Original Paper

Overexpression of M₃ Muscarinic Receptor Suppressed Adverse Electrical Remodeling in Hypertrophic Myocardium Via Increasing Repolarizing K⁺ Currents

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Key Words

Muscarinic acetylcholine receptor • Potassium currents • Electrical remodeling • Cardiomyocytes • Action potential duration

Abstract

Background/Aims: Cardiac hypertrophy (CH) is an adaptive response to diverse cardiovascular conditions, which is accompanied by adverse electrical remodeling manifested as abnormal K⁺ channel activities. M₃ subtype of muscarinic acetylcholine receptor (M₃-mAChR) is a novel regulator of cardiac electrical activity. In this study we aim to explore if the overexpression of M₃-mAChR could attenuate the adverse electrical remodeling in CH and then uncover its underlying electrophysiological mechanisms. **Methods:** Transgenic mice with M₃-mAChR overexpression (M₃-TG) and wild type (WT) mice were subjected to transverse aortic constriction (TAC) to induce CH. Myocardial hypertrophy and cardiac function were quantified by the measurement of echocardiography, electrocardiogram, heart weight and tibia length. Whole-cell and signal-cell patch-clamp were employed to record electrophysiological properties by acute isolation of acutely isolated ventricular cardiomyocytes and Western blot was carried out to evaluate the Kir_{2.1} and Kv_{4.2/4.3} protein levels in left ventricular tissue. **Results:** Compared with WT group, the elevation of cardiac index, including heart weight/body weight index and heart weight/tibia length index confirmed the myocardial hypertrophic growth induced by TAC. Echocardiography detection revealed that the TAC-treated mice showed an obvious increase in the thickness of left ventricular posterior wall (LVPW) and ejection fraction (EF) due to compensatory hypertrophy, which attenuated by the overexpression of M₃-mAChR. Pressure overload induced a prolongation of QTc interval in WT mice, an effect blunted in M₃-TG mice. Furthermore, compared with WT mice, M₃-mAChR overexpression in hypertrophic myocardium accelerated cardiac repolarization and shortened action potential duration, and

thus correcting the prolongation of QTc interval. Moreover, M₃-TG mice have the greater current density of I_{K1} and I_{to} in ventricular myocytes after TAC compared with WT mice. Finally, compared with WT mice, M₃-TG mice expressed higher levels of Kir_{2.1} in ventricular myocytes.

Conclusion: M₃-mAChR overexpression protected against adverse electrical remodeling in CH by enhancing potassium currents and promoting repolarization.

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Introduction

Adverse electrical remodeling is associated with many cardiac conditions and may predispose to arrhythmias and even sudden cardiac death, which is a major threat to human lives worldwide [1, 2]. It is particularly susceptibility in the occurrence of cardiac hypertrophy (CH) [3, 4]. CH is electrophysiologically characterized by prolongation of action potential duration (APD), as well as the temporal and spatial dispersion of membrane repolarization [5]. These electrical disturbances can result in electrical instability which eventually develops into life-threatening arrhythmias. Generally, the initial rapid phase (phase 1) of ventricular repolarization is caused by transient outward K⁺ current (I_{to}), and the terminal phase of ventricular repolarization is caused by inward rectifier K⁺ current (I_{K1}). Numerous evidences suggest that the reduction of I_{K1} and I_{to} contribute importantly to abnormal ventricular membrane repolarization [6-8]. For instance, the current densities of I_{K1} and I_{to} have been seen decreased in both experimental CH models and human CH [9-12]. These alterations favor the adverse ionic remodeling and lead to excess APD prolongation, which can further trigger the occurrence of arrhythmia. It is well known that both functional impairment and downregulation of channel proteins expression underlying these ion currents contribute to pathological decreases in their current densities. However, how these ion channels downregulate remains incompletely understood.

Cardiac ion currents are critically regulated by a wide array of factors which serve to maintain normal electrophysiological activities in the heart. Among these various regulators, the M₃ subtype of muscarinic acetylcholine receptors (M₃-mAChR) has been noticed in recent years for its role in modulating cardiac function under physiological and pathological conditions [13-15]. M₃-mAChR, which was once believed to be absent in myocardium, has not long ago been identified for its existence in the heart of various species, including man [16, 17]. More importantly, the research on the function of M₃-mAChR in myocardium reaches a new level as evidenced by its significant role in influencing many aspects of cardiac function, such as the regulation of cardiac electrophysiology [15, 18-20]. We have earlier unraveled that cardiac M₃-mAChR is coupled to a G-protein-gated delayed rectifier K⁺ current (I_{KM3}), and also demonstrated that I_{KM3} plays an important role in atrial repolarization and produces negative chronotropic and inotropic effects [20-22]. However, it remained unexplored whether M₃-mAChR is involved in the regulation of electrical remodeling in the setting of CH. Theoretically, M₃-mAChR might contribute to the remodeling process through activating its coupled outward repolarizing current I_{KM3}. This contribution nevertheless is likely limited to atrial electrophysiology because I_{KM3} was found to be present only in atrial myocytes but not in ventricular cells [23]. Given the ability of M₃-mAChR to modulate expression and function of genes relevant to cardiac pathophysiology, we proposed that it might regulate ion currents that are crucially involved in the adverse electrical remodeling in CH.

The present study was set up to prove our hypothesis by two specific goals. The first aim was to explore the potential role of M₃-mAChR in abnormal slowing of cardiac repolarization associated with CH induced by pressure-overload in transgenic mice with M₃-mAChR overexpression and in wild-type mice with M₃-mAChR stimulation. The second aim was to decipher the potential ionic mechanisms for the effects of M₃-mAChR on the adverse cardiac electrical remodeling. Our research generates strong evidence to support our hypothesis by providing insightful data for a mechanistic link between the function and the mode of action of M₃-mAChR.

Materials and Methods

Animals

All procedures for the use of animals were pre-approved by the Experimental Animal Ethic Committee of Harbin Medical University. Use of animals complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011).

Healthy male adult C57BL/6 mice (22–28 g) were obtained from the Experimental Animal Center of Harbin Medical University. The mice were received standard diet and water, and kept under standard conditions in the vivarium.

Construction of M₃-mAChR overexpression transgenic (TG) mice model

TG mice were generated in our university using a construct in which the α -myosin heavy chain (α -MHC) promoter drove the exclusive expression of M₃-mAChR in cardiomyocytes. TG mice and their wild-type (WT) littermates mice were used in our study. The mice were randomly divided into five groups: sham-operated WT control mice (WT/Sham), WT mice with CH (WT/CH), sham-operated TG control mice (M₃-TG/Sham), TG mice with CH administration (M₃-TG/CH), TG mice with CH and 4-DAMP administration (M₃-TG/CH+4DAMP).

Transverse aortic constriction (TAC)

CH model was established by pressure overload using the transverse aortic constriction (TAC) procedures [24]. Adult mice were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). After successful endotracheal intubation, the cannula was connected to a volume cy-129 cled rodent ventilator (UGO BASILE S.R.L. Italy). The transverse aorta was isolated from annexed tissue and partially ligated with 7.0 silk around a 25-gauge blunted needle, which was subsequently removed gently. Sham group mice underwent the same procedure, except that the transverse aorta was not ligated. The chest was closed and the mice were kept ventilated until the recovery of autonomic breath. Then, the mice were kept in warm environment with a breathing machine (UGO BASILE, Biological Research Apparatus, Italy) at the respiratory rate of 95 breaths/min with a tidal volume of 0.5 mL until restoration of spontaneous breathing. All experimental interventions and measurements were conducted 3 weeks after TAC.

Echocardiographic analysis, Heart weight (HW) and Tibia length (TL) measurement

After 3 weeks, the transthoracic echocardiography was performed and examined by using the high resolution little animals ultrasonic real-time video system (Vevo 2100, VisualSonics, Canada). When the anesthetic mice were prepared, place the transducer in vertical fashion with the notch pointing towards the animal's head. Then rotate the transducer approximately 35 degrees counterclockwise. Then locate diastole in the long axis loop, trace the endocardial area. Then repeat for systole. Once these measurements were complete, the available data of echocardiographic parameters could be determined at last. We have observed the LVPW (left ventricular posterior wall diameter), LVID (Left ventricular internal diameter) and EF% to evaluate the level of cardiac hypertrophy in mice.

The survived animals were then sacrificed, the hearts were quickly excised and washed by cold (4°C) PBS buffer. Another two observations were also used to evaluate the hypertrophic level, including the ratio of whole heart weight to body weight (HW/BW) and the ratio of heart weight to tibia length (TL).

Electrocardiogram analysis

QT interval was measured from ECG with the standard lead II method, using BL-420 bio-functional experiment system. ECG recording was carried out for 15 min every time and made over five recordings for each group. Electrodes were penetrated subcutaneously into four limbs and in correct positions on the chest, and then connected with electrocardiograph. We recorded the QT interval (ms) among the five groups. QTc interval was calculated with the modified Bazett formula ($QT/(RR/100)^{1/2}$, in ms), which was adjusted for mice [25]. The end of the T-wave determined as the point at which the slow component of the biphasic T-wave returned to the isoelectric line. All data were the mean of at least 50 beats per mouse and the analyzed data were carried by two independent examiners who were blinded to the genotypes. The survived animals were then sacrificed and the hearts were quickly excised for the subsequent experiments.

Ventricular cardiomyocyte isolation

The procedures used to isolate ventricular cardiomyocytes were similar to those described in detail previously [26]. Briefly, mice were anaesthetized by intraperitoneal injection of sodium pentobarbital (65 mg/kg). The hearts were excised rapidly and connected to a Langendorff apparatus with a constant flow at a rate of 3 mL/min for retrograde perfusion through the aorta, at 37°C for 5 min in Ca²⁺-free Tyrode's solution containing with (in mmol/L): NaCl 126, KCl 5.4, HEPES 10, NaH₂PO₄ 0.33, MgCl₂·6H₂O 1.0, and glucose 10 (pH was adjusted to 7.4 with NaOH). The hearts were then further digested with the same solution containing collagenase (Type II, 100 mg/L; Worthington Biochemical, Lakewood, NJ, USA) and bovine serum albumin (BSA, 100 mg/L; Gibco-BRL, Grand Island, NY, USA) at 37°C for an additional 5-10 min. Left ventricular cardiomyocytes were rapidly excised from the softened hearts, minced and placed in KB medium containing with (in mmol/L): glutamic acid 70, taurine 15, KCl 30, KH₂PO₄ 10, MgCl₂ 0.5, EGTA 0.5, HEPES 10, and glucose 10 (pH was adjusted to 7.4 with KOH) at 4°C for 1 h. All solutions were gassed with 100% oxygen and warmed to 37.0±0.5°C. Only single rod-shaped cells with clear cross-striations and without spontaneous contractions were available for patch-clamp.

Patch-clamp recording

The dissociation procedures for ventricular cardiomyocytes were similar to those described previously [27]. The cardiomyocytes were transferred to a chamber mounted on an inverted microscope (Nikon Diaphot; Nikon, Tokyo, Japan) for electrophysiological recording. The chamber was perfused with physiological salt solution containing with (mM): NaCl 136, KCl 5.4, MgCl₂·6H₂O 1, NaH₂PO₄·2H₂O 0.33, CaCl₂ 1.8, HEPES 10 and glucose 10 (pH was adjusted to 7.35 with 30 mM NaOH). I_{to} was recorded in the presence of 0.3 mM CoCl₂ to block I_{K1}, and I_{K1} was recorded in the presence of 2 mM 4-AP to suppress I_{to}. The pipette resistance was in the range 2-4 MΩ. The pipette solution contained (mM): potassium aspartate 110, KOH 110, KCl 20, MgCl₂ 1, HEPES 5, EGTA 10 and Na₂ATP 5 (pH was adjusted to 7.2 with 30 mM KOH).

To measure I_{K1}, myocytes were held at a membrane potential of -40 mV (to inactivate sodium and calcium channels) and voltage steps from -120 to +50 mV in 10 mV increments were applied for 300 ms with an inter-pulse interval of 1 s. The magnitude of I_{K1} was measured at the end of the 300 ms pulses. I_{to} was recorded from a holding potential of -80 mV and a 100 ms prepulse to -40 mV was used to inactivate the Na⁺ current followed by a 1000 ms test pulse to +50 mV. The amplitude of I_{to} was measured as the magnitude of the peak outward current. Action potential (AP) and K⁺ currents including inward rectifier K⁺ current (I_{K1}) and transient outward K⁺ current (I_{to}) were recorded under current- and voltage-clamp configurations respectively, using the whole-cell patch-clamp techniques with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA).

Western blot analysis

The procedures for membrane protein extraction from ventricular tissue and for western blot analysis have been described in detail previously [28]. Briefly, the protein samples were extracted from left ventricular tissues of mice. A total of 70 µg protein samples were fractionated by SDS PAGE (10-12 % polyacrylamide gels) and transferred to nitrocellulose membranes. The membranes were blocked with 5 % evaporated milk at room temperature for 2 h. Then the membranes were incubated overnight at 4°C with primary antibodies, including anti-Kir2.1, anti-Kv4.2, anti-Kv4.3 and anti-M₃ (Alomone Labs, Jerusalem, Israel), GAPDH (anti-GAPDH antibody from Kangcheng, Shanghai, China) was an internal control. The membranes were washed three times for 10-15 min each time with PBS-T, and incubated with secondary antibody for 1 h. The images were captured on the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). Western blot bands were quantified using Odyssey v1.2 software by measuring the band intensity (area × optical density OD) for each group.

Data analysis

Data are expressed as mean ± standard error (SEM). The significance (p<0.05) of results was assessed by one-way analysis of variance (ANOVA), followed by Post Hoc test multiple comparison and t-test when needed, using SPSS 20.0. Nonlinear least-square curve fitting was performed with CLAMPFIT in pCLAMP 10.2 or GraphPad Prism.

Results

M₃-mAChR Overexpression Attenuated Cardiac hypertrophy induced by TAC

To induce M₃-mAChR overexpression in cardiomyocytes, α -myosin heavy chain (α -MHC) promoter driving cardiac specific overexpression of M₃-mAChR in transgenic mice was established. The overexpression of M₃-mAChR in the myocardium of M₃-TG mice was verified by the data that showed the robust increase in the protein level of M₃-mAChR in M₃-TG mice (Fig. 1A). Then, we investigated the effects of M₃-mAChR overexpression on TAC-induced myocardial hypertrophy. Results showed that M₃-mAChR overexpression attenuated cardiac hypertrophy induced by TAC, as identified by the decreased ratio of heart weight to body weight (HW/BW) and heart weight to tibia length (HW/TL), which were reversed by 4-DAMP (Fig. 1B and 1C). Echocardiography detection revealed that the TAC-treated mice showed an obvious increase in the thickness of left ventricular posterior wall (LVPW) and ejection fraction (EF) due to compensatory hypertrophy and these were altered in M₃-mAChR overexpression (Table 1). On the whole, these data suggest the protective effects of M₃-mAChR overexpression against cardiac hypertrophy induced by TAC in mice.

M₃-mAChR overexpression prevented abnormal QTc prolongation in hypertrophic myocardium

To elucidate the potential role of M₃-mAChR in the regulation of cardiac electrophysiology and arrhythmogenesis, an experimental model of cardiac hypertrophy by transverse aortic constriction (TAC) was set up in M₃-mAChR transgenic mice (M₃-TG). Alterations of electrocardiogram in M₃-TG/CH mice were compared with those in sham-operated control M₃-TG mice (M₃-TG/Sham), wild-type mice with CH (WT/CH), and sham-operated control wild-type mice (WT/Sham). QTc interval was measured and calculated based on the electrocardiogram (ECG) recording by BL-420 bio-functional experiment system. (Fig. 2A) As illustrated in Fig. 2B, without TAC operation, QTc interval in M₃-TG was not significantly different from that in the WT/Sham group. While the QTc interval was significantly prolonged

Fig. 1. The M₃ protein expression level and the effects of M₃ overexpression on TAC-induced cardiac hypertrophy (A) M₃ protein expression level. (B) Comparison of HW/BW (heart weight/body weight). (C) Comparison of HW/TL (heart weight/tibia length). Data are expressed as mean $\pm$ SEM. *p < 0.05, **p < 0.01, n = 6.

Table 1. Echocardiographic measurements in mice, Data are expressed as mean $\pm$ SD. LVIDd, interventricular septum diameter in diastole; LVIDs, interventricular septum diameter in systole; LVPWd, left ventricular posterior wall diameter in diastole; LVPWs, left ventricular posterior wall diameter in systole; EF, ejection fraction. *p < 0.05 vs WT/sham, ***p < 0.001 vs WT/sham, #p < 0.05 vs WT/CH, ###p < 0.001 vs WT/CH, &p < 0.05 vs M₃-TG/CH, n = 5

Parameters	WT/Sham	M ₃ -TG/Sham	WT/CH	M ₃ -TG/CH	M ₃ -TG/CH+4DAMP
LVIDd,mm	3.42±0.46	3.32±0.38	3.23±0.56	3.29±0.41	2.91±0.22&
LVIDs,mm	2.08±0.48	2.13±0.47	1.8±0.57*	1.99±0.44	1.67±0.28&
LVPWd,mm	0.89±0.17	0.83±0.14	1.15±0.16*	1.13±0.18	1.35±0.23
LVPWs,mm	1.15±0.09	1.10±0.11	1.60±0.07***	1.3±0.09###	1.63±0.17&
EF%	71.0±8.21	70.6±8.13	80.3±7.20*	74.1±4.99#	76.1±6.03

Fig. 2. M₃-mAChR overexpression prevented abnormal QTc prolongation in CH mice. (A) Representative electrocardiogram (ECG) recorded from animals of varying groups. (B) Differences in QTc interval calculated by the formula $(QT/(RR/100)^{1/2})$. Data are expressed as mean±SEM. *p<0.05, n=6.

Fig. 3. M₃-mAChR overexpression suppressed abnormal APD lengthening in CH mice. (A) Changes of resting membrane potential (RMP). (B) Representative traces of single-cell AP recorded with or without TAC. (C) Averaged data of APD at 50% (APD₅₀) repolarization. (D) Mean data for APD at 90% (APD₉₀) repolarization. Data are expressed as mean±SEM. *p<0.05, **p<0.01, n=4.

in WT/CH mice compared with healthy counterparts (either WT/Sham or M₃-TG/Sham) and it remained shortened in M₃-TG/CH, indicating that M₃ overexpression prevented QTc lengthening induced by CH.

M₃-mAChR overexpression prevented abnormal APD lengthening in ventricular myocytes of CH mice

In order to delineate the cellular mechanisms of M₃-mAChR overexpression to shorten or prevent prolongation of QTc interval, we recorded the single-cell action potential (AP) and compared the resting membrane potential (RMP) and action potential duration (APD) in ventricular myocytes isolated from four different groups of mice. Fig. 3A showed representative AP recorded in ventricular cardiomyocytes derived from WT and M₃-TG mice with or without TAC mice as well as M₃-TG with 4DAMP mice. The resting membrane potential (RMP) was significantly depolarized in WT/CH mice, which was not seen in the M₃-TG/CH group (Fig. 3B). As depicted in Fig. 3C and 3D, APD at 50% and 90% repolarization (APD₅₀ and APD₉₀, respectively) were considerably lengthened in the cardiomyocytes isolated from WT/CH mice, but not from M₃-TG/CH in APD₅₀, relative to their respective sham controls. These effects were reversed by co-administration of 4-DAMP to selectively antagonize M₃-mAChR. These results indicated that M₃-mAChR overexpression could prevent the adverse electrophysiological changes manifested as abnormal APD lengthening and membrane depolarization in the setting of CH.

Fig. 4. M_3 -mAChR overexpression increased I_{K1} and I_{to} of ventricular cardiomyocytes. (A) Raw traces of I_{K1} recorded from ventricular myocytes of isolated from the hearts of varying groups. (B) The averaged current density-voltage (I-V) relationships of I_{K1} . (C) Raw traces of I_{to} . (D) I-V curves of I_{to} . Data are expressed as mean $\pm$ SEM. * p <0.05 vs WT/sham, # p <0.05 vs WT/CH, & p <0.05 vs M_3 -TG/CH, n =4.

Fig. 5. M_3 -mAChR overexpression upregulated the expression of Kir2.1. (A) Top panel: examples of Western blot bands of Kir2.1; bottom: mean values of Kir2.1 protein band density (n =6). (B) Top; examples of Western blot bands of Kv4.2; bottom: mean data of Kv4.2 protein (n =5). (C) Top: examples of Western blot bands of Kv4.3; bottom: Kv4.3 protein levels (n =4). Data are expressed as mean $\pm$ SEM. * p <0.05, ** p < 0.01.

M_3 -mAChR overexpression increased I_{K1} and I_{to} of ventricular cardiomyocytes

I_{K1} plays a key role in determining cardiac membrane potential and terminal phase of membrane repolarization (represented by APD_{90}) [29]. With whole-cell patch-clamp recordings, we found that I_{K1} density was substantially reduced in the ventricular myocytes isolated from WT/CH mice, relative to the sham control animals (Fig. 4A and 4B). Similar decrease was also observed in M_3 -TG/CH mice, but to a considerably less extent relative to WT/CH. On the other hand, transient outward K^+ current (I_{to}), known to be responsible for the initial phase of cardiac repolarization [7, 30], was found markedly decreased in WT/CH mice but not in M_3 -TG/CH mice as compared with their relative sham controls (Fig. 4C and 4D). These results indicate that the overexpression of M_3 -mAChR could increase I_{K1} and I_{to} in ventricular cardiomyocytes.

M_3 -mAChR overexpression upregulated the expression of Kir2.1 protein

The results above indicated that the enhancement of I_{K1} and I_{to} densities could be an ionic mechanism underlying the ability of M_3 -mAChR overexpression to prevent or correct the CH-induced QTc prolongation and APD lengthening, then we also would like to know how M_3 -mAChR overexpression act on these K^+ currents. We reckoned that M_3 -mAChR might act by upregulating the expression of same genes, which encoding the ion channel proteins for

I_{K1} and I_{to}. It is known that Kir2.1 is the main pore-forming α -subunit for the inward rectifier K⁺ channel carrying I_{K1} in cardiomyocytes [31]. On the other hand, I_{to} is a current flowing through the K⁺ channels composed of Kv4.2 and Kv4.3 as the main pore-forming α -subunits [32, 33]. We first measured the changes of Kir2.1 protein levels in cardiomyocytes from the mice of varying groups. As shown in Fig. 5A the data revealed that the Kir2.1 protein level was considerably downregulated in WT/CH mice, and it remained essentially unaltered in M₃-TG/CH mice relative to its sham control animals. This difference made the protein level of Kir2.1 far higher in M₃-TG/CH than in WT/CH. The beneficial effects of M₃-mAChR overexpression were nearly abolished by inhibition of M₃-mAChR with 4-DAMP. A similar pattern of differences in Kv4.2 and Kv4.3 protein levels was consistently observed (Fig. 5B and 5C), with downregulation of these K⁺ channel subunits in WT/CH mice but not in the M₃-TG/CH group.

Discussion

In the present study, we have investigated the role of M₃-mAChR in cardiac electrical remodeling associated with cardiac hypertrophy (CH) in transgenic mice with M₃-mAChR overexpression, and in wild-type mice with M₃-mAChR stimulation as well. The aim of our study was to elucidate the contribution of M₃-mAChR to the well-recognized slowing of repolarization as a part of the adverse electrical remodeling process in CH and their underlying ionic mechanisms. We first demonstrated that M₃-mAChR was tremendously upregulated in M₃-TG animals. Then we investigated the effects of M₃-mAChR overexpression on TAC-induced myocardial hypertrophy by cardiac index, tibia length and echocardiography analysis. We have revealed the overexpression of M₃-mAChR could prevent CH-induced prolongation of QTc interval. Further investigation unraveled the acceleration of cardiac repolarization or shortening of APD as a cellular mechanism for the correction of abnormal QTc prolongation. Finally, we deciphered the ionic mechanism for QTc shortening by detecting the enhancement of K⁺ currents (I_{K1} and I_{to}) and the molecular mechanism by the upregulation of Kir2.1 and Kv4.2/Kv4.3 protein expressions. Our findings prompted us to reach a conclusion that M₃-mAChR produces beneficial effects against the adverse electrical remodeling in CH by minimizing or correcting the expression downregulation and functional impairment of I_{to} and I_{K1}. Therefore, M₃-mAChR might be considered as a potential therapeutic target for arrhythmias occurring in CH and maybe in other cardiac conditions associated with M₃-mAChR as well.

It has been documented that M₃-mAChR offers protective effects against myocardial damages under various heart diseases by acting on multiple cellular and molecular components. For example, M₃-mAChR can interact with cardiac gap-junction channel connexin-43 to maintain normal cell-cell communication [34], activate the anti-apoptotic signal molecule Bcl-2 and p38 mitogen-activated protein kinase (MAPK) [28], enhance endogenous antioxidant capacity, reduce intracellular Ca²⁺ overload [27], and promote cardioprotection via inhibiting miR-376b-5p [35], protein kinase C- ϵ and β -catenin [36] in the setting of myocardial infarction. Atrial M₃ muscarinic receptors mediate positive inotropic effects in mouse [37]. M₃-mAChR protects the heart against hypertrophy by downregulating the expression of AT1 receptor [38], normalizing the deregulated expression of miR-133a [14], and upregulating the expression of phospho-p38 and calcineurin [39]. Probably, the most pertinent to the present study is the finding that M₃-mAChR can produce anti-arrhythmic effects in ischemic hearts through downregulating miR-1 expression along with upregulation of Kir2.1 levels and reducing Ca²⁺ overload mediated by enhanced L-type Ca²⁺ channels and NCX [13]. Here our findings add to the beneficial profile of M₃-mAChR with its anti-arrhythmic properties in the setting of CH. Although some published studies have demonstrated the role of M₃-mAChR in modulating cardiac conduction via targeting on connexin-43 and L-type Ca²⁺ channels, the present study unraveled the ability of M₃-mAChR to regulate cardiac repolarization through controlling the activities of I_{K1}/Kir2.1 and I_{to}/Kv4.2/Kv4.3.

Excessive APD prolongation is a hallmark of the abnormally altered electrophysiology or adverse electrical remodeling in CH, which is attributed to the reduction of voltage-gated Kv and inward rectifier Kir current densities. APD prolongation and a reduction of I_{K1} and I_{to} represent early electrical remodeling events in diseased myocardium [40], pointing toward a potential role in disease initiation and progression. Our single-cell current-clamp data indicated that M₃-mAChR overexpression in TG mice accelerated membrane repolarization and abbreviated APD, which counteracted with the CH-induced APD lengthening. Our voltage-clamp data further revealed that the repolarization-accelerating property of M₃-mAChR could be ascribed to its promoting effects on the expression of Kir2.1 and Kv4.2/4.3 K⁺ channel subunits, which is deemed to bring about increases of the current densities of I_{K1} and I_{to} . Enhancement of these outward repolarizing currents can then favor the membrane stability and repolarization to minimize arrhythmogenicity. These notions are readily understandable because Kir2.1 and Kv4.2/4.3 are the major pore-forming α -subunits of K⁺ channels that carry I_{K1} and I_{to} respectively [33, 41], which are the key repolarizing currents governing the length of APD thereby the likelihood of arrhythmogenesis [42]. It is interesting to note that the inhibiting effect of M₃-mAChR on L-type Ca²⁺ channels as reported previously can also contribute to APD shortening [27]. Moreover, it is also anticipated that the hyperpolarizing effects associated with the I_{K1} -enhancing effects of M₃-mAChR observed in this study would improve cardiac conduction by relieving Na⁺ channels from the inactivated state induced by membrane depolarization in CH. Together with the improving effect of M₃-mAChR on connexin-43 revealed in our previous study [34], it appears that M₃-mAChR favors both intracellular conduction (by minimizing Na⁺ channel inactivation) and intercellular excitation propagation (by enhancing connexin-43), which might also contribute to mitigating the adverse electrical remodeling.

It should be noted that there are still some limitations in the present study. Our study failed to delineate how M₃-mAChR upregulated expression of Kir2.1 and Kv4.2/4.3 in our model. It is possible that M₃-mAChR activation enhances the G_q protein/PLC/PKC signaling pathway that can ultimately activate transcription factors in nucleus to transactivate the expression of the genes coding for Kir2.1 and Kv4.2/4.3. Alternatively, M₃-mAChR could regulate expression of miRNAs, as having already been shown by previous studies, and the latter could then regulate the expression of Kir2.1 and Kv4.2/4.3 as target genes. Another limitation is that our study does not present panoramic view of the effects of M₃-mAChR. It is plausible that in addition to I_{K1} and I_{to} , M₃-mAChR might also act on other K⁺ currents critical to cardiac repolarization, such as the rapid and slow delayed rectifier K⁺ currents I_{Kr} and I_{Ks} , respectively, which are known to be responsible for termination of the plateau duration and for completing the final repolarization. These two currents can hardly be recorded in rodent species, and hence, further studies using other mammalian species such as guinea pig and rabbit are needed for determine whether M₃-mAChR also can modulate these currents.

Taken together, this is the first demonstration that M₃-mAChR overexpression plays a critical role in protecting cardiomyocytes from arrhythmogenesis in the setting of CH. The anti-arrhythmic property of M₃-mAChR is likely owing to its ability to reverse adverse electrical remodeling process by rescuing the mitigated I_{K1} and I_{to} densities to constrain excess APD/QTc prolongation. These favorable effects are conferred by the regulatory roles of M₃-mAChR on Kir2.1 and Kv4.2/4.3. It is therefore reasonable to speculate that M₃-mAChR overexpression or activation might be a worthwhile strategy for anti-arrhythmic therapy of CH patients. It is further conceivable that given the wide variety of advantageous actions afforded by M₃-mAChR, it might be applied to suppressing arrhythmogenesis under other cardiac conditions as well.

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Disclosure Statement

The authors declare no competing financial interests.

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