GPER mediated estrogenic amelioration of sodium channel dysfunction in stressed human induced pluripotent stem cell-derived cardiomyocytes

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Abstract

Στρεσσ-ινδυχεδ εξχεσσιε αχτιατιον οφ τηε αδρενεργις σψστεμ ορ χηανγες ιν εστρογεν λεελς προμοτε τηε οχχυρρενχε οφ αρρηψτημιας. Σοδιυμ χηαννελ, α ρεσπονδερ το β-αδρενεργις στιμυλατιον, ις ινολεδ ιν στρεσσ-ινδυχεδ χαρδιας ελεχτροπηψσιολογιχαλ αβνορμαλιτιες. Ηοωεερ, ιτ ηας νοτ βεεν εσταβλισηεδ ωηετηερ εστρογεν ρεγυλατες σοδιυμ χηαννελς δυρινγ αχυτε στρεσς. Ουρ στυδψ αιμεδ το εξπλορε ωηετηερ ολταγε-γατεδ σοδιυμ χηαννελς πλαψ ρολες ιν τηε ραπιδ ρεγυλατιον οφ αριους χονχεντρατιονς οφ εστρογεν ιν στρεσσεδ ηυμαν ινδυχεδ πλυριποτεντ στεμ χελλ-δεριεδ χαρδιομψοχψτες (ηιΠΣ^{n}-^{n}Μχ), ανδ ρεεαλ τηε ποσσιβλε μεχηανισμ οφ εστρογεν σιγναλινγ πατηωαψ μοδυλατινγ στρεσς. Αν ισοπροτερενολ-ινδυχεδ στρεσς μοδελ οφ ηιΠΣ^{n}-^{n}Μχ ωας πρε-ινχυβατεδ ωιτη β-Εστραδιολ ατ διφφερεντ χονχεντρατιονς (0.01 νμολ/Λ, 1 νμολ/Λ, ανδ 100 νμολ/Λ). Αχτιον ποτεντιαλ (ΑΠ) ανδ σοδιυμ χυρρεντς ωερε δετεχτεδ βψ πατχη χλαμπ. Τηε Γ προτειν-χουπλεδ εστρογεν ρεχεπτορ (ΓΠΕΡ)-σπεχιφις εφφεχτ ωας δετερμινεδ ωιτη αγονιστ Γ1, ανταγονιστ Γ15 ανδ σμαλλ ιντερφερινγ ΡΝΑ. β-Εστραδιολ ατ χονχεντρατιονς οφ 0.01 νμολ/Λ, 1 νμολ/Λ, ανδ 100 νμολ/Λ ινχρεασεδ τηε πεακ σοδιυμ χυρρεντ ανδ προλονγεδ ΑΠ δυρατιον (ΑΠΔ) ατ 1 νμολ/Λ. Στρεσς ινχρεασεδ πεακ σοδιυμ χυρρεντ, λατε σοδιυμ χυρρεντ, ανδ σηορτενεδ ΑΠΔ. Τηε εφφεχτς οφ στρεσς ον σοδιυμ χυρρεντς ανδ ΑΠΔ ωερε ελιμινατεδ βψ β-Εστραδιολ. Αχτιατιον οφ ΓΠΕΡ βψ Γ1 εξηιβιτεδ σιμιλαρ εφφεχτς ας β-Εστραδιολ, ωηιλε ινηιβιτιον οφ ΓΠΕΡ ωιτη Γ15 ανδ σμαλλ ιντερφερινγ ΡΝΑ αμελιορατεδ εστρογενις αχτιονς. Estrogen, antagonized the stress-related abnormal electrical activity, and through GPER alleviated sodium channel dysfunctions in stress state in hiPSC-CMs. These results provide a novel mechanism through which estrogenic rapid signaling against stress by regulating ion channels.

Keywords

Estrogen; G protein-coupled estrogen receptor; Sodium channel; Stress; Cardiomyocytes

Introduction

Cardiac voltage-gated sodium channels that are responsible for peak sodium current (I_{Na}) and late sodium current (I_{NaL}) are involved in the generation of action potential (AP).[1, 2] It is an important responder for physiological β-adrenergic stimulation of cardiomyocytes.[3] Catecholamine-induced overstimulation of β-adrenergic dysregulates sodium channel functions, characterized by elevated I_{Na} and I_{NaL}, as the case during cardiac failure.[3, 4] Abnormal elevations of these two currents are associated with contractile dysfunctions and arrhythmias.

Studies have revealed that sex hormones, especially estrogen, are crucial factors affecting sympathetic activity and cardiac electrophysiology. Estrogen increases delayed rectifier current and shortens QTc interval, thereby, correcting arrhythmias.[5] Compared to men, estrogen is an important cardiac stress resistance factor in women.[6, 7] Through affecting the β-adrenergic receptor (βAR) signaling, estrogen initiates cardioprotection during stress by antagonizing inhibitory contractions and decreasing the risk of stress-related cardiac arrhythmias.[8-10] However, it has not been established whether cardio-protective effects of estrogen during stress are exerted through sodium channel modulation.

Estrogen is not always beneficial to the heart. It was shown to prolong AP duration (APD) by upregulating L-type calcium currents in both women and female rabbits, thereby inducing arrhythmia.[11, 12] Fluctuations in estrogen levels during the menstrual cycle are associated with differences in the risk of arrhythmias in adult women.[13, 14] This can be explained by diverse effects of different concentrations of estrogen on ventricular electric functions in guinea pigs.[13] Studies on gender differences on cardiac sodium channels revealed that heavy dispersions of peak I_{Na} and larger I_{NaL} in females than males may be associated with sex-specific arrhythmias.[15, 16]

The different roles of estrogen in the myocardium described above could be attributed to the differences in estrogen concentrations, animal model species, or the external environment of the myocardium, such as stress. Therefore, we aimed at evaluating the impact of various concentrations of estrogen, and its underlying mechanisms on sodium channel functions in normal and stressed human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs).

Materials and Methods

Cell culture

Spontaneously-beating cardiomyocytes induced from hiPSC derived from normal female epithelial cells (IMR90-4, Generation 35 - 48, WiCell Research Institute, USA) were cultured for 40 ± 10 days and randomized for treatment with Isoprenaline hydrochloride (ISO), β-Estradiol (E2), G1, G15, and small interfering RNA. The induction, differentiation and culture procedures of IMR90-4 and hiPSC-CMs were as previously reported.[17, 18]

Chemical reagents

In this study, ISO (Sigma, USA, Lot # WXBC9656V) was dissolved in 0.01 mol/L HCl solution (36˜38%), then the PBS solution was added to prepare a 1×10^{-3} mol/L ISO stock solution. E2 (Sigma, USA, Lot # SLBT2822) was dissolved in DMSO solution to prepare a 2×10^{-1} mol/L E2 stock solution. 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 and G15 stock solutions, respectively. To avoid the adverse effects of DMSO on sodium currents, its concentration in the extracellular fluid was <0.001%.[2] These liquids were aliquoted and stored in the dark at -20 and diluted to the desired concentrations when used.

Experimental design

In this study, hiPSC-CMs were treated with 10 μmol/L ISO for 30 min to simulate cardiac stress. These procedures were performed based on modelling cardiac stress protocols of our lab, in combination with previously described methods.[19, 20] hiPSC-CMs were incubated with various concentrations of E2 (0.01, 1 and 100 nmol/L) for a short time such as 1 h.[13, 14, 21] The stress model of hiPSC-CMs was treated with E2 (0.01, 1 and 100 nmol/L, 30 min prior to ISO) for 1 h to simulate cell reactions in an estrogenic environment against stress. In addition, hiPSC-CMs were treated with G1 (11 nmol/L, 30 min) or G15 (20 nmol/L, 30 min) and small interfering RNA.[10, 22, 23] These tested drugs were not continuously present in the external solution during electrophysiological recordings.

GPER silencing by small interfering RNA

The GPER of hiPSC-CMs was silenced by siRNA.[10] Briefly, the transfection complex solution containing 16.7 nmol/L siRNA (GenePharma, China) was prepared following the manufacture's protocol (GenePharma, Suzhou, China), then it was co-cultured with hiPSC-CMs serum-free medium at 37 for 4-6 hours. Then, the culture medium was replaced with serum-free medium and cultured overnight prior to experimentation. The GPER gene silencing sequence was:

Sense (5'-3') : GGCCUCAUCUGGAUGGCAUTT;

Antisense (5'-3') : AUGCCAUCCAGAUGAGGCCTT.

Patch clamp measurements

Sodium currents

Sodium currents were recorded as described by Yang et al..[24] The intracellular solution contained (in mmol/L): 5 NaF, 110 CsF, 20 CsCl, 10 EGTA and 10 Hepes. The pH was adjusted to 7.4 using 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 using NaOH.In addition, 0.005 mmol/L Nifedipine (Sigma, USA, Lot # MKCB9232) and 0.5 mmol/L 4-Aminopyridine (MedChemExpress, USA, Lot # 27027) were used to eliminate L-type calcium currents and outward potassium currents.

Sodium currents were measured at room temperature to avoid the ion current run-down phenomenon.[24] Recording microelectrodes were made of hard glass with tip resistances between 2˜4 MΩ. Sodium currents and sodium channel dynamics were recorded as follows: i. Peak I_{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. ii. Steady state inactivation was measured using 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, followed by a single 50 ms test pulse at -30 mV. iii. Recovery from inactivation was measured by a double pulse as well. 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 at intervals of 1, 3, 5, 7, ····· 49, 51 ms, followed by a single 50 ms test pulse at -30 mV. iv. Late I_{Na} was elicited from -120 mV to -30 mV with a cycle length of 500 ms.

EPC10 patch clamp amplifier and PatchMaster version v2x73.2 software (HEKA, Germany) were used for data acquisition. Sodium currents were collected at a sampling frequency of 20 kHz without filtering to ensure the details and authenticity of such large currents. To enhance data accuracy, ˜70% of series resistance was corrected and automatic leakage compensation performed. Considering the existence of cell membrane fragments, the compensation of series resistance in some current recording is not perfect. Current data were presented as current amplitude/cell capacitance (pico Ampere/pico Farad) to reduce intercellular errors. Origin 2017 software (OriginLab, USA) was used to analyze data and prepare figures.The selection criteria of current data were: standard current shape, cell capacitance between 10-50pico Farad, with outward current of less than 10 pico Ampere/pico Farad. Activation and inactivation curves were fitted with Boltzmann function (y=$\{1+\exp[(V\text{-}V_{1/2})/k]$ $^{-1}$), while the recovery from inactivation curve was fitted using a single exponential function (y = A1*exp (-x/t1)+y0). The average I_{NaL} from 50 to150 ms after the test pulse was used.[25]

Acquisition of action potential

Spontaneously-beating hiPSC-CMs clusters with similar frequencies were used to record APs in a current-clamp mode (Free gap mode) at a physiological temperature.[24] The intracellular solution contained (in mmol/L): 150 KCl, 1 $MgCl_2$, 10 EGTA, 5 ATP Na2 and 5 Hepes. The pH was adjusted to 7.2 using 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. The pH was adjusted to 7.4 using NaOH. APs were acquired at 20 kHz sampling frequency and digitized at 100 Hz by Clampfit Software.

Statistical analysis

GraphPad Prism 7, Clampfit Software 10 and Origin 2017 were used for data analysis. Data are presented as Mean ± S.E.M. Mean differences between and among groups were compared using the unpaired student's t-test and One-way ANOVA, respectively. Unpaired t-test with Welch's correction and Kruskal-Wallis test were selectively used in cases of uneven variance. $p<0.05$ was considered statistically significant.

Results

E2 has no acute effect on I_{NaL} but suppresses the increase induced by ISO in I_{NaL}

It is still unclear whether estrogen can acutely modulate sodium currents in human induced pluripotent stem cell systems. Here, we set out to observe whether the estrogenic effects of sodium currents are dependent on the estrogen concentrations and stress in hiPSC-CMs.First, we performed the identifications of hiPSC-CMs. Elevated expressions of cardiac troponin T (Supplemental Figure 1A), which is similar with the resting membrane potential (RMP) and AP in adult cardiomyocytes,[26] revealed that hiPSC-CMs have the same characteristics as cardiomyocytes. hiPSC-CMs were incubated with E2 at concentrations of 0.01 nmol/L, 1 nmol/L and 100 nmol/L for 1 h, a short incubation time, which is enough to show the rapid effect of estrogen.[10, 12] At the three concentrations, E2 did not affect the average I_{NaL} density in hiPSC-CMs (Figure 1A and B).

It has been reported that βAR overstimulation increases sodium currents in mouse and rabbit myocytes, resulting in arrhythmias.[3, 4] In this study, a stress model of hiPSC-CMs was established using a high concentration of ISO (10 μmol/L, 30 min), a βAR agonist. Changes in sodium channels were investigated by observing I_{NaL} densities, and peak I_{Na} amplitudes and I_{Na} kinetics. ISO treatment increased I_{NaL} density (Supplemental Figure 2A). In the stress state, E2, at the concentration of 1 nmol/L, abated ISO induced increase in the average I_{NaL} density (Figure 1A and C). Supplemental Table 1-8 presents data on Mean ± S.E.M of the number of cells (the same below).

E2 suppresses ISO-induced increase in peak I_{Na} amplitude

Our results show that E2 at concentrations of 0.01 nmol/L, 1 nmol/L and 100 nmol/L increased peak I_{Na} density and depressed the I-V curve of the sodium channel (Figure 2A-C), but did not affect either the $V_{1/2}$ and k values of activation and inactivation or t value of recovery from inactivation (Figure 2D-J, Supplemental Figure 3A). It has been revealed about stress induced sodium channel dysfunction that ISO treatment significantly elevated peak I_{Na} amplitude and depressed the current-voltage curve (I-V) of the sodium channel (Supplemental Figure 2B and C). ISO elevated the t value, and shifting the recovery from inactivation curve to the right (Supplemental Figure 2D and E, Supplemental Figure 3B). Moreover, ISO resulted in a shift of the $V_{1/2}$ value of activation and inactivation towards more negative potentials (Supplemental Figure 2F-J). In the presence of stress, E2 at 1 nmol/L diminished the increase induced by ISO in peak I_{Na} density and I-V curve downshift of the sodium channels, while E2 at 0.01 nmol/L and 100 nmol/L had no obvious effect (Figure 3A-C). Moreover, E2 at the three concentrations did not rescue the increase induced by ISO in $V_{1/2}$ values of activation and inactivation or the t value of recovery from inactivation (Figure 3D-J, Supplemental Figure 3B).

The effects of E2 and ISO on AP

Stress spoils cardiac electrophysiological activities and induces arrhythmia. The results also indicates that ISO dysregulates AP parameters by reducing AP amplitude (APA), maximal action potential upstroke velocity ($\mathrm{d}V/\mathrm{d}t_{\max}$), shortening action potential duration at 10% repolarization (APD_{10}), 50% repolarization (APD_{50}) and 90% repolarization (APD_{90}), but did not change the RMP (Supplemental Figure 2K-Q). Our previous study has shown that estrogen can improve the cardiac systolic dysfunction and electrical activity disorder induced by epinephrine to protect the heart.[10] Here, we repeatedly verified whether estrogen can improve the electrophysiological dysfunction induced by ISO in hiPSC-CMs. The effect of E2 on APs of hiPSC-CMs was as shown in Supplemental Figure 4. E2 at concentrations of 1 nmol/L and 100 nmol/L reduced APA (Supplemental Figure 4D). E2 at 100 nmol/L decreased the absolute value of RMP (Supplemental Figure 4E). Moreover, E2 at the three concentrations decreased $\mathrm{d}V/\mathrm{d}t_{\max}$, especially at the concentration of 1 nmol/L (Supplemental Figure 4F). In addition, at 1 nmol/L, E2 prolonged APD_{50} and APD_{90} (Supplemental Figure 4G-I). In contrast, under stress, E2 at the three concentrations had no effects on APA, $\mathrm{d}V/\mathrm{d}t_{\max}$ and APD_{10} in ISO-treated cells (Supplemental Figure 5D, F and G). However, E2 normalized the ISO shortened APD_{90}, although at the concentrations of 0.01 and 1 nmol/L, it decreased the absolute value of RMP (Supplemental Figure 5I and E). In addition, E2 normalized APD_{50} at concentrations of 100 nmol/L (Supplemental Figure 5H).

GPER mediates the effect of E2 on increasing peak I_{Na} amplitude

E2 plays an acute role through its rapid signal receptor, GPER.[27] Our latest study also shows that GPER mediates the cardioprotective effect of estrogen on epinephrine-induced stress.[10] Therefore, to further determine whether GPER mediates the effect of E2 on increasing peak I_{Na} amplitude, hiPSC-CMs were incubated with 1 nmol/L E2 for 1 h and treated with the GPER agonist (G1), and antagonist (G15) and gene silenced using small interfering RNA (GPER-siRNA). The effect of siRNA on GPER gene silencing was verified by qRT-PCR. It was found that GPER gene expression was suppressed after siRNA interference (Supplemental Figure 1B). Moreover, negative GPER-siRNA had no effect on electrophysiological functions of the sodium channel (Supplemental Figure 7).

The results show thatG1 and G15 did not affect I_{NaL} in hiPSC-CMs (Figure 4A and C), which is same as the case of GPER-siRNA (Figure 6A and Supplemental Figure 7A). Similar with the case of E2, G1 increased peak I_{Na} density and depressed the I-V curve of sodium channel. It also shows that the effects of E2 were eliminated by G15 and GPER-siRNA (Figure 4B, D and E; Figure 6B and C; Supplemental Figure 7B). G1, G15 and GPER-siRNA did not affect the $V_{1/2}$ and k values of activation and inactivation (Figure 4F and H-K; Figure 6D; Supplemental Figure 7C-F). Interestingly, treatment with G15 alone, instead of GPER siRNA, increased thet value and caused a shift to the right in recovery from inactivation curve (Figure 4G and L; Supplemental Figure 6A; Figure 6E; Supplemental Figure 7G; Supplemental Figure 8A).

GPER mediates the action of E2 on decreasing the augmentation induced by ISO in I_{NaL} and peak I_{Na}

To determine the role of GPER in restoring sodium channel function under cardiac stress on hiPSC-CMs, G1, G15 and GPER-siRNA were pretreated before ISO treatment. G1 pretreatment suppressed the increase in I_{NaL} (Figure 5A and C) and peak I_{Na}, and upshifted the depressed I-V curve of sodium channel induced by ISO, which was similar to E2 (Figure 5B, D and E). G15 and GPER-siRNA abolished the action of E2 on reducing the increase induced by ISO in I_{NaL} and peak I_{Na} (Figure 5A-E; Figure 6F-H; Supplemental Figure 7H and I). G1 or G15 and GPER-siRNA pretreatment did not exert any effects on the $V_{1/2}$ and k values of activation and inactivation and t value of recovery from inactivation in ISO treated hiPSC-CMs (Figure 5F-L; Supplemental Figure 6B; Figure 6I and J; Supplemental Figure 7J-N; Supplemental Figure 8B).

Discussion

In this study, we found that estrogen normalized the shortened APD and attenuated the increase in peak I_{Na} and I_{NaL} induced by stress, although by itself, estrogen prolonged APD and increased peak I_{Na} in hiPSC-CMs. The effects of estrogen on I_{Na} and I_{NaL} were mediated by GPER. Our results imply that estrogen and its rapid signal receptor GPER, play an important role in regulating myocardial electrical activity and ion channels. Especially under stress, estrogen rapid signal pathway exerts protective effects for cardiomyocytes.

Recently, cell models of hiPSC-CMs have been developed to delineate the mechanisms of the ion channel.[2, 19, 24] Their APs are similar to those of humans,[26] and they exhibits good physiological responses to β-adrenergic stimulation.[19, 20] Our previous studies also showed that hiPSC-CMs expresses βAR and GPER, which provides a model guarantee for revealing the relationship between estrogen rapid receptor and stress response.[10] There are several studies that contrasting effects of estrogen on ionic currents in different animal models or levels.[13, 14, 28] Depolarizing sodium current has been found to be unevenly distributed in female dogs rather than in male dogs, but there is no gender difference in rabbits.[15, 29] It is also reported that I_{NaL} is higher in female mice than in male mice, on the contrary, it is higher in male rabbits than in female rabbits.[16, 29] These intraspecific differences suggest that further validations using human cardiomyocytes should be considered. Therefore, in this study, hiPSC-CMs were used to investigate the effects of estrogen on sodium channels of cardiomyocytes. Different concentrations of estrogen within the physiological range were selected (0.01 nmol/L, 1 nmol/L and 100 nmol/L), which corresponded to the level of estrogen in postmenopausal, premenstrual and preovulatory women, respectively.[13, 14, 21]

Acute estrogen stimulation increases peak I_{Na} and prolongs APD, but has no effect on I_{NaL}

We found that estrogen at all levels increases peak I_{Na} but with no effect on I_{NaL} in hiPSC-CMs. Moreover, estrogen at 1 nmol/L decreases APA and prolongs APD_{50} and APD_{90}. This inconsistency can be attributed to the fact that estrogen increases the peak I_{Na}, and amplifies transient outward potassium current, which exhibits a decrease in APA. The prolonged APD is not caused by the acute role of estrogen on I_{NaL}. It has been reported that estrogen increases calcium currents and inhibits rapid delay rectifier potassium current, which extends the AP plateau.[11-13] This may be the reason why estrogen prolongs APD. Lowe JS reported that I_{NaL} is larger in females.[16] The result reveals sodium channel dysfunctions induced by long-term exposures to estrogen in normal conditions. We considered that the long estrogenic state increases the expression of sodium channels to enhance I_{NaL}, though acute estrogen treatment has no effect on I_{NaL}. Therefore, in the future studies on the long-term effects of estrogen on sodium channels, we will focus on the expression level of sodium channel and the change of late sodium current.

Estrogen at the concentration of 1 nmol/L (the level of estrogen in premenstrual women) rather than other estrogen concentrations (in postmenopausal and preovulatory women) prolonged APD_{90}. This suggests that AP is more selective to the concentration of estrogen than sodium current. The concentration-dependent effects of estrogen on APD may explain cardiomyocyte repolarization fluctuation during the menstrual cycle. Occurrence time of arrhythmia in patients with congenital or drug-induced long QT syndrome might be predicted by the periodic fluctuation of estrogen.[30]

Acute estrogen treatment improves sodium channel dysfunction and electrical activity disorder under stress

A high concentration of ISO was used in this study to simulate acute cardiac stress in hiPSC-CMs. ISO increases both I_{NaL} and peak I_{Na}, impairs sodium channel kinetics and shortens APD. Stress is a risk factor for cardiovascular diseases such as Takotsubo syndrome and arrhythmias.[31] Dybkova and Hegyi found that, in mouse and rabbit cardiomyocytes, stimulation of βAR by ISO causes excessive upregulation of peak I_{Na} and I_{NaL}, leading to an increased risk of arrhythmias.[3, 4] This corresponds with our findings that stress impairs sodium channel functions in hiPSC-CMs.

When stress occurs, cardiomyocytes in an estrogenic environment initiate some beneficial responses against

stress. Estrogen at a concentration of 1 nmol/L rather than 0.01 nmol/L or 100 nmol/L, reduces the augmentation in peak I_{Na} and I_{NaL} induced by stress, while all concentrations normalize the shortened APD_{90}. It seems to imply that estrogen has greater concentration sensitivity to sodium current during stress, that is, it needs the right estrogen level. However, under the promotion of stress, almost all estrogens in physiological range regulate AP reflecting cell electrophysiological function. AP is the result of superposition of all current interactions, and its change will directly lead to cardiovascular diseases such as arrhythmia. Estrogen prolongs APD and may increase the susceptibility to arrhythmia, but under stress, almost all concentrations of estrogen improve disordered APD and protect cardiomyocytes. This may be one of the reasons why adult women are more prone to arrhythmias and less likely to have stress related cardiovascular diseases.[7, 12, 23] Therefore, inhibition of abnormal increases in peak I_{Na}, I_{NaL} and normalizing the shortened APD are potential mechanisms through which estrogen inhibits stress-related arrhythmias and contraction dysfunctions.

GPER mediates acute regulatory effects of estrogen on sodium channel function

Estrogen regulates multiple physiological processes through its receptors (classical nuclear ERα, ERβ, and membrane-associated GPER).[23, 27] GPER is a crucial receptor that mediates rapid estrogen actions. Therefore, GPER is a potential therapeutic target for cardiovascular diseases.[23, 27, 32] In this study, activation of GPER by G1 increases peak I_{Na} in normal states, but suppresses the increase in I_{NaL} and peak I_{Na} in stress state. These effects of G1 are similar to those of estrogen treatment. In addition, inhibition of GPER by siRNA and G15 eliminates the effects of estrogen. These findings highlight the importance of GPER in mediating estrogenic effects on sodium channel functions, and suggest that interventions of GPER hold great promise as druggable targets for the treatment of cardiovascular diseases related to sodium channels or stress.

Estrogen activates its rapid signaling pathway to ameliorate cardiac contractile functions and arrhythmias under stress by regulating the balance of the βAR-G protein which controls the functions of ion channels.[8-10] Herein, we also provided definitive evidence that estrogen, through GPER, improves sodium channel dysfunction in stress states, although it increases sodium channel function in normal states in hiPSC-CMs. This study elucidates on the cardioprotective mechanisms of estrogen from the ion channels angle in stress conditions that are involved in β-adrenergic stimulation. It further provides guidance for the follow-up study on the downstream signaling mechanism of GPER activation, sodium channel expression, and arrhythmia susceptibility, as well as the interaction between GPER and βAR-G protein signaling pathway.

Study limitations

In this study, some additional considerations may be required. The RMP and APs of hiPSC-CMs in this study were very similar to human adult cardiomyocytes, and sodium currents were distinct. However, the $\mathrm{d}V/\mathrm{d}t_{\mathrm{max}}$ was smaller than that of adult cardiomyocytes and the expression of sodium channels was not detected. Although studies have used hiPSC-CMs, either the reduced or no inward rectifier potassium current (I_{K1}) enabled our objective examination of its possible electrophysiological immaturity and potential changes in sodium channel availability. Further studies should consider the use of dynamic clamp technique by injecting I_{K1} to improve the electrophysiological maturity of hiPSC-CMs, so as to obtain realistic APs and sodium currents.[2]

Clinical implications

Different estrogen concentrations are an important variable that influence decisions regarding therapeutic strategy for preventing and treating estrogen or stress-induced cardiovascular diseases. Estrogen promotes the occurrence of arrhythmias and resists stress-induced arrhythmias by altering action potentials. Through GPER, estrogen improves sodium channel functions in stress conditions, and strengthens sodium channel function in normal states. Therefore, rational use of estrogen and targeting GPER may be an effective approach for treating sodium channel, estrogen or stress-related cardiac diseases.

In conclusion, our results show that estrogen, antagonized the stress-related abnormal electrical activity,

and through its rapid signal receptor GPER, alleviated sodium channel dysfunctions in stress state, and enhanced sodium channel functions in normal state in hiPSC-CMs. These results elucidate the mechanism through which the estrogenic rapid signaling pathway regulates ion channels. There is a need to establish the role of sex hormones in cardiovascular diseases.

Conflict of interest statement

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

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Author contributions

Xide Hu and Hong Sun contributed to the study conception and design. Material preparation, data collection and analysis were performed by Xide Hu, Lu Fu, Mingming Zhao, Hongyuan Zhang, Zheng Gong, Tongtong Ma. The first draft of the manuscript was written by Xide Hu. The revision in language and logic of the manuscript was undertook by Xide Hu, Hong Sun, Jeremiah Ong'achwa Machuki, Gabriel Komla Adzika, Xiaomei Liu and Renxian Tang. All authors read and approved the final manuscript.

Availability of data and material

The data underlying this article are available in the article and its online supplementary materials.

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Figure legends

Figure 1 E2 inhibites the increase in I_{NaL} in hiPSC-CMs stimulated by ISO. (A) Representative I_{NaL} of hiPSC-CMs incubated with E2 (0.01 nmol/L, 1 nmol/L and 100 nmol/L, 1h) or ISO (10 μmol/L, 30 min). (B and C) Average I_{NaL} density. n=8-26 cells, from 3-10 petri dishes. One-way ANOVA was used for data analysis. Kruskal-Wallis test was selectively used in cases of uneven variance. $^{*}p < 0.05$.E2, β-Εστραδιολ. Ινσετς: ολταγε ςλαμπ προτοςολς.

Figure 2 E2 increases peak I_{Na} but did not alter I_{Na} kinetics. (A) Representative peak I_{Na} in hiPSC-CMs incubated with various concentrations of E2 (0.01 nmol/L, 1 nmol/L, and 100 nmol/L, 1h). (B) Current-voltage curve of sodium channel. (C) Peak I_{Na} density. n=10-32 cells, from 4-11 petri dishes. (D) Activation and inactivation curve of the sodium channel. (E)Time constant of recovery from inactivation curve of the sodium channel. (F-I) Values for k and $V_{1/2}$ of (in) activation. n=8-26 cells, from 4-11 petri dishes. (J) t value of recovery from inactivation. n=8-17 cells, from 4-10 petri dishes.$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Insets: voltage clamp protocols. Statistical analysis and other abbreviations are as shown above (the same below).

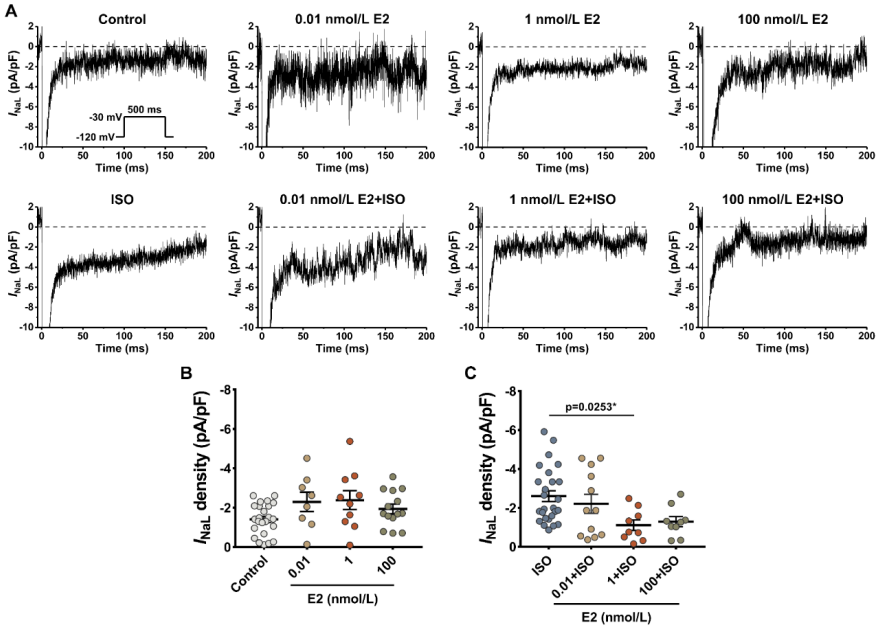
Figure 3 E2 reduces ISO induced increase in peak I_{Na} without altering I_{Na} kinetics. (A) Representative peak I_{Na} in hiPSC-CMs pre-treated with various concentrations of E2 (0.01, 1, and 100 nmol/L, 30 min before ISO treatment). (B) Current-voltage curve of the sodium channel. (C) Peak I_{Na} density. n=12-37 cells, from 4-11 petri dishes. (D) Activation and inactivation curve of the sodium channel. (E) The time constant of recovery from inactivation curve of the sodium channel. (F-I) Values for k and $V_{1/2}$ of (in) activation. n=8-34 cells, from 4-11 petri dishes. (J) t value of recovery from inactivation. n=5-32 cells, from 3-11 petri dishes. $^{**}p < 0.01$, $^{****}p < 0.0001$.

Figure 4 GPER mediates the effect of estrogen on increasing peak I_{Na} but did not alter I_{NaL} and I_{Na} kinetics. (A) Representative I_{NaL} in hiPSC-CMs. (B) Representative I_{Na} in hiPSC-CMs. (C) Average I_{NaL} density. n=10-25 cells, from 4-9 petri dishes. (D) Current-voltage curve of the sodium channel. (E) Peak I_{Na} density. n=9-32 cells, from 4-11 petri dishes. (F) Activation and inactivation curve of the sodium channel. (G) The time constant of recovery from inactivation curve of the sodium channel. (H-K) Values for k and $V_{1/2}$ of (in) activation. n=8-26 cells, from 4-11 petri dishes. (L) t value of recovery from inactivation. n=7-17 cells, from 3-10 petri dishes. $^{*}p < 0.05$, $^{**}p < 0.01$,$^{***}p < 0.001$. G1, G protein-coupled estrogen receptor agonist; G15, G protein-coupled estrogen receptor antagonist.

Figure 5 Estrogen through GPER reduces the increase induced by ISO in sodium currents but does not alter I_{Na} kinetics. (A) Representative I_{NaL} in hiPSC-CMs. (B) Representative I_{Na} in hiPSC-CMs. (C) Average I_{NaL} density. n=9-26 cells, from 4-10 petri dishes. (D) Current-voltage curve of the sodium channel. (E) Peak I_{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 the sodium channel. (H-K) Values for k and $\mathrm{V}_{1/2}$ of (in) activation. n=8-34 cells, from 4-11 petri dishes. (L) t value of recovery from inactivation. n=5-32 cells, from 4-11 petri dishes. $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

*Figure 6*Silence of GPER cancels the effect of estrogen on regulating peak I_{Na} and I_{NaL}. (A and F) Representative I_{NaL} in hiPSC-CMs. (B and G) Representative peak I_{Na} in hiPSC-CMs. (C and H) Current-voltage curve of sodium channel. (D and I) Activation and inactivation curve of sodium channel. (E and J) The time constant of recovery from inactivation curve of sodium channel. GPER, G protein coupled estrogen receptor. Insets: voltage clamp protocols.

Figure 1



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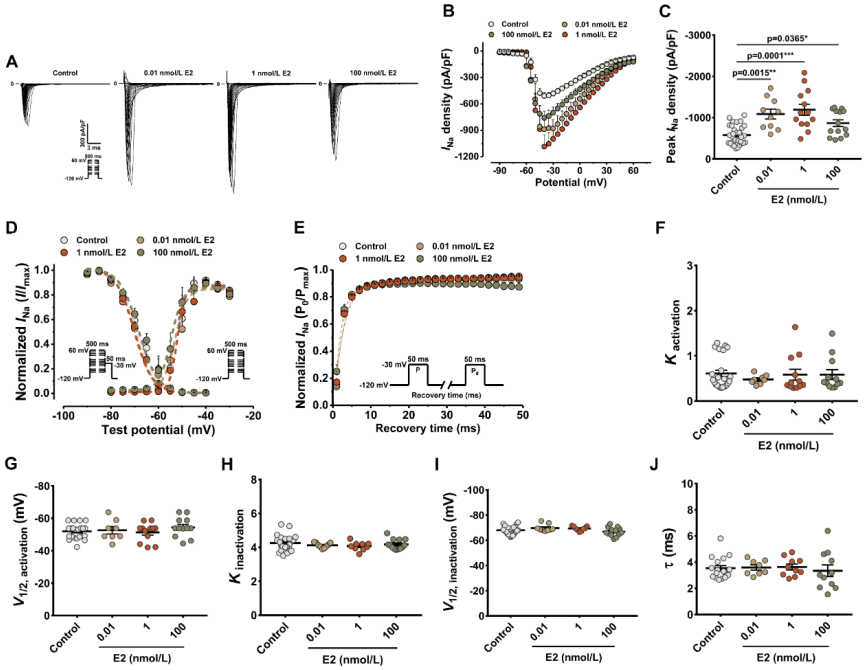

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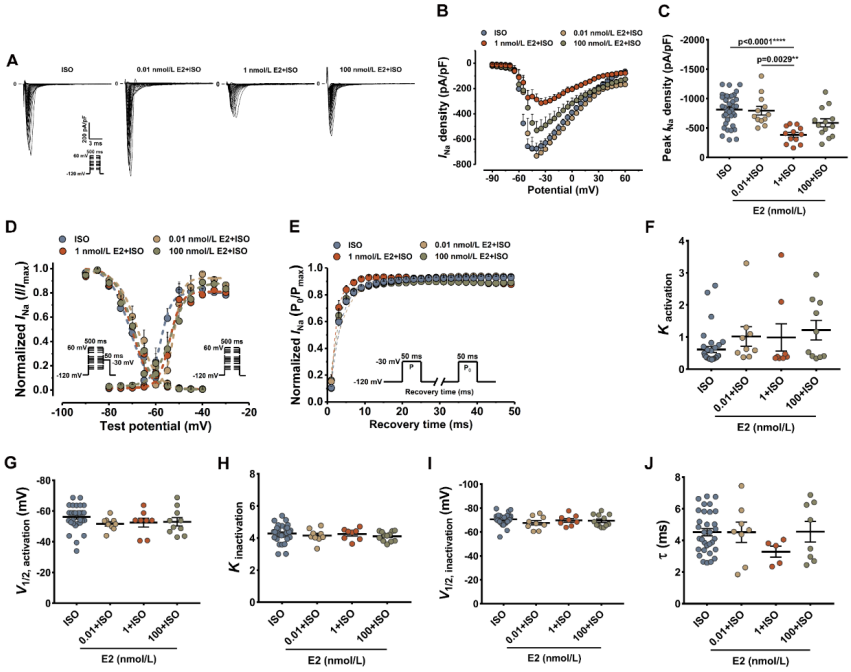

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Figure 4

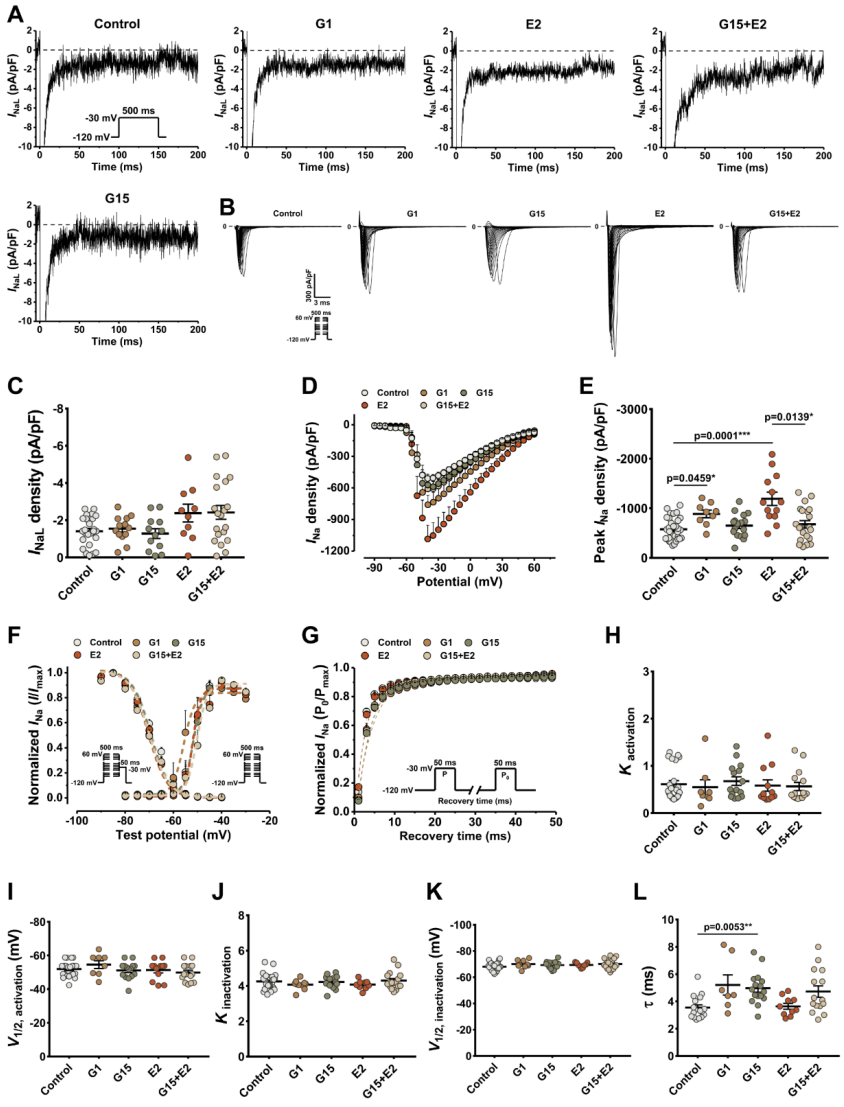

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Figure 5

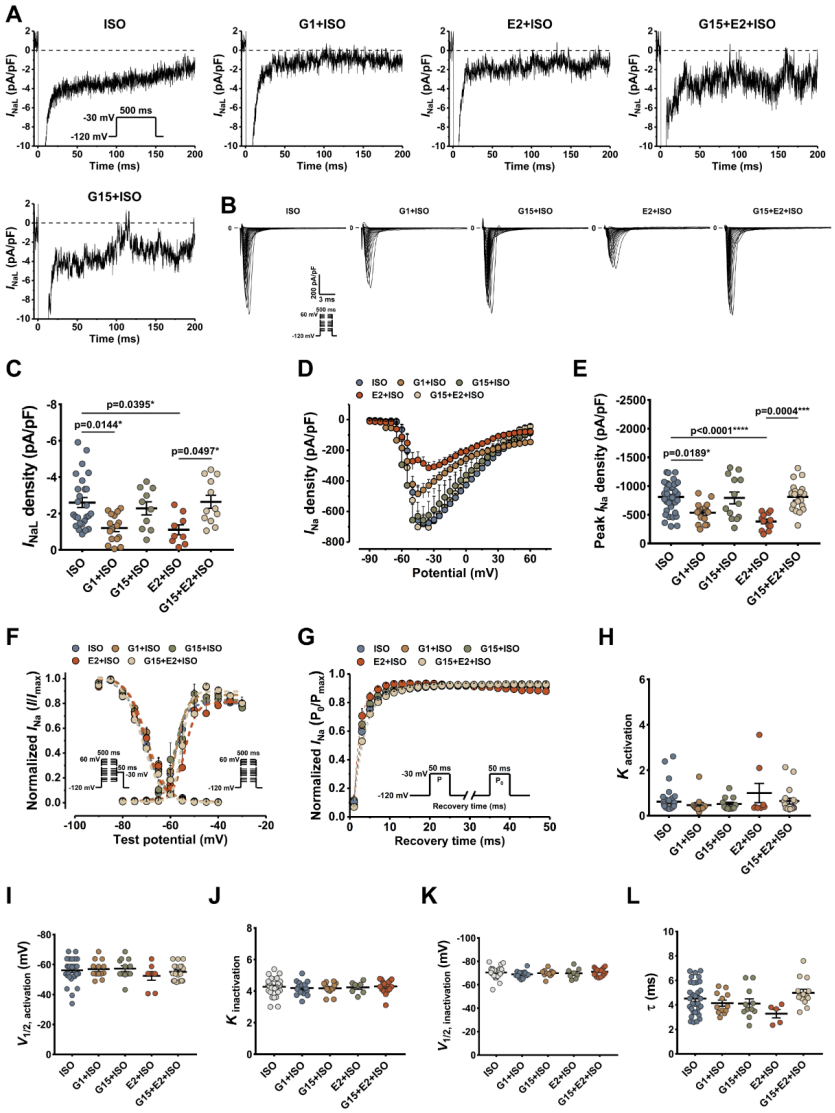

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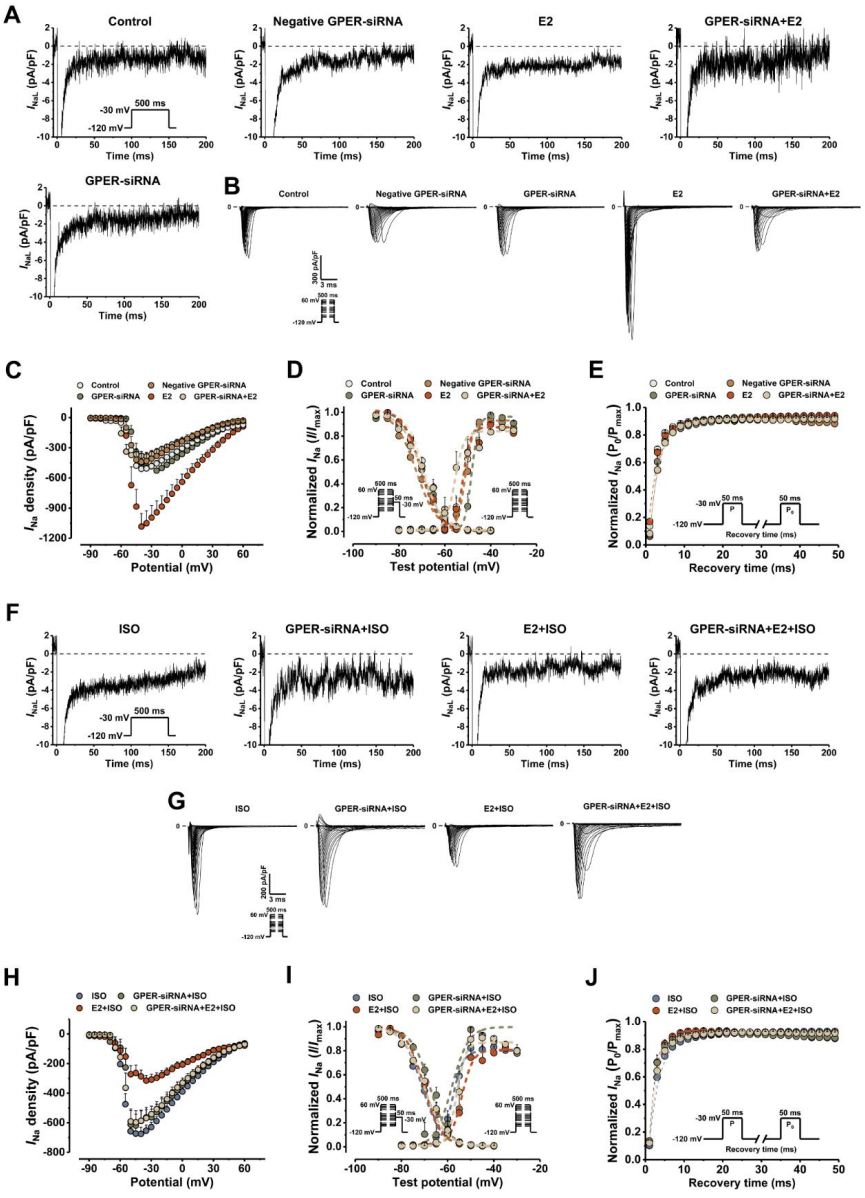
Figure 6



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