Sex hormones ameliorated sodium channel dysfunction induced by β-adrenergic overstimulation: the role of estrogen and G protein-coupled estrogen receptor

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Abstract

Background and Purpose: Sex hormones affect heart rhythm by regulating ion channels, but the effect of estrogen on cardiac voltage-gated sodium channel in stress-induced pathological conditions is currently not well defined. In this study, we explored the impact of various concentrations of estrogen and the role of its rapid receptor G protein-coupled estrogen receptor (GPER) on sodium channel function in a simulated cardiac stress model of human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs). Experimental Approach: Isoproterenol treated hiPSC-CMs were pre-incubated with various concentrations of β-Estradiol. Their sodium channel electrophysiological function and action potential were dissected by patch clamp and the content of sodium channel was observed by immunohistochemical method combined with laser scanning confocal microscopy. The GPER-specific effect was determined with agonists G1, antagonists G15 and small interfering RNA and sodium channel electrophysiology was selectively detected. Key Results: Isoproterenol-induced stress increased peak sodium current and late sodium current, and shortened action potential duration but the effects of stress were eliminated by β-Estradiol. Pearson Correlation analysis demonstrated no association between estrogenic effects on sodium currents versus content of sodium channel. Activation of GPER produced similar effects as β-Estradiol, while inhibition of GPER cancelled the effects induced by β-Estradiol. Conclusion and Implications: Estrogen through its rapid signal receptor GPER ameliorated the detrimental effects of β-adrenergic overstimulation like in cardiac stress on sodium channel dysfunction in hiPSC-CMs. These results are of great clinical significance as we need to understand the role of sex hormones in cardiovascular disease.

Introduction

Cardiac voltage-gated sodium channel responsible for the peak sodium current (I_{Na}) and late sodium current (I_{NaL}) contributes to the generation of the action potential (AP) that maintains normal heart function (Portero et al., 2017; Vikram et al., 2017). It is a necessary responser for physiological β-adrenergic stimulation resulting in increased conductivity and excitability of cardiomyocytes (Dybkova N, 2014; Grinshpon & Bondarenko, 2016). However, evidence suggests that β-adrenergic overstimulation induced by catecholamines as is the case during arrhythmia and heart failure disturbed sodium channel function characterized by the increase in I_{Na} andI_{NaL} (Dybkova N, 2014; Bence Hegyi et al., 2018). Abnormal elevation of these two currents has been associated with arrhythmias and contractile dysfunction (Dybkova N, 2014; Eiringhaus et al., 2019).

Numerous studies have confirmed that sex hormones, especially estrogen, regulated cardiac contraction

and rhythm which are controlled by ion channels. It was reported that estrogen upregulated L-type calcium current in women and female rabbits thereby prolonged AP duration (Papp et al., 2017; X. Yang et al., 2018). Moreover, it increased the delayed rectifier current shortening QTc interval hence corrected arrhythmia (Anneken et al., 2016). Some studies focused on sodium currents in male and female, suggested that the larger dispersion ofI_{Na} in female ventricle may be related to sex-specific arrhythmias (Barajas-Martinez et al., 2009). Female had larger I_{NaL} than male, which may be the cause of long QT-related arrhythmia in female (Lowe et al., 2012).

We have previously demonstrated that estrogen antagonized the inhibitory contraction in Takotsubo (stress) cardiomyopathy and decreased the risk of stress-related cardiac arrhythmia by influencing β-adrenergic receptor (βAR) signaling (Cao X, 2015; Hou et al., 2018). Currently, the cellular mechanisms through which estrogen modulates sodium channel in the context of stress are elusive. We, therefore, hypothesized that estrogen may correct the abnormal elevation ofI_{NaL} and peak I_{Na} caused by β-adrenergic overstimulation, thereby normalize electrical activity of cardiomyocytes.

A stress model of human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) was established using isoproterenol (ISO) treatment. G protein-coupled estrogen receptor (GPER) agonists and antagonists and small interfering RNA technology were used to manipulate and dissect GPER-specific effects onI_{NaL} and peak I_{Na}.

Our data indicate that β-adrenergic overstimulation caused abnormal increase in I_{NaL} and peakI_{Na} and this effect was abolished by estrogen through GPER. We conclude that targeting GPER may be a novel strategy for maintaining sodium channel function under cardiac stress situations.

Methods

Cell culture

Spontaneously-beating hiPSC-CMs derived from normal female epithelial cells (IMR90, Wicell, USA) were cultured for 40±10 days. The induction, differentiation and culture procedures of hiPSC-CMs awere as previously reported (Burridge et al., 2014; Lian et al., 2013).

Materials

Isoprenaline hydrochloride (ISO, Sigma, USA, Lot # WXBC9656V) was dissolved in 0.01 mol/L HCl solution (36˜38%), and then PBS solution was added to prepare 10^{-3} mol/L ISO mother liquor. β-Estradiol (E2, Sigma, USA, Lot # SLBT2822) was dissolved in DMSO solution (the concentration of DMSO in extracellular fluid was not higher than 0.1%) to prepare 2×10^{-1}mol/L E2 mother liquor. G1 (Cayman, USA, Lot # 0459034-3) and G15 (Cayman, USA, Lot # 0458784-13) were dissolved in DMSO solution, and then PBS solution was added to prepare G1 mother liquor and G15 mother liquor respectively. These liquids were sub packed and stored in dark at negative 20 and dilute to the desired concentration when used.

Establishment of a cardiac stress model in hiPSC-CMs

By using protocols of modeling cardiac stress of our lab, together with methods described previously (Borchert et al., 2017; Zhao et al., 2018), 10 μmol/L ISO was used as the stress stimuli. In summary, hiPSC-CMs were incubated with 10 μmol/L ISO for 30 min to simulate cardiac stress.

GPER silencing by small interfering RNA

According to the procedure given by GenePharma (Suzhou, China), the transfection complex solution containing 16.7 nmol/L siRNA (GenePharma, China) was prepared, and then co-cultured with hiPSC-CMs serum-free medium at 37 for 4-6 hours. After that, the culture medium was replaced with serum-free medium and then cultured overnight prior to experimentation. Detail in GPER gene silencing sequence:

sense (5'-3') : GGCCUCAUCUGGAUGGCAUTT;

antisense (5'-3') : AUGCCAUCCAGAUGAGGCCTT.

Patch clamp measurements

Recording of sodium currents

The recording of sodium currents was performed as previously reported (T. Yang et al., 2014). The intracellular solution contained (in mmol/L): 5 NaF, 110 CsF, 20 CsCl, 10 EGTA and 10 Hepes. Adjusted the pH to 7.4 with CsOH. The external solution contained (in mmol/L): 135 NaCl, 4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 $C_6H_{12}O_6$ and 10 Hepes. The pH was adjusted to 7.4 with NaOH. In addition, 0.005 mmol/L Nifedipine (Sigma, USA, Lot # MKCB9232) and 0.5 mmol/L 4-aminopyride (MedChemExpress, USA, Lot # 27027) were used to eliminate L-type calcium current and outward potassium currents.

Sodium currents were measured at room temperature (22-24). Recording microelectrodes were made of hard glass and their tip resistances were between 2˜4 MΩ. The protocol for recording sodium current and sodium channel dynamics is as follows: (1) PeakI_{Na}, current-voltage curve and activation were measured from a holding potential of -120 mV, and were elicited with steps of 5 mV from -90 mV to +60 mV with a cycle length of 500 ms. (2) Steady state inactivation was measured with a double pulse. The conditioning pulse was held at -120 mV, with steps of 5 mV from -90 mV to +60 mV with a cycle length of 500 ms, and followed a single 50 ms test pulse at -30 mV. (3) Recovery from inactivation was measured by double pulse as well. The conditioning pulse was held at -120 mV, and the first test pulse was kept at -30 mV for 50 ms, then recovered to -120 mV, with the interval of 2, 4, 6, 8, ····· 49, 51 ms, and this was followed by a single 50 ms test pulse at -30 mV. (4) LateI_{Na} was elicited from -120 mV to -30 mV with a cycle length of 500 ms.

Voltage clamp protocols are inset in figures. EPC10 patch clamp amplifier and PatchMaster version v2x73.2 software (HEKA, Germany) are used for data acquisition. The stimulation frequency was 1 Hz and the sampling frequency was 20 kHz. To increase data accuracy, ˜70% of the series resistance was corrected and automatic leakage compensation was carried out. Origin 2017 software (OriginLab, USA) was used to analyze data and prepare figures. Data were presented as the current amplitude / cell capacitance to reduce intercellular error. Activation and inactivation curves were fitted with Boltzmann function ($y=\{1+\exp[(V - V_{1/2})/k]\}^{-1}$), and recovery from inactivation curve was fitted with single exponential function ($y = A1*\exp(-x/t1)+y0$). The averageI_{NaL} from 50 to150 ms after the test pulse was used (Glynn et al., 2015).

Acquisition of action potential

Spontaneously-beating clusters hiPSC-CMs were used to record action potentials (APs) in current-clamp mode (Free gap mode). The intracellular solution contained (in mmol/L): 150 KCl, 1 $MgCl_2$, 10 EGTA, 5 ATP Na2 and 5 Hepes. Adjusted the pH to 7.2 with KOH. The external solution contained (in mmol/L): 135 NaCl, 4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 $C_6H_{12}O_6$ and 5 Hepes. Adjusted the pH to 7.4 with NaOH. Data were analyzed by ABF Utility and MiniAnalysis software. APs were filtered at 2.9 kHz and digitized at 100 Hz respectively.

Immunohistochemical analysis combined with laser scanning confocal microscopy

hiPSC-CMs were fixed in 4% paraformaldehyde, treated with Triton X-100 and blocked with 3% BSA (sigma, USA). The cells were incubated with anti-$Na_v1.5$ (Alomone, Israel, Lot # ASC005AN4202) diluted in 1% BSA (1:100) overnight at 4, in darkness. The cell was washed with PBS and incubated with Peroxidase-conjugated Goat Anti-Rabbit IgG (H+L) diluted in 1% BSA (1:400) at room temperature in the dark for 1 hour. Subsequently, DAPI was added to hiPSC-CMs, covered with a glass slide, fixed around nail polish, and photographed by Olympus FV1000 (Olympus, Japan). The images were analyzed with OLYMPUS FLUOVIEW Ver.3.1b software (Olympus, Japan).

Experimental Design

On the basis of many studies, target group size (n) [?] 5 were calculated (Portero et al., 2017; T. Yang et al., 2014). The number of small dishes used in each group is at least 3 independent dishes. The exact group size (n) for each experimental group is found in supplementary material online.

The study did not contain clinical studies or patient data or animal experiments. hiPSC-CMs were randomised for treatment. In this study, hiPSC-CMs were incubated with various concentrations of E2 (0.01, 1 and 100 nmol/L, 1 h). The stress model of hiPSC-CMs was pre-treated with E2 (0.01, 1 and 100 nmol/L, 30 min prior to ISO). hiPSC-CMs were treated with G1 (11 nmol/L, 30 min) or G15 (20 nmol/L, 30 min) and small interfering RNA.

Data and Statistical Analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2018). GraphPad Prism, Clampfit, Origin 2017, and ImageJ software were used for data analysis. Data are shown as Mean +- S.E.M. Mean difference between groups were compared with Unpaired Student's t -test and One-way ANOVA. Unpaired t -test with Welch's correction and Kruskal-Wallis test were used selectively in the case of uneven variance. Pearson Correlation Coefficient was conducted to determine the correlation between content of $Na_v1.5$ and sodium current. P <0.05 was considered significant.

Results

Estrogen reduced the ISO-induced increase in I_{NaL}

hiPSC-CMs were incubated with different concentrations of E2 (0.01 nmol/L, 1 nmol/L and 100 nmol/L, 1h) to dissect estrogenic effects on cardiacI_{NaL}. Results showed that E2 at any concentration did not affect the average I_{NaL} density in hiPSC-CMs (Figure 2A and B). ISO treatment significantly elevatedI_{NaL} density (Figure 1A and Figure 2A), but pre-treatment with 1 nmol/L E2 abated the increase (Figure 2A and C). Detailed data on Mean +- S.E.M and the number of cells are found in supplementary material online, Supplemental Table 1.

Estrogen suppressed the ISO-induced increase in peak I_{Na} amplitude but did not alter I_{Na}kinetics

Exposure of hiPSC-CMs to different concentrations of E2 (0.01 nmol/L, 1 nmol/L and 100 nmol/L, 1h) increased peak I_{Na}density (Figure 3A-C), but did not affect the $V_{1/2}$ and$k$ values of activation and inactivation or τ value of recovery from inactivation (Figure 3F-L, Supplemental Figure 2A). Treatment of hiPSC-CMs with ISO resulted to an increase in peakI_{Na} (Figure 1B and C, Figure 3A), and the effect was blunted by 1 nmol/L E2 (Figure 3A, D and E). Interestingly, a significant difference was observed in peakI_{Na} between 0.01 nmol/L E2 and 1 nmol/L E2 in cells exposed to ISO indicating that the effects of E2 were concentration dependent (Figure 3A, D and E). However, E2 at any concentration did not rescue the increase of the $V_{1/2}$values of activation and inactivation and the τ value of recovery from inactivation induced by ISO (Figure 3M-S, Supplemental Figure 2B). Full statistical details on Mean ± S.E.M and the number of cells are found in supplementary material online, Supplemental Table 2-7.

Estrogen normalized action potential parameters of hiPSC-CMs under stress conditions

To assess the effect of E2 on electrophysiological function of hiPSC-CMs, we recorded APs of cells exposed to ISO under various concentrations of E2. Representative APs and slope curves of hiPSC-CMs are shown (Figure 4A). Notably, E2 (1 nmol/L and 100 nmol/L) decreased AP amplitude (APA) in hiPSC-CMs (Figure 4B). E2 (100 nmol/L) decreased resting membrane potential (RMP) (Figure 4C). Moreover, E2 at all concentrations decreased maximal action potential upstroke velocity ($\mathrm{d}V/\mathrm{d}t_{\mathrm{max}}$) (Figure 4D). In addition, action potential duration at 50% repolarization (APD_{50}) and action potential duration at 90% repolarization (APD_{90}) were prolonged by 1 nmol/L E2 (Figure 4E-G). In contrast, ISO stimulation decreased APA, $\mathrm{d}V/\mathrm{d}t_{\mathrm{max}}$ and shortened action potential duration at 10% repolarization (APD_{10}), APD_{50} and APD_{90} but had no effect on RMP (Figure 1K-Q). In cells treated with ISO, E2 had no effects on APA, $\mathrm{d}V/\mathrm{d}t_{\mathrm{max}}$ and APD_{10} (Figure 4H, J and K). In comparison, E2 prolonged APD_{50} (at only 100 nmol/L) and APD_{90}(Figure 4L and M). Interestingly, although E2 at 0.01 nmol/L and 1 nmol/L had no effect on RMP, they markedly decreased RMP under stress state (Figure 4C and I). Complete data on Mean ± S.E.M are provided in supplementary material online, Supplemental Table 8.

No correlation between sodium currents versus content of $Na_v1.5$ under estrogen treatment

Immunohistochemical staining performed to explore the effect of E2 on protein content of $Na_v1.5$ revealed that E2 at 1 nmol/L decreased whereas 100 nmol/L increased the $Na_v1.5$ protein staining signal (Supplemental Figure 6A and B; Supplemental Table 9). To determine the correlation between content of $Na_v1.5$ and sodium currents, Pearson Correlation Coefficient was conducted. The results showed that there was no significant relationship between the effect of E2 on $Na_v1.5$ content and currents (Supplemental Figure 6A, B, E and F). However, after ISO stimulation, E2 at all concentrations reversed the increase in the content of $Na_v1.5$ induced by ISO (Supplemental Figure 6A, C and D; Supplemental Table 9). Pearson Correlation analysis demonstrated no association between the influence of E2 on $Na_v1.5$ content and currents after ISO treatment (Supplemental Figure 6A, C-F).

Activation of GPER increased peak I_{Na} but had no effect on I_{NaL} and I_{Na} kinetics

To further investigate whether the effect of estrogen is mediated by GPER, hiPSC-CMs were treated with GPER agonists (G1, 11 nmol/L) and antagonists (G15, 20 nmol/L) for 30 min. Activation and inhibition of GPER did not affectI_{NaL} in hiPSC-CMs (Figure 5A and C). G1 increased peak I_{Na} density similar to E2, and the effects of E2 were eliminated by G15 which indicates that GPER mediated the effect of estrogen on peak I_{Na} (Figure 5B, D and E). GPER did not affect the $V_{1/2}$ and k values of activation and inactivation (Figure 5F and H-K). Interestingly, treatment with G15 alone increased the τ value of recovery from inactivation (Figure 5G and L; Supplemental Figure 3A). Data values for these results are provided in supplementary material online, Supplemental Table 1-7.

Activation of GPER decreased the increase in $I_{NaL}$and peak I_{Na} induced by ISO but did not affect I_{Na} kinetics

To establish whether GPER will restore the effects of ISO on hiPSC-CMs, we characterized sodium channel function under G1 and G15 treatment. We observed that G1 pretreatment eliminated the increase inI_{NaL} and peak I_{Na} induced by ISO which was similar to E2. Notably, the effects of E2 were abolished by G15 pretreatment (Figure 6A-E). Activation or inhibition of GPER did not affect the $V_{1/2}$ and k values of activation and inactivation in hiPSC-CMs treated with ISO as described before (Figure 6F and H-K). G1 or G15 pretreatment had no effects on the τ value of recovery from inactivation caused by ISO (Figure 6G and L; Supplemental Figure 3B). The data of Mean ± S.E.M are found in supplementary material online, Supplemental Table 1-7.

Silencing GPER abolished the effect of estrogen on peak I_{Na} and altered I_{Na} kinetics

Having found that GPER affected sodium currents using G1 and G15, we tested whether gene silencing with small interfering RNA (GPER-siRNA, 16.7 nmol/L, 4-6 h) could produce similar results. Knockdown of GPER did not change I_{NaL} in hiPSC-CMs (Figure 7A and C), but it canceled the increase in peak I_{Na} caused by E2 (Figure 7B, D and E). More interestingly, silencing GPER increased the k value of inactivation and decreased the $V_{1/2}$ values of activation and inactivation (Supplemental Figure 5A and C-F). Unexpectedly, negative GPER-siRNA decreased the $V_{1/2}$ value of inactivation (Supplemental Figure 5F) and increased the τ value of recovery from inactivation (Supplemental Figure 4A, Supplemental Figure 5B and G). Altogether, these results demonstrated that gene silencing of GPER affected the function of sodium channel. Data of Mean ± S.E.M are presented in supplementary material online, Supplemental Table 1-7.

Silencing GPER suppressed the effect of estrogen on I_{NaL} and altered I_{Na} kinetics under stress

In hiPSC-CMs treated with ISO, silencing GPER canceled the inhibitory effects of E2 on I_{NaL} (Figure 7F and H). Despite E2 having no influence on the $V_{1/2}$ values of inactivation, GPER silencing decreased it under stress challenge (Supplemental Figure 5H and J-M). GPER silencing did not affect the τ value of recovery from inactivation caused by ISO (Supplemental Figure 4B, Supplemental Figure 5I and N). Detailed statistical data are available in supplementary material online, Supplemental Table 1-7.

Discussion

This study explored whether estrogen regulates cardiac voltage-gated sodium channel in hiPSC-CMs. Results show that estrogen reduced the increase in peak I_{Na} andI_{NaL} caused by β-adrenergic stimulation via its

receptor GPER. Several studies have also used hiPSC-CMs as a cell model to delineate the mechanisms of ion channels and carry out pharmacological research (Portero et al., 2017; T. Yang et al., 2014; Zhao et al., 2018). In addition, hiPSC-CMs exhibited a good physiological response to β-adrenergic stimulation, which provides a unique in vitro simulated cardiac stress model for understanding the estrogenic cardioprotection under stress-induced pathological conditions (Bekhite et al., 2020; Borchert et al., 2017; Zhao et al., 2018). hiPSC-CMs acted abundant expressions of cardiac troponin T (Supplemental Figure 1) and exhibited characteristics similar to native cardiomyocytes (Ma et al., 2011; Zhao et al., 2018) in terms of AP shape and sodium currents, indicating that they are ideal for the purpose of this study.

Stress increased sodium currents and altered I_{Na}kinetics

Stress has been recognized as a risk factor of cardiovascular diseases such as Takotsubo cardiomyopathy and arrhythmia (Stiermaier et al., 2015; Templin et al., 2015). Stimulation of βAR located in cardiomyocyte membranes by catecholamines regulates ion channels to maintain normal cardiac function. For instance, stimulation of βAR-Gs signaling pathway enhances peak I_{Na} and the subsequent conductivity and excitability of cardiac myocytes (Aflaki M, 2014; Grinshpon & Bondarenko, 2016; Szentmiklosi AJ, 2015). However, overstimulation of βAR impairs ion channels function causing myocardial injury and abnormal rhythm (Parati & Esler, 2012). This is because excessive upregulation of I_{NaL} increases the risk of arrhythmia (Dybkova N, 2014; Bence Hegyi et al., 2018). In this study, ISO was used as a stimulus to simulate cardiac stress, increasingI_{NaL} and peak I_{Na}, which is consistent with previous studies (Dybkova N, 2014; Bence Hegyi et al., 2018). It was also found that that sodium channel is easily activated but difficult to be inactivated or recover from inactivation after a stress stimulus. Given the lack of effective treatments for ventricular arrhythmias caused by sodium channel dysfunction, it is important to investigate the regulation of sodium currents in order to uncover novel I_{NaL} blockers for the prevention and treatment of arrhythmias (Remme & Wilde, 2014).

Estrogen increased peak I_{Na} but reduced both of I_{NaL} and peak I_{Na} after stress

Some studies have shown that estrogen exert concentration-dependent dual effects on ventricular electrical activity in guinea pigs (Kurokawa, Kodama, Clancy, & Furukawa, 2016; Kurokawa et al., 2008). To test the rapid effect of estrogen on sodium channel and whether it is dependent on concentration, hiPSC-CMs were cultured with low, medium, and high concentrations of estrogen for 1 hour. In our study, estrogen did not alter I_{NaL} at any concentration under normal condition. In contrast, under stress state, estrogen at medium concentration inhibited I_{NaL} and this effect was not observed for the low and high concentrations. Estrogen increased peak I_{Na} at all concentrations, but reduced the augmentation in peak I_{Na} at medium concentration under stress state. Estrogen did not affect activation, inactivation and recovery from inactivation of sodium channel under stress or basal state. Although previous studies have revealed gender specific differences in peak I_{Na} andI_{NaL}, the effect of estrogen on those sodium currents is still unclear. Therefore, our results further complement the role of sex hormone in regulating sodium currents, and suggest for the first time that estrogen blunts the increase in sodium currents caused by β-adrenergic overstimulation.

Prolonged AP duration by estrogen is independent of I_{NaL}

The rapid and inward Na^+ currents determine the dynamics of the ascending branch of AP (Vikram et al., 2017). On the other hand, I_{NaL}, a small and sustained Na^+ influx, lasts for hundreds of milliseconds and maintains the plateau phase of AP with inward calcium current (Portero et al., 2017; Zaza & Rocchetti, 2013). Therefore, we analyzed the AP to explore the relationship between estrogen, sodium currents and AP. Estrogen at medium concentration prolonged the AP duration, which may explain why women have longer AP duration and longer QTc interval than men of the same age (Salem, Alexandre, Bachelot, & Funck-Brentano, 2016). It seems that the prolonged AP duration caused by estrogen was independent of I_{NaL} which is likely that the treatment time of estrogen is too short to increase theI_{NaL}. Short-term estrogen treatment failed to amplify I_{NaL}, but increased a certain amount ofI_{NaL}. With the prolongation of estrogen treatment time, I_{NaL} increased significantly, just as theI_{NaL} in female mice exposed to estrogen for a long time is larger than that in male mice (Lowe et al., 2012). In addition, the prolonged AP duration induced by

estrogen may be associated with the increase in calcium currents (Papp et al., 2017; X. Yang et al., 2018).

Notably, the shortened AP duration induced by stress was abolished by estrogen. Although β-adrenergic overstimulation increasedI_{NaL}, however, it did not prolong the AP duration. The shortened AP duration might be caused by the increase and rapid inactivation of the L-type calcium current in the early stage of repolarization following β-adrenergic activation (Sala et al., 2018). Also, ISO treatment increased slow delayed rectifier potassium current which will shorten the AP duration (Banyasz et al., 2014; Gong JQX, 2020). During the occurrence of heart failure, the electrophysiological remodeling of the myocardium is manifested by an increase inI_{NaL} and the prolongation of AP duration, which may be related to time and the degree of stress (B. Hegyi et al., 2019) . So, under acute stress, it is likely that estrogen may inversely regulates L-type calcium current or potassium currents to prolong the AP duration while reducing the increase in I_{NaL}. Therefore, we will further study the regulation of estrogen on calcium and potassium channels under stress.

Effects of estrogen on sodium currents and $Na_v1.5$ expression are inconsistent

Sodium currents are mainly generated by $\mathrm{Na_v}1.5$ (Jiang et al., 2020). Here, we examined the relationship between effects of estrogen on sodium currents and $\mathrm{Na_v}1.5$. Notably, low and medium concentrations of estrogen decreased the content of $\mathrm{Na_v}1.5$, while high concentrations upregulated the content of $\mathrm{Na_v}1.5$. This partly indicates that the effects of estrogen were concentration-dependent as reported previously (Kurokawa et al., 2016; Kurokawa et al., 2008). Under stress state, estrogen at all concentrations reversed the increase in content of $\mathrm{Na_v}1.5$ caused by ISO. However, the changes in the content of $\mathrm{Na_v}1.5$ did not match with the changes inI_{NaL} and I_{Na}.

GPER mediates the acute effect of estrogen on sodium currents

Estrogen regulates multiple physiological processes through its receptors (ERα, ERβ, and GPER). GPER is reported to be the key receptor mediating the rapid action of estrogen (Revankar et al., 2019). In the cardiovascular system, estrogen via GPER confers protection (Menazza & Murphy, 2016). In this study, estrogen treatment was carried out for 1 hour, and thus we speculated that its effects could be mediated by GPER. Activation of GPER with G1 increased peak I_{Na}similar to estrogen. Moreover, siRNA-driven silencing of GPER or inhibition with G15 abolished the effects of estrogen. This shows that GPER mediated the effect of estrogen on peak I_{Na}. Under stress challenge, G1 suppressed the increase inI_{NaL} and peak I_{Na} caused by ISO consistent with the effect of estrogen. These effects were eliminated by siRNA-driven silencing of GPER or inhibition with G15, suggesting that GPER mediates the rapid effects of estrogen on sodium currents under stress. Another interesting finding in this study is that inhibition of GPER alone affected the recovery from inactivation of sodium channel. This suggests that GPER could be structurally or homologically similar to sodium channel, hence G15 may directly alter the function of sodium channel. This speculation deserves further clarification.

The present results show that various concentrations of estrogen enhanced the peak I_{Na}, whereas medium concentration of estrogen, that is, physiological concentration, effectively antagonized the functional changes of sodium channels caused by stress through GPER. These findings underscore the importance of estrogen and its receptor GPER on sodium channel function. We show that they hold great promise as druggable targets for the treatment of cardiovascular diseases related to sodium channel or stress.

Conclusion

On this basis, we conclude that estrogen increased the peak sodium current through GPER. However, under pathological conditions such as stress, estrogen attenuated the increase in both of the peak sodium current and late sodium current induced by β-adrenergic overstimulation thereby conferring protection in hiPSC-CMs. GPER mediated the protective effects of estrogen on cardiomyocytes under stress state, indicating that the application of estrogen and targeting GPER may be an effective approach to treat sodium channelopathies and stress-related heart diseases. Estrogen and GPER have very important role, based on which more drugs can be developed to prevent and treat these diseases. These results are of great clinical significance as we

address an important unmet clinical need to understand the role of sex hormones in caridiovacular disease.

Novelty and therapeutic implications

This work provides novel insights into the cardioprotective effect of estrogen in the condition of excessive stimulation of β-adrenergic receptors. Our data reveals that GPER activation mediates estrogenic effects on cardiomyocytes to attenuate sodium current and maintain normal action potential duration under stress state. Thus, GPER may be a novel target to treat stress-related heart diseases.

Author contributions

X.H. and H.S. contributed to the study conception and design. Material preparation, data collection and analysis were performed by X.H., L.F., M.Z., D.W., H.Z., Z.G., T.M., Y.Z., X.P., J.G., C.X., L.H., X.M. and L.J.. The first draft of the manuscript was written by X.H.. The revision in language and logic of the manuscript was undertook by H.S., J.O.M., G.K.A. and A.O.A.. All authors read and approved the final manuscript.

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Figure legends

Figure 1 ISO increased sodium currents and alteredI_{Na} kinetics as well as action potential parameters in hiPSC-CMs. (A) Average I_{NaL}density. (B) Current-voltage curve of sodium channel. (C) PeakI_{Na} density. (D) The time constant of recovery from inactivation curve of sodium channel. (E) τ value of recovery from inactivation. (F) Activation and inactivation curve of sodium channel of hiPSC-CMs. (G-J) Values for k and $V_{1/2}$ of (in) activation. n=17-37 cells, from 9-11 Petri dishes. (K) Representative single AP and slope curve of hiPSC-CMs. (L-Q) Values for APA, RMP, $\mathrm{d}V/\mathrm{d}t_{\mathrm{max}}$, APD_{10}, APD_{50} and APD_{90}. n=20-34 cells, from 4 Petri dishes separately. Unpaired t test was used. Welch's correction was used selectively in the case of uneven variance. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. ISO, isoproterenol; I_{NaL}, late sodium current;I_{Na}, sodium current; k of (in) activation, slope factor of (in) activation; $V_{1/2}$ of (in) activation, half-voltage of (in) activation; τ, time constant of recovery from inactivation; APA, action potential amplitude; RMP, resting membrane potential; $\mathrm{d}V/\mathrm{d}t_{\mathrm{max}}$, maximal action potential upstroke

velocity; APD_{10}, action potential duration at 10% repolarization; APD_{50}, action potential duration at 50% repolarization; APD_{90}, action potential duration at 90% repolarization. pA, pico Ampere; pF, pico Farad. Insets: voltage clamp protocols.

Figure 2 Estrogen inhibited the increase ofI_{NaL} in hiPSC-CMs stimulated by ISO. (A) Representative I_{NaL} of hiPSC-CMs. (B and C) Average I_{NaL} density. n=8-26 cells, from 3-10 Petri dishes. One-way ANOVA was used. Kruskal-Wallis test was used selectively in the case of uneven variance. $^{*}P < 0.05$. E2, β-Estradiol. Insets: voltage clamp protocols.

Figure 3 Estrogen increased peak I_{Na} but reduced the increase of peak I_{Na} induced by ISO without altering I_{Na} kinetics. (A) Representative peakI_{Na} in hiPSC-CMs. (B and D) Current-voltage curve of sodium channel. (C and E) Peak I_{Na} density. n=10-37 cells, from 4-11 Petri dishes. (F and M) Activation and inactivation curve of sodium channel. (G and N) The time constant of recovery from inactivation curve of sodium channel. (H-K and O-R) Values for k and $V_{1/2}$ of (in) activation. n=8-34 cells, from 4-11 Petri dishes. (L and S) τ value of recovery from inactivation. n=5-32 cells, from 3-11 Petri dishes. One-way ANOVA was used. Kruskal-Wallis test was used selectively in the case of uneven variance. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P< 0.001$, $^{****}P < 0.0001$. Insets: voltage clamp protocols. Other abbreviations are as shown above.

Figure 4 Estrogen modified action potential parameters of hiPSC-CMs. (A) Typical APs and slope curve of hiPSC-CMs. (B-M) Values for APA, RMP, $\mathrm{d}V/\mathrm{d}t_{max}$, APD_{10}, APD_{50} and APD_{90}. n=19-40 cells, from 4-6 Petri dishes. One-way ANOVA was used. Kruskal-Wallis test was used selectively in the case of uneven variance. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P< 0.001$, $^{****}P < 0.0001$. Other abbreviations are as shown above.

Figure 5 GPER mediated the effect of estrogen on increasing peakI_{Na} but did not alter $I_{NaL}$and I_{Na} kinetics. (A) RepresentativeI_{NaL} in hiPSC-CMs. (B) Representative peakI_{Na} in hiPSC-CMs. (C) AverageI_{NaL} density. n=10-25 cells, from 4-9 Petri dishes. (D) Current-voltage curve of sodium channel. (E) PeakI_{Na} density. n=9-32 cells, from 4-11 Petri dishes. (F) Activation and inactivation curve of sodium channel. (G) The time constant of recovery from inactivation curve of sodium channel. (H-K) Values for k and $V_{1/2}$ of (in) activation. n=8-26 cells, from 4-11 Petri dishes. (L) τ value of recovery from inactivation. n=7-17 cells, from 3-10 Petri dishes. One-way ANOVA was used. Kruskal-Wallis test was used selectively in the case of uneven variance. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P< 0.001$. G1, G protein coupled estrogen receptor agonists; G15, G protein coupled estrogen receptor antagonists. Other abbreviations are as shown above.

Figure 6 Estrogen through GPER reduced the increase of sodium currents induced by ISO but did not alter I_{Na}kinetics. (A) Representative I_{NaL} in hiPSC-CMs. (B) Representative peak I_{Na} in hiPSC-CMs. (C) Average I_{NaL} density. n=9-26 cells, from 4-10 Petri dishes. (D) Current-voltage curve of sodium channel. (E) PeakI_{Na} density. n=12-37 cells, from 4-11 Petri dishes. (F) Activation and inactivation curve of sodium channel. (G) The time constant of recovery from inactivation curve of sodium channel. (H-K) Values for k and $V_{1/2}$ of (in) activation. n=8-34 cells, from 4-11 Petri dishes. (L) τ value of recovery from inactivation. n=5-32 cells, from 4-11 Petri dishes. $^{*}P< 0.05$, $^{***}P < 0.001$, $^{****}P< 0.0001$. Statistical analysis and other abbreviations are as shown above.

Figure 7 Silence of GPER cancled the effect of estrogen on increasing peak I_{Na} and reducingI_{NaL} under stress. (A) RepresentativeI_{NaL} in hiPSC-CMs. (B) Representative peakI_{Na} in hiPSC-CMs. (C) AverageI_{NaL} density. n=10-25 cells, from 3-9 Petri dishes. (D) Current-voltage curve of sodium channel. (E) PeakI_{Na} density. n=10-32 cells, from 3-11 Petri dishes. (F) RepresentativeI_{NaL} in hiPSC-CMs. (G) Representative peakI_{Na} in hiPSC-CMs. (H) AverageI_{NaL} density. n=9-26 cells, from 3-10 Petri dishes. (I) Current-voltage curve of sodium channel. (J) PeakI_{Na} density. n=9-37 cells, from 3-11 Petri dishes. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$. GPER, G protein coupled estrogen receptor. Statistical analysis and other abbreviations are as shown above.

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