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Down-regulation of *ether-a-go-go-related* gene potassium channel protein through sustained stimulation of AT_1 receptor by angiotensin II

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ABSTRACT

We investigated the effects of AT_1 receptor stimulation by angiotensin II (Ang II) on human *ether-a-go-go-related* gene (hERG) potassium channel protein in a heterogeneous expression system with the human embryonic kidney (HEK) 293 cells which stably expressed hERG channel protein and were transiently transfected with the human AT_1 receptors (HEK293/hERG). Western-blot analysis showed that Ang II significantly decreased the expression of mature hERG channel protein (155-kDa band) in a time- and dose-dependent manner without affecting the level of immature hERG channel protein (135-kDa band). The relative intensity of 155-kDa band was 64.7 ± 6.8% of control ($P < 0.01$) after treatment of Ang II at 100 nM for 24 h. To investigate the effect of Ang II on the degradation of mature hERG channel protein, we blocked forward trafficking from ER to Golgi with a Golgi transit inhibitor brefeldin A (10 μM). Ang II significantly enhanced the time-dependent reduction of mature hERG channel protein. In addition, the proteasomal inhibitor lactacystin (5 μM) inhibited Ang II-mediated the reduction of mature hERG channel protein, but the lysosomal inhibitor bafilomycin A1 (1 μM) had no effect on the protein. The protein kinase C (PKC) inhibitor bisindolylmaleimide 1 (1 μM) antagonized the reduction of mature hERG channel protein induced by Ang II. The results indicate that sustained stimulation of AT_1 receptors by Ang II reduces the mature hERG channel protein via accelerating channel proteasomal degradation involving the PKC pathway.

1. Introduction

The human *ether-a-go-go-related* gene (*hERG*) encodes the pore-forming subunit of the channel protein responsible for the rapidly activating delayed rectifier K^+ current, I_{Kr}, which is crucial for terminal repolarization in the heart [1]. Reduction of hERG currents caused by mutations or drug-induced blockade of hERG channel produces hereditary or acquired long QT syndrome, a potentially lethal repolarization disorder associated with syncope, torsade de points arrhythmias, and sudden cardiac death [2–5]. In addition, hERG channel dysfunction is associated with heart diseases such as myocardial infarction, heart failure and atrial fibrillation [6,7]. However, the molecular mechanism underlying the pathologic change of hERG channel in chronic heart disease is not fully understood.

The renin-angiotensin system (RAS) plays a pivotal role in maintaining cardiovascular homeostasis. Emerging evidences indicate that over-activation of RAS is associated with the development of atrial and ventricular arrhythmias. At present, angiotensin-converting enzyme (ACE) inhibitors and AT_1 receptor blockers (ARBs) are the most effective therapeutic agents in the prevention of arrhythmias in patients with heart failure [8,9]. Different mechanisms have been postulated to explain the clinically beneficial effects of RAS inhibition [9]. For example, a recent study has demonstrated that AT_1 receptor-mediated signaling directly contributed to the increase in arrhythmogenicity in hypertrophied hearts independently of structural remodeling [10]. The major effector of RAS, angiotensin II (Ang II), has been shown to directly modulate ion channel function of cardiac myocytes [11–15]. However, available information is limited regarding the direct modulation of Ang II on hERG channel. We previously reported that Ang II exhibited an acute inhibitory effect on I_{Kr} in cardiac ventricular myocytes or hERG currents in a heterogeneous expression system through AT_1 receptor by PKC activation [13]. It is known that the abundance of hERG channel protein in the plasma membrane is a key determinant of hERG channel functionality. Ion channel protein forward trafficking and degradation are the targets of

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posttranslational modifications under pathophysiological conditions [16]. So far, the possible effect of Ang II on hERG channel protein expression still remains unknown. In the present study, we examined the effects of Ang II on hERG channel protein expression and two posttranslational processes of forward trafficking and degradation in the human embryonic kidney (HEK) 293 cells which stably expressed hERG channel proteins and were transiently transfected with the human AT_1 receptors (HEK293/hERG). The results may provide new insights into the mechanism underlying hERG channel remodeling in chronic heart disease.

2. Materials and methods

2.1. Cell culture

HEK293 cell line stably expressing hERG WT channel protein was established as previously described [17]. The cells were maintained at 37 °C, 5% CO_2 in DMEM supplemented with 10% fetal bovine serum, 2 mM/ml L-glutamine, 100 U/ml penicillin/streptomycin, and 200 μM/ml G418. The cells were grown in 35-mm dishes at 60–70% confluence, the human AT_1 receptor cDNA (kindly provided by Dr. Vauquelin, Vrije University of Brussel, Brussel, Belgium) and the plasmid pcDNA3-WT-hERG, pcDNA3-A422T-hERG or pcDNA3-H562P-hERG cDNA (gifts from Dr. Chiamvimonvat, University of California at Davis, CA, USA) were transiently transfected into the cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), respectively. Drug treatment was initiated after 24 h of transfection.

2.2. Western blot analysis

Whole-cell lysates prepared from the HEK293/hERG cells were used for analysis. In brief, cells were solubilized for 1 h at 4 °C in lysis buffer containing 150 mM NaCl, 1 mM EDTA, 50 mM Tris, pH 7.5, 1% Triton X-100, and protease inhibitors cocktail (Sigma, St. Louis, MO, USA). Protein concentrations were measured with a BCA protein kit (Thermo Scientific, Waltham, MA, USA). For detection of hERG channel protein levels, protein was separated on 7.0% SDS polyacrylamide gels, transferred to polyvinylidene difluoride membrane. The membrane was then blocked using 10% non-fat milk and 0.08% Tween 20 in Tris-buffered saline and immunoblotted with a rabbit anti-hERG primary antibody (anti-Kv11.1, 1:200, Alomone, Jerusalem, Israel) overnight at 4 °C. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference for loading control. The protein signals were detected using fluorescently labeled secondary antibody. For quantitative analysis, signals were captured directly on Odyssey Infrared Imaging System.

2.3. In-cell Western

For detection of surface expression of hERG channel protein in HEK293cells, the same method was used as described by Chang and colleagues [18]. The cells were transiently transfected with AT_1 receptor, grown on 96-well plates, and treated with Ang II (100 nM) or vehicle control (medium) for 24 h. The cells were washed and fixed with 4% ice-cold paraformaldehyde for 10 min, and blocked with 5% bovine serum albumin in phosphate-buffered saline solution directly in 96-well plates. The cells were subsequently incubated for 60 min at 4 °C with anti-hERG antibody (1:200), rinsed three times in PBS at 4 °C with 1% BSA/PBS, and incubated with fluorochrome-conjugated secondary antibody in 1% BSA/PBS for 60 min at room temperature in the dark. The cells were washed thoroughly 6 times at 4 °C (initially 3 times in 1% BSA/PBS, and then 3 times in PBS without BSA) and scanned using an Odyssey Infrared Imaging System.

2.4. Data analysis

Data are expressed as the mean ± SEM. A one way analysis of variance in conjunction with two-tailed Student's *t*-test was used to determine the statistical significance between two groups. A *P*-value of 0.05 or less was considered statistically significant.

3. Results

3.1. Ang II selectively down-regulates mature hERG channel protein expression

The effect of Ang II on hERG channel protein expression in HEK293/hERG cells was examined using Western blot analysis and the data are shown in Fig. 1. The hERG channel protein displayed two bands with molecular masses of 135-kDa and 155-kDa, which represented the immature core-glycosylated form residing in the endoplasmic reticulum and the mature fully glycosylated form in the plasma membrane, respectively [17,19,20].

Our previous patch-clamp recordings showed that the inhibitory action of Ang II on hERG reached saturation at 10 min [13]. A recent study has demonstrated that activation of protein kinases can acutely regulate the intracellular distribution of SCN5A channels and thus change the number of functional Na^+ channels on the plasma membrane [21]. To investigate the possible change of hERG channel protein abundance on the plasma membrane involved in the functional regulation of hERG channel by Ang II, we first studied the effects of Ang II on hERG channel protein levels at 10 min and 24 h after continuous exposures. Ang II (10–1000 nM) did not affect the intensities of both 135-kDa and 155-kDa bands after 10 min exposure (Fig. 1A), however, the intensity of the 155-kDa hERG band, but not the 135-kDa hERG band was significantly decreased after 24 h exposure (Fig. 1B). The relative values of mature protein (155-KDa) are 75.2 ± 5.1%, 64.7 ± 6.8%, and 57.2 ± 3.6%, respectively, compared to those of controls ($P < 0.01$) after treatment of Ang II at 10, 100 and 1000 nM for 24 h (Fig. 1B). The time course of protein expression after treatment of Ang II at 100 nM is shown in Fig. 1C and it showed that the 155-kDa band was significantly decreased ($P < 0.01$) as early as 12 h. The results suggest that the selective inhibition of mature protein expression by Ang II exhibits time- and dose-dependent manners.

3.2. Reduction of mature hERG channel protein expression may not be caused by disruptive forward trafficking

Increasing evidence has indicated that defective hERG protein trafficking contributes to the down-regulation of channel function in drug- and mutation-causing LQT [21–23]. To investigate the possible mechanism underlying the hERG channel protein reduction induced by Ang II, we performed parallel experiment to compare the Western blots phenotypes of fluoxetine treatment, channel mutants and Ang II treatment. Previous studies have demonstrated that fluoxetine [24] or mutants of A422T-hERG [25] and H562P-hERG [26,27] could cause trafficking disturbance. The data showed that fluoxetine (3 μM) decreased the intensity of the 155-kDa band to 61.5 ± 3.6% of control ($P < 0.01$, Fig 2A) and the intensity of 155-kDa band was also significantly reduced in A422T-hERG and H562P-hERG mutants (Fig 2B). Concomitantly, the 135-kDa band was remarkably increased with fluoxetine treatment or mutations, indicating that blockade of hERG channel protein forward trafficking resulted in ER retention of the protein.

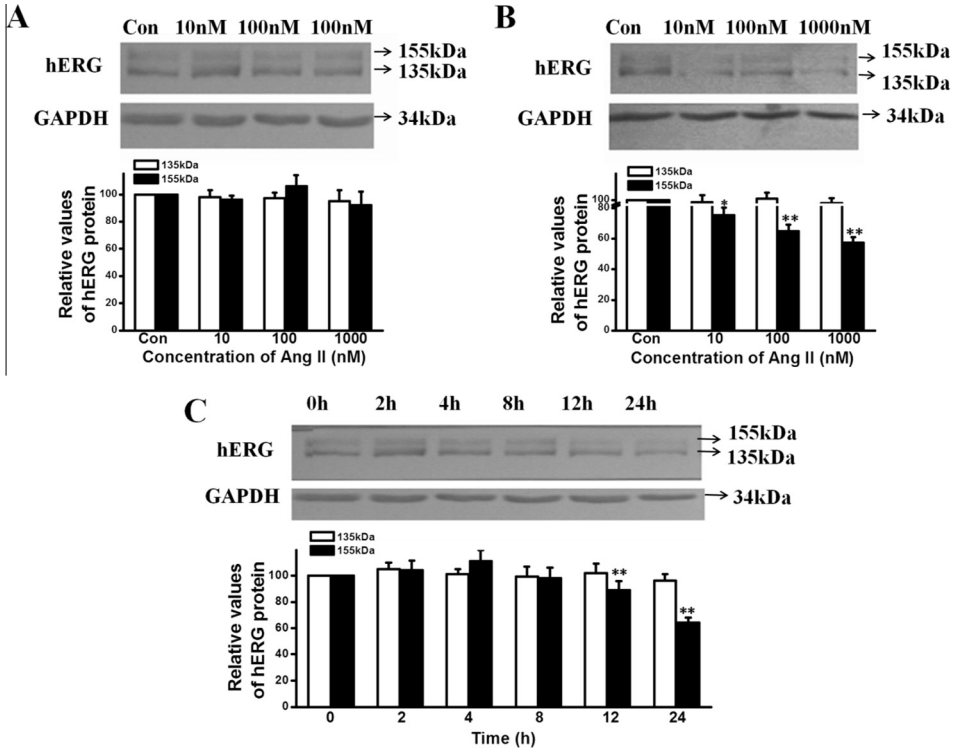

Fig. 1. Effect of Ang II on the expression of hERG channel protein. (A) and (B) Representative Western blots bands in control (Con) and after exposure to different concentrations of Ang II for 10 min (A) or 24 h (B) and summary data for relative intensity of 135-, 155-kDa band from four independent experiments; (C) Time course of response to Ang II (100 nM) and summary data are shown from four representative independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to control.

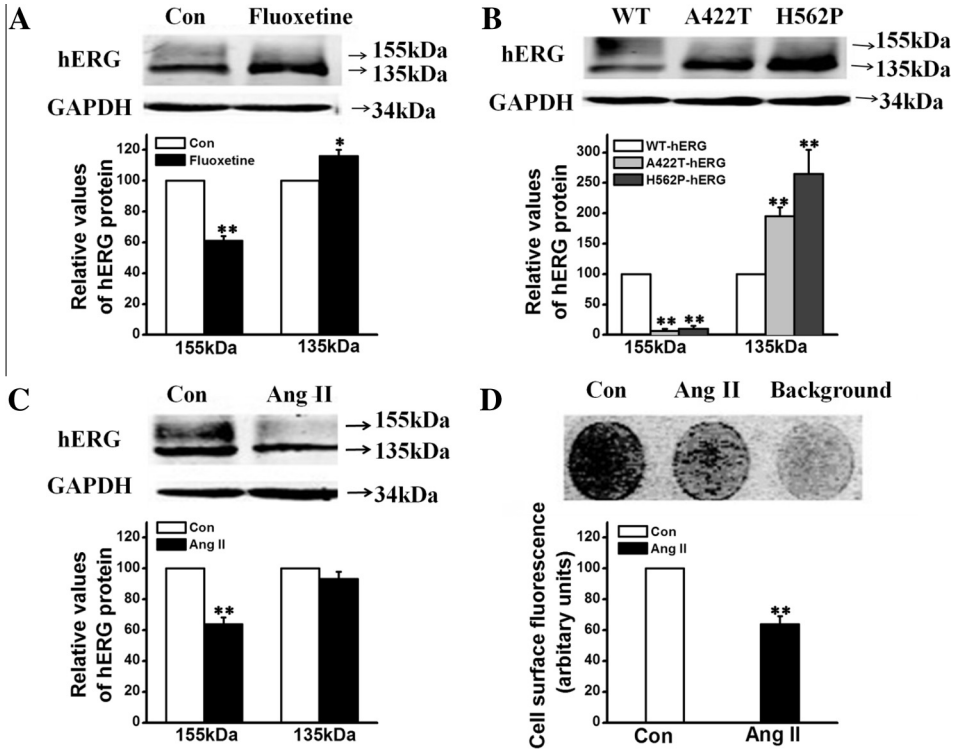

Fig. 2. Comparision of Western blots phenotypes. (A) Representative Western blots bands in the presence of Fluoxetine (3 μM) and corresponding summary data from five independent experiments; (B) Western blot analysis for WT, and A422T and H562P mutant hERG channels. Summary data are shown from three independent experiments; (C) Representative Western blots bands in the presence of Ang II (100 nM) and corresponding summary data from four independent experiments; (D) Cell surface fluorescence of hERG mature protein after 24 h treatment of Ang II (100 nM) by in-cell Western from three representative independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to control.

However, Ang II (100 nM) decreased the 155-kDa band to 64.7 ± 6.8% of control without affecting the 135-kDa band (Fig. 2C). Similar degree of decrease in mature hERG channel protein of cell surface hERG channel on the plasma membrane was also observed by in-cell Western analysis (Fig. 2D). The results suggest that Ang II-induced reduction of the mature hERG channels may not be caused by disruptive forward trafficking.

3.3. Ang II enhances the degradation of mature hERG channel protein via proteasome pathway

The abundance of the mature hERG channel protein on the plasma membrane depends on the balance between the forward trafficking and retrograde degradation. In order to examine the effects of Ang II on degradation of mature hERG channel protein in HEK293/hERG cells, we blocked forward trafficking with brefeldin A (BFA), an inhibitor of protein transport from the endoplasmic reticulum to the Golgi [20,28]. The cells were pretreated with BFA (10 μM) for 1 h and then added Ang II (100 nM) or vehicle (as control) in the continued presence of BFA for Western blot analysis. The data showed that BFA alone resulted in a time-dependent reduction of the 155-kDa band (Fig. 3A), reflecting the dynamic degradation of mature hERG protein [29]. Ang II further significantly accelerated the degradation of the mature hERG protein (Fig. 3B). The data in Fig. 3C summarized the time course of relative values of mature hERG channel protein and showed that Ang II significantly decreased the mature hERG channel protein compared to the control from 4 to 24 h. Thus, the results indicate that Ang II promotes the degradation of mature hERG channel protein by sustained stimulation of AT_1 receptor.

Intracellular proteolysis of channel protein can take place either in the endosome/lysosome or in the cytosol by the proteasome [30]. Therefore, we tried to identify the possible contribution of proteasome or lysosome pathway in protein degradation. The data in Fig. 4A illustrates the effects of lactacystin, a proteasome inhibitor, and bafilomycin A1, a lysosomal inhibitor, on hERG channel protein degradation induced by Ang II in HEK293/hERG cells. Lactacystin (5 μM) significantly inhibited the effect of Ang II in the reduction of 155-kDa band ($P < 0.05$), whereas, bafilomycin A1 (1 μM) did not significantly affect the effect of Ang II on this band ($P > 0.05$) (Fig. 4B). The findings suggest that the effect of Ang II on the hERG channel protein degradation is mainly through the proteasome pathway.

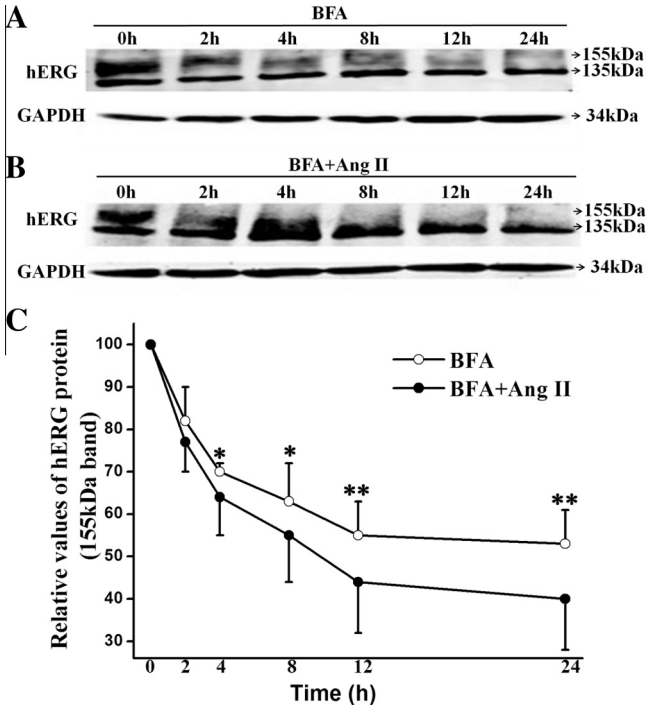

Fig. 3. Effect of Ang II on hERG channel protein degradation. (A and B) Representative Western blot analysis showing a dynamic degradation of hERG channel protein in the presence of forward trafficking blocker brefeldin A (BFA, 10 μM) alone or with Ang II (100 nM). (C) Summary data from three independent experiments showing time-course response. The hERG channel protein values were normalized by control. $^*P < 0.05$ and $^{**}P < 0.01$ compared to control.

3.4. Ang II reduces the mature hERG channel protein via activation of PKC pathway

Activation of AT_1 receptor by Ang II is classically coupled to Gαq/Gα11 to further activate phospholipase C, Ca^{2+}, and PKC signaling pathways. To determine whether the reduction of hERG channel protein by activation of AT_1 receptor is mediated by PKC, bisindolylmaleimide 1 (Bis-1), an inhibitor of PKC, was used for the study (Fig. 4C). Bis-1 (10 μM) slightly decreased the levels of both mature and immature hERG channel proteins (data not shown). Ang II (100 nM) significantly decreased the intensity of 155-kDa band ($P < 0.01$) in HEK293/hERG cells, however, the effect of Ang II was abolished when Bis-1 (10 μM) was pretreated 1 h before Ang II (Fig. 4D). The results suggest that the effect of Ang II on the mature hERG channel protein expression is involved in PKC pathway.

4. Discussion

The present study was designed to investigate the effects of activation of AT1 receptor upon hERG protein expression levels. Our data demonstrated that Ang II produced an inhibitory action on hERG protein expression, which manifested by a selective reduction of mature hERG abundance. The inhibition took place within hours, indicating it was a chronic response. The abundance of hERG in the plasma membrane is a key determinant of the hERG current. Thus, it is speculated that by down-regulation of ion channel abundance, Ang II contributes to cardiac electrical remodeling in chronic heart disease. The result may provide an explanation for the clinical beneficial effects of RAS inhibition. However, since our experiments have been done in HEK cells, further investigation is warranted to demonstrate the effect of Ang II on hERG channel protein expression in the native cardiomyocytes and to elucidate whether Ang II-mediated regulation contributes to in vivo cardiac electrical remodeling under pathophysiological conditions.

The expression of hERG channel protein on the plasma membrane is a balance between anterograde trafficking and retrograde degradation from the cell surface. A selective reduction of mature hERG protein on the membrane by sustained stimulation of AT1 receptor may prompt a defective trafficking and/or an enhanced degradation. Our data show that the defects in forward trafficking causes by mutant channels or drug-treatment is characterized by an remarkable increase in the immature protein in addition to the reduction of mature protein. The results are consistent with the previous reports [24,25,27]. Whereas, Ang II displays a different phenotype, decrease in the 155-kDa band without a concomitant increase in the 135-kDa band. The results suggest that the effects of Ang II may be not due to blocking hERG channel protein forward trafficking. Correspondingly, we find that Ang II significantly enhances the time-dependent reduction of mature hERG channel protein under the condition of inhibiting forward protein trafficking. The result indicates that Ang II accelerates the degradation of mature hERG channel protein.

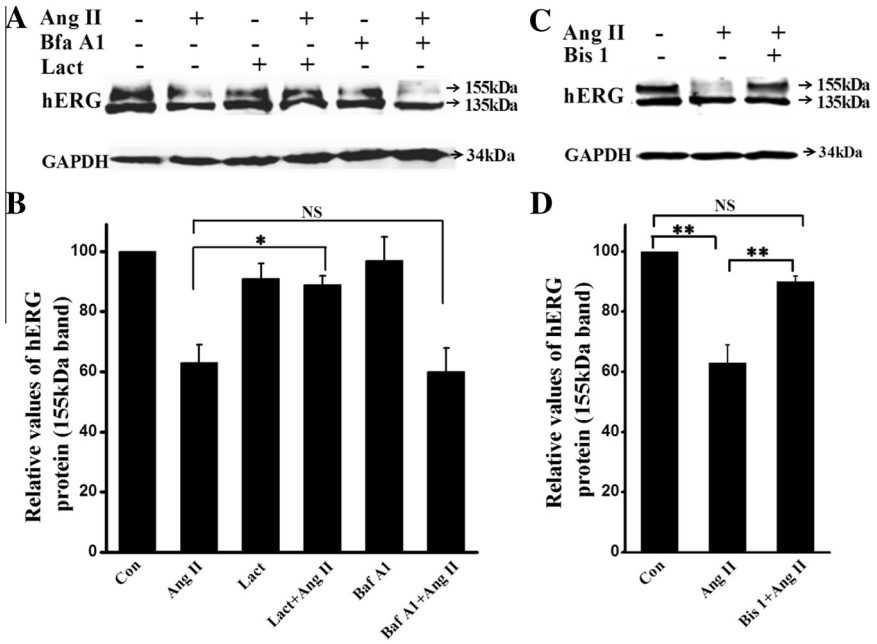

Fig. 4. Effect of proteasome inhibitor lactacystin (Lact), lysosomal inhibitor bafilomycin A1 (Baf A1), and PKC inhibitor bisindolylmaleimide 1 (Bis-1) on the action of Ang II (100 nM). (A) and (B) Representative Western blot analysis in the presence of Lact (5 μM) or Baf A1 (1 μM) and corresponding summary data. (C) and (D) Representative Western blot analysis in the presence of Bis-1 (100 nM) and corresponding summary data. The levels of hERG channel protein were normalized by control. $^{*}P < 0.05$; $^{**}P < 0.01$. All summary data are from three independent experiments.

At present, the mechanism for degradation of mature hERG channel protein on the plasma membrane is largely unknown. Eukaryotic cells exhibit at least two degradation pathways: a clathrin-dependent lysosome pathway and caveolin-dependent ubiquitin proteasome pathway. A recent study has demonstrated that ubiquitin-proteasome pathway plays a role in the degradation of hERG channel protein [31]. A reduction in extracellular K^+ concentration has been shown to induce endocytic degradation of the cell surface hERG channel protein, which is mediated by caveolin, but not by clathrin. It seems that proteasomal degradation pathway is an important mechanism for control of hERG channel protein. Consistent with these observations, our study finds that the reduction of mature hERG channel protein by Ang II is attenuated by a proteasomal inhibitor lactacystin, but not by a lysosomal inhibitor bafilomycin A1, suggesting that Ang II may enhance hERG channel protein degradation through proteasomal pathway and thus down-regulate hERG channel protein expression at the cell surface. In addition, a non-selective PKC inhibitor Bis-1 almost completely abolishes the action of Ang II, suggesting that the effects of Ang II are largely PKC-dependent.

Up to date, most studies have been focused on PKC acute regulation on the hERG channel function. The studies of regulation of PKC on surface expression of the hERG channel protein are limited with controversial data. It has been reported that sustained α_{1A}-adrenergic stimulation produced a profound augmentation of hERG channel protein mediated by PKC [32]. PKC can directly phosphorylate hERG channel protein and thus enhance synthesis and/or translation rates of the channel proteins [32]. A recent report has revealed that chronic activation of muscarinic receptor increases the mature hERG channel protein expression by slowing protein degradation via PKC pathway [33]. In hERG-expressing cells, diacylglycerol causes a reduction of surface hERG channel protein due to a rapid PKC-evoked internalization via dynamin-dependent endocytosis without affecting synthesis and/or trafficking of the protein [34]. Our results suggest that PKC may enhance hERG channel protein degradation via proteasomal pathway. Obviously, G-protein-coupled receptors produce distinct effect on surface expression of the hERG channel protein through activation of PKC. The discrepancy of PKC effect may, at least in part, result from activation of specific PKC-isoforms by distinct G-protein-coupled receptors and thus activation of separate signaling pathways. However, the differential effects of specific PKC subtypes on the abundance of the channel protein are unknown. Future studies are necessary to determine which isoform of PKC is involved with sustained stimulation of AT_1 receptor on mature hERG channel protein.

In summary, our data demonstrate that the sustained stimulation of AT_1 receptor by Ang II reduces the mature hERG channel protein through degradation by PKC activation. The finding may help us to better understand the regulation of ion channel and the degradation of plasma membrane protein.

Conflict of interest

Authors state no conflicts of interest.

Acknowledgments

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