in Cav1 expression. Western blot analyses showing hERG and Cav1 expression levels in control (Ctl), 100 μM probucol (Prob), 0.3 mg/ml LDL (LDL), or 100 μM probucol plus 0.3 mg/ml LDL (Prob + LDL). Actin was used as the loading control ($n = 3$).

Physical Association and Colocalization between hERG and Cav1. To determine interactions between hERG and Cav1, we conducted confocal microscopy experiments to examine the colocalization between hERG and Cav1. hERG-HEK cells were cultured in the absence or presence of 100 μM probucol for 48 h. The cells were fixed and permeabilized. hERG channels were labeled using an anti-hERG antibody that targets the C-terminal region of the hERG protein. The antibody recognizes both mature hERG channels in the plasma membrane and immature proteins in the endoplasmic reticulum and Golgi apparatus. Cav1 was labeled using appropriate primary and secondary antibodies. Under control conditions, hERG and Cav1 were both detected on the cell membrane (Fig. 4A). Exposure of hERG-HEK cells to 100 μM probucol for 48 h resulted in a significant reduction of cell surface hERG channels and surface Cav1 expression. Furthermore, the two proteins demonstrated strong colocalization (Fig. 4A).

The association between hERG and Cav1 proteins was examined using CoIP analysis. Cell lysates were extracted from hERG-HEK cells. An anti-Cav1 antibody was used to immunoprecipitate Cav1 and its associated proteins, whereas an anti-hERG antibody was used to immunoprecipitate hERG

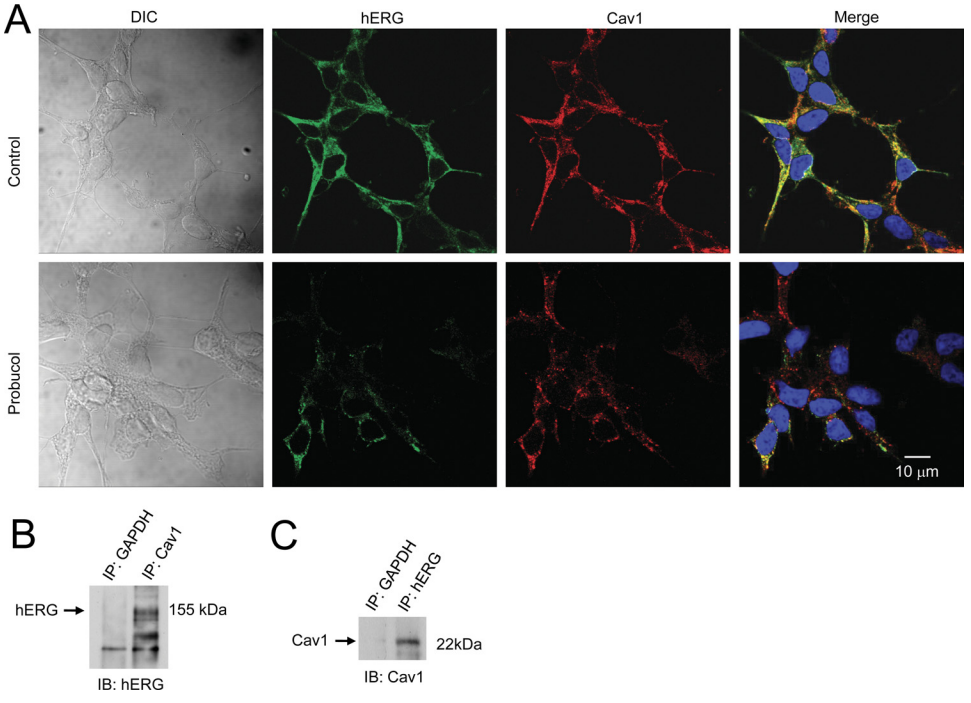

Fig. 4. The hERG channel is associated with Cav1. A, confocal images showing colocalization between hERG and Cav1. hERG-HEK cells were immunofluorescently labeled to show hERG and Cav1; the hERG channel was labeled with an anti-hERG antibody and an Alexa Fluor 488–conjugated (green) secondary antibody. Cav1 was labeled with an anti-Cav1 primary antibody and an Alexa Fluor 594-conjugated (red) secondary antibody. The cell nuclei were stained with Hoechst 33342 (blue). The first photo of each row shows differential interference contrast (DIC) image of the same cells. Scale bar, 10 μm. B and C, CoIP between hERG and Cav1. hERG-HEK cells were lysed and samples of 0.5 mg of protein were used for immunoprecipitation with Cav1 antibody (B) or anti-hERG (C) antibody. The immunoprecipitated protein was then immunoblotted (IB) using anti-hERG (B) or anti-Cav1 antibody (C). GAPDH was used as the control for immunoprecipitation.

and its associated proteins. The immunoprecipitated proteins were detected using Western blot analysis with anti-hERG or anti-Cav1 antibodies, respectively. As shown in Fig. 4, B and C, the hERG band was detected in the anti-Cav1 antibody-precipitated proteins, whereas the Cav1 expression was detected in the anti-hERG antibody-precipitated proteins. These results indicate an interaction between hERG and Cav1.

Cav1 Facilitates Probucol-Induced hERG Degradation. To further our understanding of the role of Cav1 in probucol-induced hERG reduction, we overexpressed Cav1 in hERG-HEK cells by transfecting the cells with Cav1 plasmids. Twenty-four hours after transfection, cells were cultured in either control medium or 100 μM probucol-containing medium for 24 h; hERG expression levels and I_{hERG} were then examined. As shown in Fig. 5, whereas overexpression of Cav1 did not significantly affect the 155-kDa hERG expression level in control cells, it enhanced probucol-induced reduction of the 155-kDa hERG expression (Fig. 5A). Likewise, Cav1 overexpression significantly promoted probucol-induced I_{hERG} reduction (Fig. 5B). Thus, hERG-HEK cells overexpressing Cav1 were much more sensitive to probucol exposure than control hERG-HEK cells.

The hERG-HEK cells express endogenous Cav1. We knocked down endogenous Cav1 expression with siRNA and examined the effects of probucol on hERG expression. Forty-eight hours after transfecting hERG-HEK cells with either Cav1 siRNA or control siRNA, the cells were passaged and exposed to 100 μM probucol for 48 h, and hERG expression levels and I_{hERG} were examined. Cav1 siRNA transfection reduced the endogenous Cav1 expression and significantly impeded the reduction of the 155-kDa hERG expression induced by probucol (Fig. 6A). In addition, Cav1 siRNA transfection significantly impeded probucol-induced I_{hERG} reduction (Fig. 6B). After 100 μM probucol-treatment for 48 h, I_{hERG} in cells transfected with Cav1 siRNA was significantly larger than I_{hERG} in cells transfected with control siRNA. Altogether, these data indicate that Cav1 is involved in probucol-induced degradation of mature hERG channels.

Probucol Increases the Turnover Rate of Cav1. Our data so far raised a possibility that probucol destabilizes Cav1 in the plasma membrane and promotes its degradation. Because Cav1 associates with mature hERG channels, the accelerated Cav1 turnover may consequently increase hERG degradation by dragging hERG for internalization, thus decreasing hERG expression in the plasma membrane. We tested this possibility by examining the effects of probucol on Cav1 expression in HEK cells without hERG channels. Probucol treatment significantly reduced Cav1 expression levels. Moreover, this effect was completely rescued by LDL (0.3 mg/ml) treatment (Fig. 7A). To test the notion that probucol destabilizes Cav1, we examined the effects of probucol on the degradation rate of Cav1 in HEK cells whose protein synthesis was inhibited by 10 μg/ml cycloheximide (CHX) treatment. As shown in Fig. 7, B and C, the expression level of Cav1 declined with time in HEK cells treated with CHX; the Cav1 decay rate was significantly accelerated by probucol. For example, Cav1 expression level in the cells treated with CHX plus probucol (100 μM) decreased by 74.8 $\pm$ 8.7% in 24 h, which is significantly greater than the Cav1 decline in

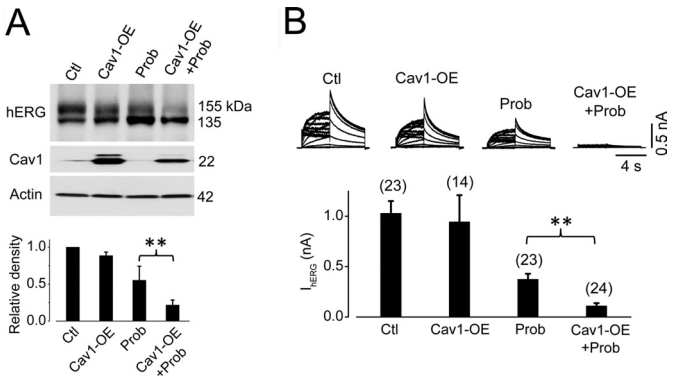

Fig. 5. Overexpression of Cav1 accelerates probucol-induced reduction of mature hERG channels. A, top, hERG expression levels in hERG-HEK cells without (Ctl) or with Cav1 overexpression (Cav1-OE) under control conditions or under 100 μM probucol treatment for 24 h (Prob). Bottom, the density of the 155-kDa band under each condition was normalized to the value of control (Ctl) (n = 3). B, I_{hERG} in hERG-HEK cells without (Ctl) or with Cav1 overexpression (Cav1-OE) under control conditions (Ctl) or with 100 μM probucol treatment for 24 h (Prob). The summarized tail current amplitudes are shown at the bottom. The number above each column indicates the number of cells examined from three independent treatments. **, $P < 0.01$.

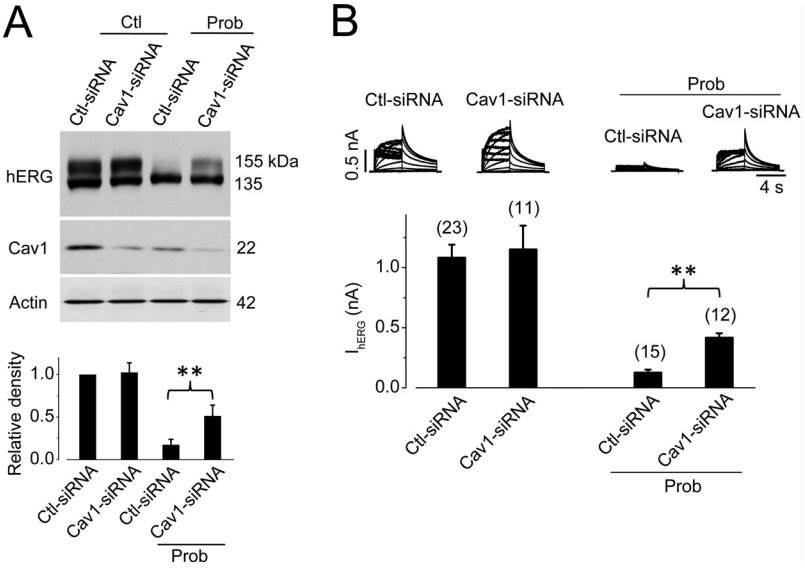

Fig. 6. Knockdown of Cav1 impedes probucol-induced reduction of mature hERG channels. A, top, hERG expression levels in hERG-HEK cells transfected with control (Ctl) siRNA or Cav1 siRNA under control conditions (Ctl) or with 100 μM probucol treatment for 48 h (Prob). Bottom, the density of the 155-kDa band under each condition normalized to the value of control (Ctl) (n = 4). B, I_{hERG} in hERG-HEK cells transfected with control (Ctl) siRNA or Cav1 siRNA under control conditions or with 100 μM probucol treatment for 48 h (Prob). The summarized tail current amplitudes are shown at the bottom. The number above each column indicates the number of cells examined from four independent treatments. **, $P < 0.01$.

cells treated with CHX alone for the same period (decreased by 46 ± 11%, $n = 6$, $P < 0.01$). Thus, probucol promotes Cav1 degradation.

To examine the effects of probucol on degradation of mature hERG channels, we blocked forward trafficking with a Golgi transit inhibitor brefeldin A (BFA), which inhibits protein transport from the endoplasmic reticulum to the Golgi (Guo et al., 2009). The hERG-HEK cells were pretreated with BFA (10 μM) for 1 h and then cultured in the absence or presence of probucol in the continued presence of BFA. BFA blocked the conversion of hERG from its immature 135-kDa form into its mature 155-kDa form (Guo et al., 2009). Thus, the decrease in the 155-kDa band in the presence of BFA represents the rate of degradation of the pre-existing mature channels. As can be seen from the Western blot analysis and the normalized 155-kDa band intensity (Fig. 8A), in control cells with BFA, the 155-kDa band decreased by approximately half in 6 h. In contrast, in probucol-treated cells, the 155-kDa band decreased by approximately half in only 3 h (Fig. 8A). Probucol also concomitantly accelerated the decay of Cav1 expression in these cells (Fig. 8A). The function of hERG channels was also evaluated by whole-cell patch-clamp method. Blockade of forward trafficking by BFA (10 μM) resulted in a time-dependent reduction in I_{hERG} (Guo et al., 2009). As shown in Fig. 8B, under conditions in which protein forward trafficking was blocked by BFA, probucol significantly accelerated I_{hERG} reduction.

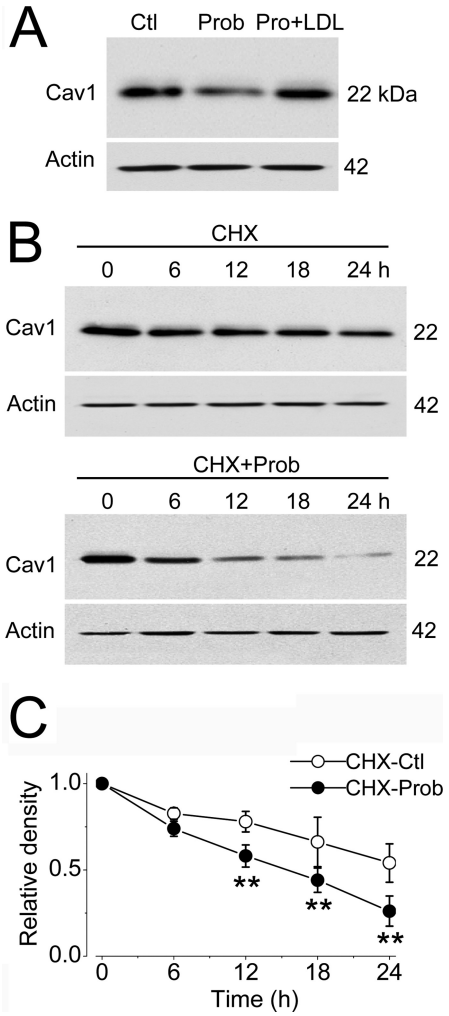

Fig. 7. Probucol reduces Cav1 expression in HEK 293 cells. A, Western blots showing Cav1 expression in HEK cells under control conditions (Ctl), with 100 μM probucol treatment for 48 h (Prob) or 100 μM probucol plus 0.3 mg/ml LDL treatment for 48 h (Prob + LDL). B, Western blots showing time-dependent effects of 100 μM probucol (Prob) on Cav1 expressions levels in CHX (10 μg/ml)-treated HEK 293 cells. Top, time-dependent reduction in Cav1 expression in cells treated with CHX; bottom: acceleration of time-dependent reduction in Cav1 expression in cells treated with CHX plus 100 μM probucol (CHX + Prob). CHX (10 μg/ml) was applied 1 h before and was present throughout the experiments to inhibit protein synthesis. At each time point, protein was extracted for Cav1 detection. C, summarized relative Cav1 expression levels at each time point. The Cav1 band intensities at each time point were normalized to the initial values and summarized ($n = 6$). **, $P < 0.01$ versus control.

Discussion

A large variety of compounds interferes with hERG function and has the potential to cause long QT syndrome by blocking the channel or reducing the channel membrane expression (Roy et al., 1996; Zhang et al., 2001; Ficker et al., 2004; Kuryshev et al., 2005; Rajamani et al., 2006; Guo et al., 2007; Wang et al., 2007). Probucol is a cholesterol-lowering drug that causes long QT syndrome and torsades de pointes arrhythmia in patients (Hayashi et al., 2004). We demonstrated previously that probucol reduces the expression levels of the mature form of ERG (I_{Kr} protein) in cultured cardiomyocytes and of 155-kDa hERG channels stably expressed in HEK 293 cells (Guo et al., 2007). However, the mechanisms of probucol-reduced hERG expression remain unknown.

Drug-induced reduction in cell-surface hERG density has been attributed to defects in hERG forward trafficking (Ficker et al., 2004). Blockade of hERG forward trafficking usually leads to an increase in the expression of the 135-kDa

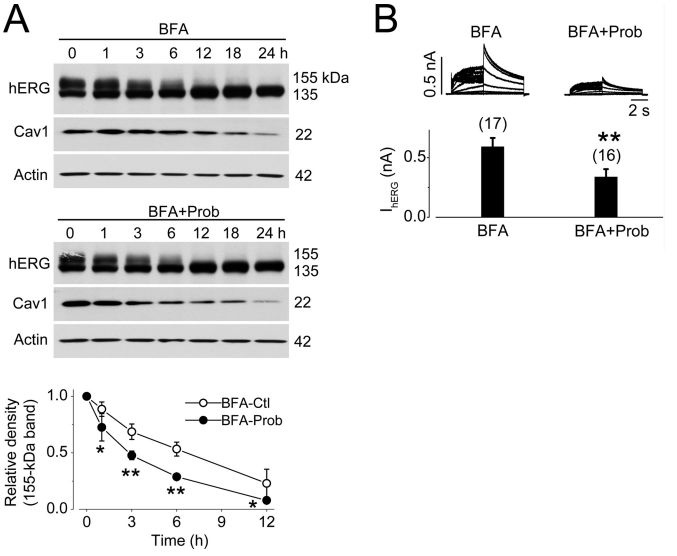

Fig. 8. Effects of probucol on hERG degradation. A, Western blots showing hERG and Cav1 expression levels in BFA (10 μM)-treated hERG-HEK cells cultured in the absence (BFA) or presence of 100 μM probucol (BFA + Prob). The intensity of the 155-kDa hERG band at each time point was normalized to its initial value and plotted against time ($n = 3$). *, $P < 0.05$, and **, $P < 0.01$ versus control. B, families of hERG currents at 6 h after BFA (10 μM) treatment in the absence or presence of 100 μM probucol (BFA + Prob). Bottom, the summarized hERG tail current amplitudes under each condition. The number above each column indicates the number of cells examined from three independent treatments. **, $P < 0.01$ versus control.

immature hERG band as shown in Fig. 8A. Our data show that although probucol decreases the upper (155 kDa) band, it does not concomitantly increase the lower (135 kDa) band (Fig. 3), suggesting that probucol may not block hERG forward trafficking. On the other hand, we demonstrated that accelerated hERG protein degradation from the cell membrane represents an alternative mechanism for drug-induced reduction of hERG channels. We show that probucol reduces the expression of mature hERG channels through a Cav1-dependent pathway, and such reductions can be rescued by the addition of LDL, a potent cholesterol carrier.

Caveolin and cholesterol are two important components of caveolae, which are specialized lipid rafts, forming 50- to 100-nm "flask-shaped" invaginations of the plasma-membrane of many cell types (Simons and Ikonen, 1997). Caveolae provide platforms for membrane protein interaction, signaling, trafficking, and degradation (Brown and London, 1998; Simons and Toomre, 2000; Helms and Zurzolo, 2004). Caveolin is a cholesterol-binding integral membrane protein essential for the formation of caveolae (Rothberg et al., 1992; Murata et al., 1995; Drab et al., 2001), Because of its close association with cholesterol, the expression level of caveolin is up-regulated in response to increased levels of cellular cholesterol (Fielding et al., 1997; Hailstones et al., 1998) and down-regulated in response to cholesterol depletion (Hailstones et al., 1998; Thorn et al., 2003).

Probucol inhibits cholesterol synthesis in cells and affects cell membrane lipid composition (Favari et al., 2004). Our data suggest that, by disrupting cholesterol level, probucol destabilizes Cav1 membrane expression and accelerates Cav1 turnover. Because of the physical association between mature hERG and Cav1 (Fig. 4), accelerated turnover of Cav1 may essentially drag in hERG for degradation, leading to an accelerated degradation rate and a decreased expression level of hERG in the plasma membrane. This notion is directly supported by our data; probucol accelerates Cav1 degradation (Fig. 7, B and C); LDL, a cholesterol carrier, effectively prevented probucol-induced reduction of Cav1 in HEK cells (Fig. 7A). LDL also prevented probucol-induced reduction of Cav1 and 155-kDa hERG in hERG-HEK cells (Fig. 3). Overexpression of Cav1 accelerated the probucol-induced hERG reductions (Fig. 5), whereas knockdown of Cav1 impeded the probucol effects (Fig. 6). In addition, when protein forward trafficking was blocked, probucol accelerated degradation of both Cav1 and 155-kDa hERG in hERG-HEK cells (Fig. 8A).

Because of the difficulties in culturing cardiomyocytes where caveolin-3 (Cav3) is innately expressed, we did not examine the time-dependent effects of probucol on the expression levels of Cav3 and rat *ether-à-go-go*-related gene-encoded protein (the I_{Kr} channel) in cardiomyocytes. However, we demonstrated previously that probucol reduces the 155-kDa band of rat *ether-à-go-go*-related gene protein in cultured neonatal rat cardiomyocytes (Guo et al., 2007). Cav3 is the structurally related muscle-specific caveolin gene family member whose role in striated muscle cells is largely analogous to that of Cav1 in nonmuscle cells (Rybin et al., 2003). We have demonstrated previously that Cav3 associates with the 155-kDa form of I_{Kr} (Massaeli et al., 2010). We showed that exposure of hERG-HEK cells to 0 mM K^+ medium triggers rapid internalization of membrane hERG channels and that Cav1 is involved in this process in a stable hERG cell line, and Cav3 is involved in the process in cultured neonatal rat ventricular myocytes (Massaeli et al., 2010).

In summary, our data demonstrated that probucol promoted Cav1 turnover rate, which accelerates hERG degradation, leading to a decreased hERG expression level in the plasma membrane. Furthermore, increasing cholesterol contents by the addition of LDL can efficiently impede the probucol effects. Our data provide evidence that by altering the membrane stability and turnover of hERG-interacting protein Cav1, probucol reduces hERG membrane expression, which represents a new mechanism for drugs to disrupt hERG channel function.

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Authorship Contributions

Participated in research design: Guo, Massaeli, and Zhang.
Conducted experiments: Guo, X. Li, Shallow, Xu, Yang, Massaeli, W. Li, and Sun.
Contributed new reagents or analytic tools: Pierce.
Performed data analysis: Guo, Yang, Massaeli, and Zhang.
Wrote or contributed to the writing of the manuscript: Guo, X Li, Shallow, Massaeli, W. Li, and Zhang.
Other: Zhang acquired funding for the research.

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