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Estradiol protection against toxic effects of catecholamine on electrical properties in human-induced pluripotent stem cell derived cardiomyocytes

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ABSTRACT

Background and purpose: Previous studies revealed that Takotsubo cardiomyopathy (TTC), a transient disorder of ventricular dysfunction affecting predominantly postmenopausal women, is associated with acquired long QT syndrome and arrhythmias, but the exact pathophysiologic mechanism is unknown. Our aim is to investigate the electrophysiological mechanism for QT-prolongation in TTC-patients by using human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs).
Methods: hiPSC-CMs, which were generated from human skin fibroblasts of three healthy donors, were treated by estradiol (10 μM for one week) and a toxic concentration of isoprenaline (Iso, 1 mM for 2 h). Patch clamp techniques, qPCR and fluorescence-activated cell sorting (FACS) were employed for the study.
Key results: Iso enhanced late I_{Na} and suppressed I_{to} and thus prolonged the action potential duration (APD), suggesting possible reasons for arrhythmias in TTC. Iso elevated the production of reactive oxygen species (ROS). *N*-acetylcystein (1 mM), a ROS-blocker, abolished the effects of Iso on late I_{Na} and I_{to}. H_2O_2 (100 μM) mimicked Iso effects on late I_{Na} and I_{to}. These data indicate that the effects of Iso were mediated by ROS. Metoprolol (1 mM), a beta-blocker, prevented the effects of Iso on late I_{Na} and APD, confirming the adrenoceptor-dependent effects of Iso. Estradiol treatment prevented the APD-prolongation, attenuated the enhancement of I_{Na}, diminished the reduction of I_{to}, suppressed ROS-production induced by Iso and reduced the expression levels of adrenoceptors, suggesting protective effects of estragon against toxic effects of catecholamine.
Conclusions: Estradiol has protective effects against catecholamine excess and hence reduction in estrogen level may increase the risk of acquired long QT syndrome in TTC.

1. Introduction

Takotsubo Cardiomyopathy (TTC), being first described in 1990, is a transient disorder of ventricular wall dysfunction characterized by regional wall motion abnormalities and clinically representative of an acute heart failure syndrome with substantial risk for adverse events [1–3]. TTC-Patients present with symptoms such as chest pain and dyspnea, which may mimic an acute coronary syndrome (ACS). TTC may be associated with some critical complications such as heart failure, life-threatening arrhythmias, atrial fibrillation, long QT syndrome (LQTs), thromboembolic events, left ventricular outflow obstruction, mitral valve regurgitation and cardiac rupture [4–11]. Acquired long

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QT syndrome has been described in up to 50% of TTC cases and related to life-threatening arrhythmias [7].The exact pathophysiological mechanism for selective wall motion abnormality in the absence of significant coronary artery stenosis remains unknown. Likewise, the underlying mechanism of acquired long QT syndrome in TTC is not thoroughly understood. The facts that TTC occurs frequently in patients under stress and postmenopausal women are usually more affected by the disease than premenopausal women, point to the roles of catecholamine and estrogens, respectively [12,13]. The effects of catecholamine excess and protective effects of estrogen against the toxic effects have been investigated in some animals or animal cells [14,15]. However, studies in human cardiomyocytes on cellular electrophysiology in TTC, especially the studies on mechanisms of long-QT in TTC, are still lacking. Given that oxidative-stress has been implicated in the pathogenesis of heart diseases including arrhythmias and heart failure [16]. We hypothesized that high concentration of catecholamine may elevate intracellular reactive oxygen species (ROS) production impairing cell function and estrogen may protect the cell from the impairment.

Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) have important advantages over heterologous expression systems like Xenopus oocytes, human embryonic kidney (HEK) cells and Chinese Hamster Ovary (CHO) cells lacking important constituents of cardiac ion channel macromolecular complexes that might be necessary for the normal electrophysiological characteristics, and also over transgenic animals possessing cardiac electrophysiological properties crucially different from that in humans. In addition, emerging evidences indicate that the hiPSC-CMs derived from patients with genetic heart diseases recapitulated the phenotype of the disease [17–21]. In our group, by using hiPSC-CMs we established successfully an inflammatory model of LPS-induced inflammation [22]. Therefore, we used hiPSC-CMs to investigate the toxic effects of catecholamine on cellular electrophysiological properties and the protective effects of estrogen.

2. Methods

A detailed description of all methods is provided in the online-only Supplemental Data. Key aspects are summarized below.

2.1. Ethics statement

The skin biopsy from three healthy donors was obtained with written informed consent. The study was approved by the Ethics Committee of Medical Faculty Mannheim, Heidelberg University (approval number: 2009-350N-MA) and conducted in accordance with the Helsinki Declaration of 1975, as revised in 1983.

2.2. Generation of human iPS cells

The human iPS cells (hiPSCs) were generated from primary human fibroblasts derived from skin biopsies of three healthy donors (D1, D2 and D3). D1 cells were generated by using lentiviral particles carrying the transactivator rtTA and an inducible polycistronic cassette containing the reprogramming factors OCT4, SOX2, KLF4 and c-MYC as previously described [23,24]. D2 and D3 cells were generated in feeder free culture conditions using the integration-free episomal 4-in-1 CoMiP reprogramming plasmid (Addgene, #63726) with the reprogramming factors OCT4, KLF4, SOX2, c-MYC and short hairpin RNA against p53, as described previously with modifications [25]. Generated hiPSCs were cultured under feeder free conditions. To investigate pluripotency, hiPSCs were subjected to a teratoma-formation assay [23].

2.3. Generation of hiPSC-CMs

Frozen aliquots of hiPSCs were thawed and cultured without feeder cells and differentiated into hiPSC-CMs as described with some modifications [26]. At 30 to 60 days of culture with basic culture medium, cardiomyocytes were dissociated from 24 well plates and plated on matrigel-coated 3.5 cm petri dishes for patch-clamp measurements.

2.4. Polymerase-chain-reaction assays

Details are provided in the online-only Supplemental Data. The primers that were used are listed in Table S1. RNA was reverse transcribed and converted to cDNA with oligo$(dT)_{15}$ primers using AMV reverse transcriptase according to standard protocols. Relative quantification of mRNA expression was calculated as follows: The expression of the mRNA of the gene of interest relative to the housekeeping gene GAPDH in samples from treated or untreated (Control) cells was calculated by the ΔΔCT method, based on the threshold cycle (CT), as fold change $= 2^{-\Delta(\Delta CT)}$, where $\Delta CT = CT_{\text{gene of interest}} - CT_{GAPDH}$ and $\Delta(\Delta CT) = \Delta CT_{\text{treated}} - \Delta CT_{\text{Control}}$ [27]. To ensure reproducibility the experiment was repeated once. From each experiment the cDNA of three cell culture wells were measured as biological replicates of each treatment. Each cell culture well was measured in duplicate as technical replicates.

2.5. Immunofluorescence staining

Immunofluorescence staining was performed using appropriate primary antibodies and AlexaFluor conjugated secondary antibodies (ThermoFisher). The primary antibodies used in this study were Tra-1-60 (StemGent), SSEA-3 (StemGent), alpha actinin (Sigma Aldrich), and TNNT (Sigma Aldrich).

2.6. Flow cytometry

Reactive intracellular oxygen species (ROS) levels were measured by using the dye 2,7 f-Dichlorofluorescin diacetate (DCF-DA, Sigma Aldrich). Cardiomyocytes were treated with either 1 mM isoprenaline or the same amount of water (vehicle control) for 2 h. After the treatment period, cells were harvested, washed with PBS and subsequently loaded with 41 μmol/L DCF-DA for 15 min at 37 °C in 5% CO_2. Cells were then washed with PBS followed by flow cytometer (BD FACS Canto II, BD Sciences, USA) analysis to quantify intracellular ROS levels. Fluorescence was measured at 488 nm (excitation) and at 530 nm (emission). At least 25,000 events were acquired and analyzed per experimental condition.

2.7. Patch-clamp

Standard patch-clamp recording techniques were used to measure the sodium (I_{Na}), L-type calcium (I_{CaL}), transient outward potassium (I_{to}), rapidly delayed rectifier potassium (I_{Kr}) and slowly delayed rectifier potassium (I_{Ks}) channel currents as well as action potential (AP) in the whole-cell configuration at room temperature.

2.8. Statistics

Data are shown as mean ± SEM and were analyzed using InStat (GraphPad, San Diego, USA). By analyzing the data with the Kolmogorov Smirnov test it was decided whether parametric or non-parametric tests were used for analysis. For parametric data one-way ANOVA and Dunnett's (all vs. control) or Bonferroni (selected groups) multiple comparisons post-tests were performed. For non-parametric data the Kruskal-Wallis test with Dunn's (selected groups) multiple comparisons post-test was used. Unpaired Student's t-test was used for comparisons of two independent groups with normal distribution. $p < 0.05$ (two-tailed) was considered significant.

3. Results

3.1. Characterizations of hiPSC-CMs

Cardiomyocytes were generated from hiPS-cells derived from skin fibroblasts of three healthy donors. To confirm the successful generation of hiPSC-CMs, cells were characterized at the beginning (day 0) and at different time points after onset of differentiation by immunostaining and qPCR analysis. The selected iPSC colonies displayed characteristic human embryonic stem cell (ESC) morphology, exhibited positive immunostaining for ESC markers SSEA-3 (StemGent) (Fig. S1A) and TRA-1-60 (StemGent) (Fig. S1C) prior beginning the differentiation but not at day 35 (Figs. S1B and 1D). At Day 35, the cardiac marker TNNT2 and alpha-actinin2 were expressed in the cells (Fig. S1E-H). qPRC analysis showed that the mRNA expression of the pluripotency gene POU5F1 (POU class 5 homeobox 1, RefSeq NM_002701.5) decreased by a factor of 10^3 from day 0 to day 35, while the typical cardiac genes, TNNT2 (Troponin T type 2, cardiac, RefSeq NM_000364) and MYBPC3 (Myosin-binding protein C, cardiac-type, RefSeq NM_000256), increased over time after onset of differentiation by a factor of 10^3 to 10^4 from day 0 to day 35 (Fig. S1I-K). Decreasing mRNA expression of pluripotency genes and increasing of cardiac genes as well as the spontaneous beating of cells starting around days 8–12 indicate the successful differentiation of cardiomyocytes from iPS-cells.

The electrophysiological properties of the hiPS-CMs from 30 to 40 days after onset of differentiation were also characterized. The cells displayed cardiac APs and functional I_{Na}, $I_{Ca\text{-}L}$, I_{Kr}, I_{Ks} and I_{to} that are planned to be investigated in this study.