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Insulin acts as an atypical KCNQ1/KCNE1-current activator and reverses long QT in insulin-resistant aged rats by accelerating the ventricular action potential repolarization through affecting the β_3-adrenergic receptor signaling pathway

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Abstract

Insufficient-heart function is associated with myocardial insulin resistance in the elderly, particularly associated with long-QT, in a dependency on dysfunctional KCNQ1/KCNE1-channels. So, we aimed to examine the contribution of alterations in KCNQ1/KCNE1-current (I_{Ks}) to the aging-related remodeling of the heart as well as the role of insulin treatment on I_{Ks} in the aged rats. Prolonged late-phase action potential (AP) repolarization of ventricular cardiomyocytes from insulin-resistant 24-month-old rats was significantly reversed by in vitro treatment of insulin or PKG inhibitor (in vivo, as well) via recovery in depressed I_{Ks}. Although the protein level of either KCNQ1 or KCNE1 in cardiomyocytes was not affected with aging, PKG level was significantly increased in those cells. The inhibited I_{Ks} in β_3-ARs-stimulated cells could be reversed with a PKG inhibitor, indicating the correlation between PKG-activation and β_3-ARs activation. Furthermore, in vivo treatment of aged rats, characterized by β_3-ARs activation, with either insulin or a PKG inhibitor for 2 weeks provided significant recoveries in I_{Ks}, prolonged late phases of APs, prolonged QT-intervals, and low heart rates without no effect on insulin resistance. In vivo insulin treatment provided also significant recovery in increased PKG and decreased PIP2 level, without the insulin effect on the KCNQ1 level in β_3-ARs overexpressed cells. The inhibition of I_{Ks} in aged-rat cardiomyocytes seems to be associated with activated β_3-ARs dependent remodeling in the interaction between KCNQ1 and KCNE1. Significant recoveries in ventricular-repolarization of insulin-treated aged cardiomyocytes via recovery in I_{Ks} strongly emphasize two important issues: (1) I_{Ks} can be a novel target in aging-associated remodeling in the heart and insulin may be a cardioprotective agent in the maintenance of normal heart function during the aging process. (2) This study is one of the first to demonstrate insulin's benefits on long-QT in insulin-resistant aged rats by accelerating the ventricular AP

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repolarization through reversing the depressed I_{Ks} via affecting the β_3-ARs signaling pathway and particularly affecting activated PKG.

KEYWORDS

ATP dependent K^+-channels, cardiomyocytes, electrical activity, heart dysfunction, insulin resistance, intracellular ionic homeostasis, voltage-dependent ionic channels

1 | INTRODUCTION

Aging is a physiological adaptation through the inevitable functional decline with time in the biological processes of mammals. This process continues parallel to increasing the propensity of the development of age-related diseases, including cardiovascular diseases (CVDs; Chason et al., 2018). It is clinically significant that both cardiac dysfunction and systemic insulin resistance are mostly observed together with the advancing of aging (Boudina, 2013; Van Noord, Sturkenboom, et al., 2010). Cardiac aging is defined as a progressive and intrinsic decline in heart function, which is mainly characterized by a long QT-interval in surface electrocardiograms (ECGs), a reduction in the maximum heart rate, and a decrease in the contractile activity (Lakatta et al., 2001). Correspondingly, the age-dependent alterations in heart function are also characterized by structural abnormalities impeding the heart's responsiveness to several intra- and extracellular stimulations, including cardiometabolic disturbances. Cardiometabolic disturbances include a combination of metabolic dysfunctions mainly characterized by insulin resistance, impaired glucose tolerance, and hypertension (de Waard et al., 2018). Furthermore, the aggregate of these risk factors leads to impairment in electrophysiological activities, and myocardial cell death (Boudina, 2013; Olgar et al., 2018; Olgar, Billur, et al., 2020; Olgar, Tuncay, et al., 2020; Van Noord, Sturkenboom, et al., 2010). In a mouse model with cardiac-specific insulin receptor deletion, it has been shown that the absence of insulin signaling in the heart induces changes in voltage-dependent K^+-channel expression/function and prolongation in action potential duration (APD) of isolated ventricular cells parallel to long-QT (Lopez-Izquierdo et al., 2014). However, these results support the notion of the leading cause of insulin signaling disturbance in the heart of the repolarization abnormalities (at most long-QT) in animal models of diabetes as well as aged-mammals (Bertrand et al., 2008; Durak et al., 2018; Olgar, Billur, et al., 2020; Olgar, Tuncay, et al., 2020). Therefore, understanding the molecular mechanisms linking insulin resistance and CVDs will help to design new and more effective mechanism-based agents to improve the myocardial performance of aged humans. Supporting these statements, the efficacy of insulin in preventing long-QT and the associated arrhythmias in diabetic rabbits through reduction of the rapid delayed rectifier K^+-current (Y. Zhang et al., 2006) has been demonstrated. Dubó et al. (2016) further widely discussed the relationships between impaired insulin signaling and decreases in transient outward K^+-currents and the ultra-rapid delayed rectifier-currents, and their contribution to long-QT in mammalian cardiomyocytes. These results support the hypothesis that lack of insulin signaling can produce abnormal repolarization in cardiomyocytes, having an important impact on the development of the arrhythmogenic potential, further leading to an increase in the incidence of sudden cardiac death. Altogether, therefore, already known studies strongly pointed out the regulatory function of insulin in cardiac electrophysiology. Although altered cardiac function with age can be exacerbated by several risk factors including insulin resistance, intrinsic cardiac aging is an independent risk factor for the development of CVDs.

Even early findings have shown how the changes in the expression level of a slow delayed rectifier channel can contribute to AP repolarization in the human heart (Abbott, 2020; Dixit et al., 2020; Marx et al., 2002). This type of K^+-channel has two components, such as a major pore-forming component, KCNQ1, and a small auxiliary component, KCNE1, and carries a slow outward K^+-current (I_{KS}; Abbott, 2020; Chan et al., 2020). Moreover, the important contribution of changes in these channel subunits to the induction of prolonged APD, leading to further cardiac arrhythmias (Huang et al., 2018) has been shown. On the other hand, the sympathetic nervous system regulation of cardiac APD is mediated by β-adrenergic receptors (βARs), which in turn increases the slow outward K^+-current (Leineweber et al., 2004; Xiao et al., 2006). Hence, although the main cellular mechanism for reduced cardiac β-AR responsiveness during aging is not very clear, targeting β-AR signaling seems to be effective for the aging heart's insufficient function (Lucia et al., 2018). Moreover, it has been also shown that phosphatidylinositol-4,5-biphosphate, PIP_2, regulates I_{KS}, which can also mediate the effects of cardiac arrhythmia-associated gene variants (Liu et al., 2020; Taylor & Sanders, 2017; Zaydman & Cui, 2014; H. Zhang et al., 2003). In addition to the above, it is mentioned that the regulation of KCNQ1 is performed by non-KCNE protein subunits, such as βAR-signaling hub (Marx et al., 2002). Moreover, some published data indicate the close relationship between the alterations in I_{Ks} and not only the long-QT and but also short-QT in humans (Bellocq et al., 2004; Ghosh et al., 2006; Park et al., 2005; Schwartz et al., 2012; Shamgar et al., 2006; Wulff et al., 2009; Yang et al., 1997; Y. Zhang et al., 2006). However, although there are contradictory data related to insulin action on K^+-channel currents, including I_{Ks} (Durak et al., 2018; Wu & Sanguinetti, 2016; Wu et al., 2014, 2017), the question of whether any change in I_{Ks} has the role in aging heart function as well as whether insulin can have a protective effect on the aging heart through affecting the I_{Ks} are not yet clear.

Insulin is a key component of GIK-cocktail (glucose-insulin-potassium) through modulation of PI3K-Akt-eNOS-dependent signal mechanism (Alburquerque-Bejar et al., 2015; Boucher et al., 2014; Lopez-Izquierdo et al., 2014; Yu et al., 2011). Taking into consideration the role of cAMP and PKA activation on I_{Ks} (Kanda et al., 2011), one can hypothesize their contribution to this current. However, previous studies

demonstrated that both cAMP and PKA were found to be depressed in insulin-resistant hearts, in a manner parallel to activation of β_3-AR with inhibition of β_1-AR (Dincer et al., 2001; Okatan et al., 2015). More importantly, it is today a commonly accepted issue that ion channelopathies, induced by dysfunctional ion channels, are important diseases associated with even underlying the sudden death. Therefore, considering the role of activated PKG on the modulation of PIP2 in the inner side of membranes (Kirk et al., 2016), we hypothesized that PKG can be activated through the activation of β_3-AR in insulin-resistant aged cardiomyocytes, which in turn, inactivate I_{Ks} also leading to long QT, and therefore play an important role in the remodeling of the aging heart. Furthermore, taking into consideration the possible relations either between PKG and PIP2, PIP2 and IR/IGF-IR, or both, we hypothesized that insulin treatment of aged rats can provide cardioprotection via affecting either activated β_3-AR, activated PKG, or both, which further leads to recovery in the depressed I_{Ks} and thereby ECGs in the aged rat heart. For validation of this hypothesis, we also used metabolic syndrome rats parallel to the aged rats to investigate our hypothesis, because the MetS is characterized by marked insulin resistance.

2 | MATERIALS AND METHODS

2.1 | Animals

Male Wistar rats with an age of 24-month-old (Aged group) and age of 8-month-old (Adult group) were used. All rats were given free access to tap water and exposed to a 12-h light–dark cycle. They were fed standard chow ad libitum daily. All animals were housed in the standard rat cages as three per cage. All experiments were approved by the Institutional Animal Care and Use Committee at the Ankara University, Ankara (No: 2016-18-165).

For in vivo treatment, one group of aged rats received insulin (Chr-Ins; 2 IU/kg/day, Sigma-Aldrich I0516, for 2 weeks, i.p.; (Plum et al., 2000), while another group was treated with a PKG inhibitor (Chr-PKG; 1 mg/kg/day, Cayman 159995, for 2 weeks, i.p.; Das et al., 2008), as well. The third group aged rats received vehicles during the same period.

To validate insulin resistance in the aged rats, we determined the blood insulin level, HOMA-IR index, and the oral glucose tolerance test (OGTT), as described previously (Olgar et al., 2021). We kept aged animals for experiments that have approximately 25% high serum insulin level and 80% increased HOMA-IR index in comparison to the others.

2.2 | In situ ECG recordings

Electrical activity of the heart in situ assessed by surface electrocardiography (ECG) recordings and data were acquired by using an analog-to-digital converter BIOPAC MP35 (Goleta California) and processed with a high-cut (low-pass) filter at 50–500 Hz. Experimental animals underwent ether inhalation anesthesia during the recordings and then bipolar limb leads (lead I, II, III) were carried with carefully placed 20 gauge needles to forearms and hind limb. ECG recordings were done for 10 min from every animal.

2.3 | Cardiomyocyte isolation

Freshly cardiomyocyte isolation was performed by using the enzymatic method, as described previously (Olgar et al., 2021). Briefly, hearts were cannulated in a Langendorff-perfusion system and followed with 1-mg/ml collagenase (Type IV) for 30–35 min. Following digestion with collagenase, the left ventricular part was used for cells and we used only Ca^{2+}-tolerant rod-shaped cells.

2.4 | Patch-clamp experiments

APs and ionic currents in freshly isolated left ventricular cardiomyocytes were examined by using an Axopatch 200B amplifier (software: pCLAMP 10.0 Axon Instruments and analog-to-digital converter: Digidata 1440A). All recordings were sampled and digitized at 5 kHz and filtered at 3 kHz with the Digidata. Liquid-junction-potential was compensated before establishing the gigaseal on the cell and no leak or capacitance subtractions were performed in the current and voltage recordings. All records were measured with borosilicate glass capillary tubes with 2–3 MΩ to avoid internal dialysis. The cell capacitance of cardiomyocytes was measured to determine cell size. All recordings were performed at room temperature (22 ± 2°C).

APs were determined under electrical stimulation at 0.5 Hz frequency by using the whole-cell configuration of patch-clamping at the current-clamping mode, as described previously (Degirmenci et al., 2018). Cells were placed in a HEPES-buffered bathing solution containing (in mmol/L); NaCl 137, KCl 4, $MgCl_2$ 1, CaC_{l2} 1.8, Na-HEPES 10, and glucose 10 at pH = 7.40. The pipette solution for AP recording contained a solution including (in mmol/L) KCl 140, HEPES 25, Mg-ATP 3, EGTA 5, and Na GTP 0.4 at pH = 7.2 with a resistance of 2–3 MΩ.

Whole-cell voltage-dependent total K^+-channel currents in cardiomyocytes were recorded as described previously (Degirmenci et al., 2018). Briefly, to record the total K^+-channel currents, a prepulse protocol was used to inactivate the Na^+-currents (from −70 to −50 mV, followed by 3-s depolarizing voltage). To block L-type Ca^{2+}-channel currents, we used Cd^{2+} (100 μM) in the recording solution. Total K^+-channel currents were recorded by using depolarizing pulses with 3 s duration at between −120 and +70 mV (with 10 mV steps) were applied. The interval between pulses was 7 s and the current traces were filtered at 3 kHz and were digitized at 5 kHz.

2.5 | Measurement of KCNQ1/KCNE1-channel current

Sarcolemmal KCNQ1/KCNE1-channel currents were isolated from total delayed rectifying K-channels (IKs) by pharmacological sensitivity to ML-277 (Sigma-Aldrich, SML0524) and XE-991 (Sigma-Aldrich, X2254),

respectively. Cells were placed in a HEPES-buffered bathing solution contained (in mmol/L); NaCl 137, KCl 4, $MgCl_2$ 1, $CaCl_2$ 1.8, $CdCl_2$ 0.1, Na-HEPES 10, and glucose 10 at pH = 7.40. The pipette solution for KCNQ recording contained (in mmol/L); KCl 140, HEPES 25, Mg-ATP 3, EGTA 5, and Na GTP 0.4 at pH = 7.20. KCNQ1/KCNE1-channel currents were evoked by applying a ramp protocol ranging from +40 to −100 mV with 28 mV/s frequency while the holding potential was −80 mV. KCNQ currents were obtained after calculating the difference of the pharmacological sensitivity to ML-277 (1 μM) and Xe-991 (1 μM), respectively.

2.6 | Western blot and co-immunoprecipitation analysis

To perform Western blot analysis, proteins were obtained from H9C2 and left ventricular cells using a RIPA lysis buffer (Sigma-Aldrich). The protein concentration of the samples was determined using the BCA Assay Kit (Pierce). An equal amount of lysates were run on SDS-polyacrylamide gels and incubated with antibodies against β_1-AR (sc-568 1:400; Santa Cruz), β_2-AR (sc-569 1:300; Santa Cruz, β_3-AR (sc-50436 1:400; Santa Cruz), KCNQ1 (sc-365 1:300; Santa Cruz), KCNE1 (sc-393575 1:400; Santa Cruz), NOS3 (sc-654 1:500; Santa Cruz), pNOS3 (sc-19827 1:300; Santa Cruz), PKG-1 (T.344.1 1: 1000; Thermo Fisher Scientific), and PIP2 (sc-53412 1:500; Santa Cruz) in BSA and TBS-T (Tween 20 1:1000) solution. For quantitative purposes, GAPDH (sc-365062 1:1000; Santa Cruz), β-actin (sc-47778 1:500; Santa Cruz) were used; then incubated with secondary antibodies (anti-mouse 1:2000, anti-goat 1:7500, anti-rabbit 1:7500), and visualized with Immuno Cruz Western blot analysis Luminol Reagent (sc-2048; Santa Cruz). ImageJ software was used to determine band density.

For co-immunoprecipitation (IP) examinations, cells were incubated with IP lysis buffer (pH 7.4, 0.025 M Tris, 0.15 M NaCl, 0.001 M EDTA, 1% NP40, and 5% glycerol) on ice for 5 min with periodic mixing. The supernatant was obtained by centrifuging the lysate at ~13,000*g* for 10 min. Combined supernatant (500 μg) with KCNQ1 antibody (10 μg) and incubated overnight at 4°C. Magnetic beads (0.25 mg) added microcentrifuge tube and washed with IP Lysis Buffer. Supernatant-antibody combination was added magnetic beads and incubated at room temperature for 1-h with mixing. The beads were collected using a magnetic stand then washed with IP lysis buffer and the target supernatant was obtained. Samples were separated using 10% SDS-PAGE on SDS-polyacrylamide gels.

2.7 | Immunofluorescence experiments

As previously described (Tuncay et al., 2017), isolated cardiomyocytes from rat hearts were fixed with 4% paraformaldehyde, and cells were permeabilized with 0.25% Triton X-100 and blocked with 3% BSA in PBS. KCNE1 and KCNQ1 localizations were determined by using anti-KCNE1 and anti- KCNQ1 antibodies (sc-365186; Santa Cruz and sc-393575; Santa Cruz at 1:50, respectively) in confocal microscopy. Cells were incubated with primary antibodies overnight separately. Then cells were incubated with appropriate secondary antibodies in %3 BSA (Alexa Fluor 488 goat anti-mouse and donkey anti-goat 1:1000 for KCNQ1 and KCNE1, respectively). After washing and mounting procedures, images were obtained by confocal microscope (Zeiss 800, Germany).

2.8 | Determination of the cellular ROS level

The cellular ROS level was measured from isolated cardiomyocytes from rat hearts, as described elsewhere (Griendling & FitzGerald, 2003). The cardiomyocytes were loaded with a ROS indicator chloromethyl-2′,7′-dichlorodihydrofluoroscein diacetate, DCFDA (5 μM for 1-h incubation) and then the fluorescent intensity was measured with a confocal microscope (LEICA TCS SP5). Maximal fluorescence intensity associated with ROS production was measured after the cells were exposed to H_2O_2 (100 μM). The ROS production was compared between groups as % respond to H_2O_2.

2.9 | β_3-ARs overexpression in H9c2 cell-line with stable lentiviral-infection

H9c2 cells were stably transfected with cDNA construct in lentiviral pCMV6-Entry vector tagged with the C-terminal Myc-DDK tags (Origene, NM-013108). Approximately 72-h before transfection, ~3×10^5 cells were seeded per well to obtain 70%–90% confluency. Turbofectin 8.0 (TF81001)/DNA (100 ng/μl) complexes were prepared in Opti-MEM I (Gibco 51985) media immediately before transfection. The turbofectin 8.0/DNA mixture was added drop-wise to every well. About 72-h later, the media was changed with fresh growth media containing kanamycin (25 μg/ml) as a selective antibiotic. After 2 weeks, the cells that remain growing in the selective medium were harvested for determination of β_3-AR protein level.

2.10 | Reagents and statistical analysis

Chemicals were obtained from Sigma-Aldrich unless otherwise stated. Data are presented as mean ± *SEM* of at least three independent observations for Western blot analysis with GraphPad Prism 8.1 (GraphPad Software, Inc). To provide better presentation and distribution of the data in terms of median and interquartile range, we used a box and whisker graph. Comparisons between quantitative variables were assessed by either the unpaired two-sided Student's *t*-test or one-way ANOVA-test for multiple comparisons at the $p < 0.05$ significance level.

3 | RESULTS

3.1 | General parameters of experimental animals

We used insulin-resistant 24-month-old male rats (Aged group) compared to those of adult rats (8-month-old) without insulin

resistance, as described previously (Olgar, Billur, et al., 2020). To validate insulin resistance in the rats, we determined blood insulin level, HOMA-IR index, and the OGTT, as described previously (Olgar, Billur, et al., 2020). We kept aged animals that have approximately 25% high serum insulin level and 80% increased HOMA-IR index for experiments in comparison to those of adults.

We also used male metabolic syndrome (MetS) rats with insulin resistance to compare their heart functional data with the aged rat data (with insulin resistance). As shown in Figure S1 and Table S1 associated with data obtained with echocardiographic parameters of rats from micro-CT imaging, the parameters of heart function of these two groups are significantly different from those of adults. In addition, the parameters of these two experimental groups are very similar and there are no significant differences between them. These parameters can confirm the similar cardiac functional changes in both aged and MetS rats. The light microscopy examinations of the hearts from these experimental groups can also confirm similar structural changes, basically, increase in the thickness of tunica media, foamy cells, and irregular elastic lamellae.

3.2 | Acute insulin application shortened the action potential duration in ventricular cardiomyocytes from both adult and aged rats

Our recent electrophysiological recordings from rat ventricular myocytes have shown that there is a marked increase in the APD, mostly through depression in voltage-dependent K^+-channel currents (Olgar, Billur, et al., 2020; Olgar, Tuncay, et al., 2020; Olgar et al., 2021). Specifically, prolongation of the QT interval has been reported as a consequence of an increase in ventricular APD (Gallego et al., 2000). Here, we determined also a similar increase in APD of ventricular cardiomyocytes from 24-month-old aged rats compared to that of adult rats (8-month-old). Under insulin exposure (100 nM, Shimoni et al., 1998) the APD of cardiomyocytes from either adult or aged rats is shortened, significantly without any changes in both resting membrane potential and amplitude of APs (Figure 1a–e). The repolarization phases of APs were analyzed at different time points such as 25%, 50%, 75%, and 90% of repolarization (APD_{25}, APD_{50}, APD_{75}, and APD_{90}). As shown from both representative original AP traces and the calculated values in Figure 1, the effects of insulin application are very significant at all these repolarization time points in both adult and aged rat cardiomyocytes.

To validate the effect of insulin application on prolonged APD in aged insulin-resistant rat cardiomyocytes, we also used the ventricular cardiomyocytes from insulin-resistant metabolic syndrome (MetS) rats. As shown in Figure S2, the effect of acute insulin application (100 nM for 30 min) induced a similar shortening effect on every phase of repolarization to those of aged cardiomyocytes.

3.3 | Acute insulin application augmented the inhibited voltage-dependent total K^+-channel currents in ventricular cardiomyocytes from aged rats

Electrophysiological studies have shown that a reduction in K^+-repolarizing currents is responsible for the APD prolongation in cardiomyocytes isolated from diabetic animals (Gallego et al., 2000; Lopez-Izquierdo et al., 2014; Shimoni et al., 1998). Furthermore, the significantly depressed peak amplitude of voltage-dependent total K^+-channel currents at +70 mV has been shown and their contribution to prolonged APD in aged rat cardiomyocytes, similar to those of insulin-resistant metabolic syndrome rat findings (Durak et al., 2020; Olgar, Billur, et al., 2020; Shimoni et al., 1998), Therefore, here, we next sought to determine the effect of insulin application on voltage-dependent total K^+-channel currents in the aged cardiomyocytes. The representative original current traces are given in Figure 2. The current density (as pA/pF) versus applied potential characteristics of the groups determined at between −120 and +70 mV are given in Figure 2b. The peak current density measured at +70 mV of aged cardiomyocytes is significantly depressed in the aged group compared to that of the adult group (Figure 2c). Insulin application induced significant increases in not only the aged group but also in the adult group, as well. However, neither aging nor insulin application (100 nM for 30 min) could significantly affect the peak current amplitude measured at −120 mV (Figure 2d).

3.4 | Validation of contribution of aging-associated inhibited KCNQ1/KCNE1-channel current, at least, through PKG activation to prolonged action potential duration in ventricular cardiomyocytes

As the changes in a slow delayed rectifier channel can contribute to AP repolarization (Marx et al., 2002) and among them, a type of K^+-channel (carries slow outward K^+-current, I_{Ks}; Abbott, 2020; Chan et al., 2020) can contribute to the induction of prolonged APD (leading to further cardiac arrhythmias through long-QT; Huang et al., 2018; Wu & Sanguinetti, 2016), in this group of investigations, we first recorded the APs under KCNQ1/KCNE1-channel current activator (1 μM for 30 min, ML-277) in the aged cardiomyocytes. The representative original AP traces are given in Figure 3a. As shown in Figure 3b–e, this activator has no significant effects on the 25% and 50% of repolarization of the APs, while it affects 75% and 90% of repolarization of the APs. More importantly, the effect of this activator is much higher in 90% of repolarization than that of 75% of the repolarization.

In another group of experiments, we applied a PKG inhibitor (100 nM for 30 min, C-159995) to the aged cardiomyocytes during AP recording. As shown in Figure 3b–e, this inhibitor provided similar effects on the repolarization phases of the APs.

We also examined the validation of contribution of aging-associated inhibited KCNQ1/KCNE1-channel current, at least through PKG activation to prolonged APD in ventricular cardiomyocytes, we performed similar experiments in ventricular

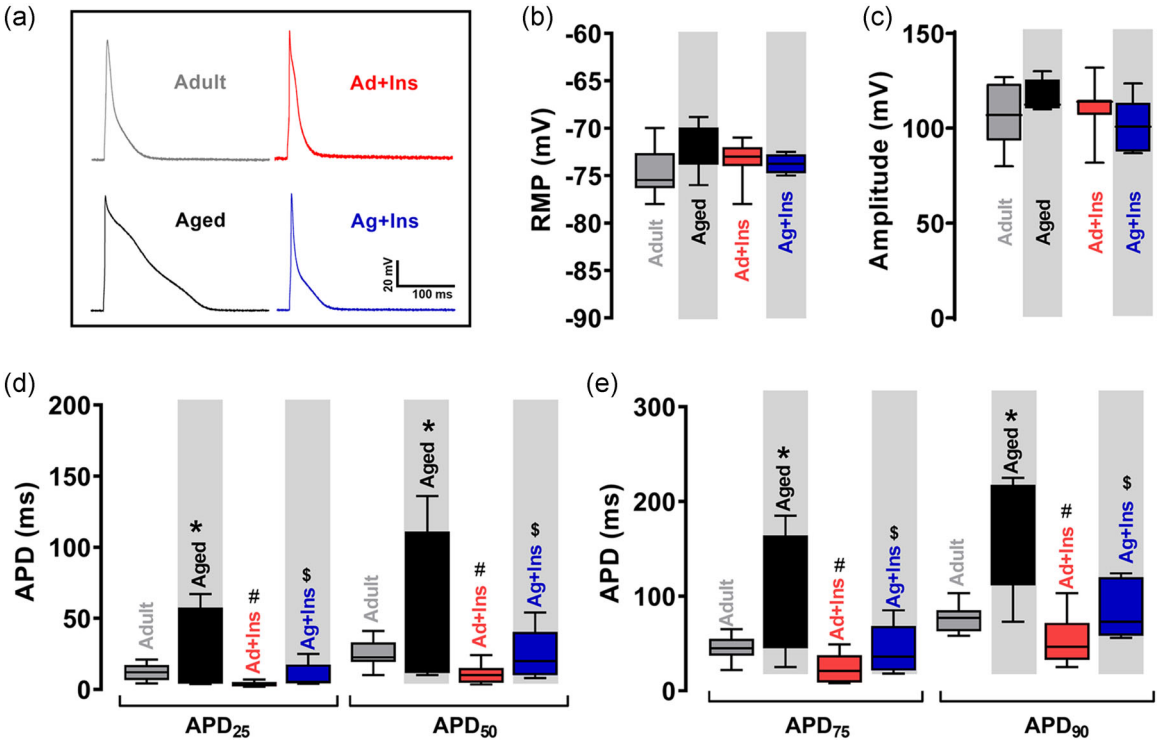

FIGURE 1 In vitro insulin application induces shortening in action potential (AP) duration of left ventricular cardiomyocytes. (a) Representative APs recorded in freshly isolated cardiomyocytes from left ventricles of hearts from adult rats (8-month-old) and aged rats (24-month-old), incubated either in the absence or presence of insulin (+Ins; 100 nM for 30-min incubation, Sigma-Aldrich I0516). Scale bars represent the time and voltage values of APs recorded with whole-cell patch-clamping with current-clamp mode under electrical stimulation with a frequency of 0.5 Hz by injecting small depolarizing pulses (5 nA with 4 ms duration) at room temperature (22 ± 2°C). The resting membrane potentials, RMP (b), and amplitude of APs (c) of the groups. The durations of APs at 25% and 50% of repolarizations (APD_{25} and APD_{50}, respectively) in (d) and 75% and 90% of repolarizations (APD_{75} and APD_{90}, respectively) in (e) Here, every group has records from 15 to 20 cells isolated from 5 to 6 hearts. Data presented as box-and-whisker plots. $^{*}p < 0.05$ versus Adult; $^{\#}p < 0.05$ versus Adult; $^{\$}p < 0.05$ versus Aged by one-way ANOVA-test for multiple comparison

cardiomyocytes from insulin-resistant MetS rats (8-month-old) in comparison to those of controls (Con; 8-month-old). As shown in Figure S2, either a KCNQ1/KCNE1-channel current activator (1 μM for 30 min, ML-277) or a PKG inhibitor (100 nM for 30 min, C-159995) application induced similar effects to those of the aged cardiomyocytes in the repolarization phases of the APs.

3.5 | Not only insulin but also PKG inhibitor application reverses the inhibited KCNQ1/KCNE1-channel current in the aged cardiomyocytes

We first determined the KCNQ1/KCNE1-channel current in either the aged or the adult rat cardiomyocytes at voltages between −100 and +40 mV with 3-mV voltage steps. The current density versus voltage characteristics of this current is given in Figure 4a. The peak amplitude of this current +40 mV is significantly high in the aged cardiomyocytes compared to those of adults (Figure 4b). Following these recordings, we applied either insulin (100 nM for 30 min) or a PKG inhibitor (100 nM for 30 min, C-159995) during the current recodings. These two applications reversed the inhibited current measured at +40 mV in the aged insulin-resistant rat cardiomyocytes (Figure 4b). Interestingly, we observed about twofold increase in the adult rat cardiomyocytes following insulin application.

We also examined the status of KCNQ1/KCNE1-channel current in ventricular cardiomyocytes from insulin-resistant MetS rats (8-month-old) comparison to those of controls. As shown in Figure S3, this current is significantly depressed at +40 mV stimulation in the MetS group compared to those of controls. Either insulin (100 nM for 30 min) or a PKG inhibitor (100 nM for 30 min, C-159995) application reversed that inhibited current, similarly, significantly.

In another group of experiments, we examined the effect of either a KCNQ1/KCNE1-channel activator (1 μM, Sigma-Aldrich ML-277) or its inhibitor (5 μM, Sigma-Aldrich XE-991) on voltage-dependent total K^+-channel currents in comparison to the insulin (INS; 100 nM, Sigma-Aldrich I0516) action on these currents. The representative voltage-dependent total K^+-channel currents are given in Figure 4c. The effects of either a KCNQ1/KCNE1-channel inhibitor (5 μM, Sigma-Aldrich XE-991) or a KCNQ1/KCNE1-channel activator (1 μM, Sigma-Aldrich ML-277) in comparison to that of insulin (100 nM) effect as the current density versus voltages are given in Figure 4d. The peak current densities measured at +70 mV are given in Figure 4e, as well.

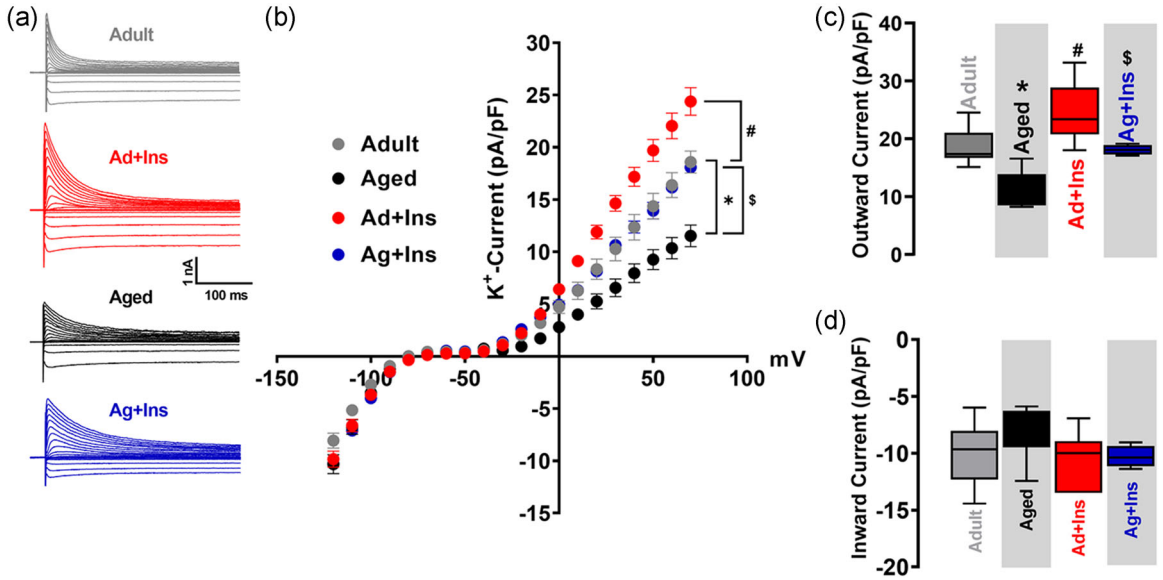

FIGURE 2 Effects of in vitroinsulin application on total voltage-dependent K^+-channel currents in left ventricular cardiomyocytes. (a) Representative total voltage-dependent K^+-channel currents recorded by whole-cell patch-clamping in freshly isolated cardiomyocytes from left ventricles of hearts from adult rats (8-month-old) and aged rats (24-month-old) with (+Ins; 100 nm for 30 min, Sigma-Aldrich I0516) or without insulin. Scale bars represent the time and current values recorded with whole-cell patch-clamping with voltage-clamp mode by applying prepulse ramp protocol to inactivate voltage-dependent Na^+-channel currents and Cd^{2+} (200 μM) to block voltage-dependent Ca^{2+}-channel currents at room temperature (22 ± 2°C). (b) The current-voltage characteristics of K^+-channel current density elicited from −120 to +70 mV electrical stimulations (with square pulses of 200 ms duration and 5 mV amplitude) at −80 mV holding potential. Both peak outward parts (c) and inward parts (d) of the currents (as pA/pF) were calculated at +70 and −120 mV, respectively. Every group has records from 15 to 20 cells isolated from 5 to 6 hearts. Data presented as box-and-whisker plots. $^*p < 0.05$ versus Adult; $^\#p < 0.05$ versus Adult; $^\$p < 0.05$ versus Aged by one-way ANOVA-test for multiple comparison

3.6 | Biochemical analysis and confocal imaging demonstrate the co-localization of KCNQ1 and KCNE1 in left ventricular cardiomyocytes from aged rats

In the examination of these groups of cardiomyocytes, we sought to demonstrate the co-localization of KCNQ1 and KCNE1 in left ventricular cardiomyocytes from aged rats in comparison to the adult cells. We first performed Western blot analysis to demonstrate the protein levels of KCNQ1 and KCNE1 in the cardiomyocytes isolated from aged or adult rats. As shown in Figure 5a,b, the protein levels of both KCNQ1 and KCNE1 are similar in both groups of cardiomyocytes. However, the protein level of PKG was about 50% increased in the aged cardiomyocytes compared to the adults, whereas the protein level of PIP2 was about 50% decreased in the aged cardiomyocytes compared to the adults (Figure 5c,d respectively).

In another group of experiments, we aimed to quantify the co-localization of KCNQ1/KCNE1-channel current related proteins and performed co-IP to demonstrate the co-localizations of KCNQ1 with either KCNE1, PKG, or PIP2 in the left ventricular aged cardiomyocytes comparison to those of adults (Figure 5e). The co-localization of KCNQ1with KCNE1 was found to be about 90% less in the aged cardiomyocytes in comparison to those of adults, while the co-localization of KCNQ1 with PIP2 was not significantly different among these two groups. However, the co-localization of KCNQ1 with PKG was fourfold higher in the aged cardiomyocytes concerning the adults. Insulin treatment of the aged cardiomyocytes (100 nM for 3–4 h) provided an 80% reversing in the co-localization of KCNQ1with KCNE1 with an about 2.5-fold decrease in the co-localization of KCNQ1 with PKG, while no significant effect on the localization of KCNQ1 with PIP2.

We also performed confocal imaging of these two proteins KCNQ1 and KCNE1, with and without insulin treatment in the adult or the aged cardiomyocytes (Figure 5f, left). Further quantification of these co-localization associated images with Pearson correlation coefficients, calculated by using Huygens software has shown no significant differences between the co-localizations of those images (Figure 5f, right).

We further performed some more investigations to confirm the unchanged protein levels of KCNQ1 and KCNE1 in insulin-resistant heart ventricular cardiomyocytes, we determined these proteins in the cardiomyocytes from insulin-resistant MetS rats. As shown in Figure S4a,b, these proteins levels were found to be not significantly different from those of their age-matched controls (8-month-old male rats). For further confirmation, we also determined the protein level of PKG in these cardiomyocytes. Its level was about fourfold higher in the MetS cardiomyocytes compared to those of controls (Figure S4c). More importantly, insulin treatment of these cardiomyocytes with insulin (100 nM for 3–4 h incubation) provided a full recovery in the level of PKG.

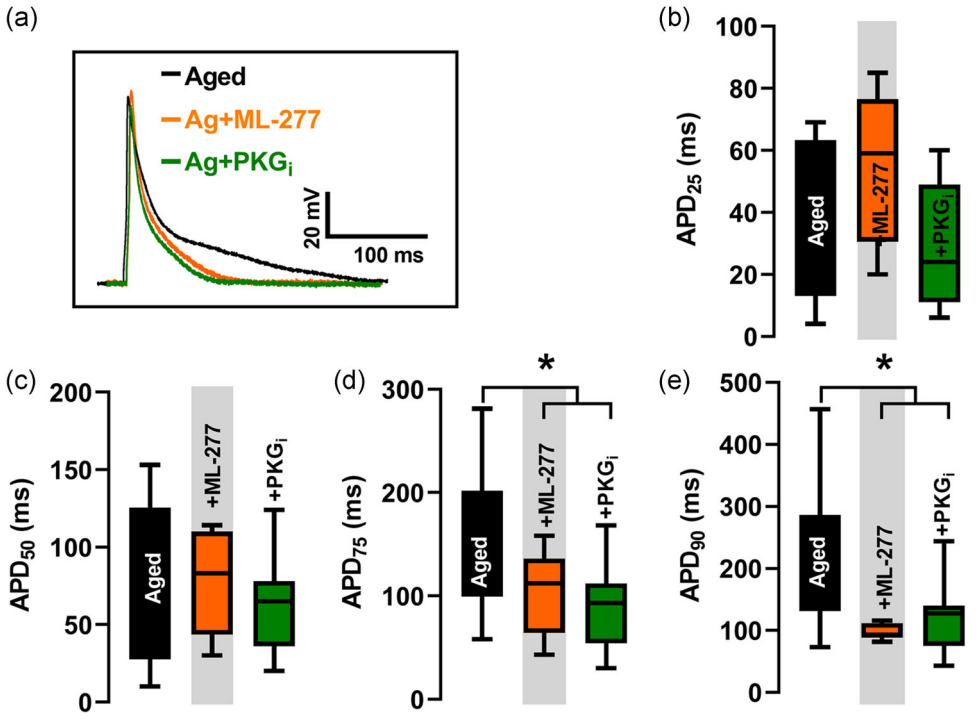

FIGURE 3 The effects of either a PKG inhibitor or a KCNQ1-channel activator on action potentials (APs) comparison to insulin in left ventricular cardiomyocytes from aged rats. (a) Representative APs recorded in freshly isolated cardiomyocytes from left ventricles of hearts from aged rats (24-month-old) with either a PKG inhibitor (100 nM; Cayman, 159995) or a KCNQ1-channel activator (1 μM, Sigma-Aldrich ML-277) comparison to insulin (100 nM, Sigma-Aldrich I0516) application for 30 min. Scale bars represent the time and voltage values of APs recorded with whole-cell patch-clamping with current-clamp mode under electrical stimulation with a frequency of 0.5 Hz by injecting small depolarizing pulses (5 nA with 4 ms duration) at room temperature (22 ± 2°C). The durations of APs at 25% and 50% of repolarizations (APD_{25} and APD_{50}, respectively) in (d) and 75% and 90% of repolarizations (APD_{75} and APD_{90}, respectively) in (e). Here, every group has records from 15 to 20 cells isolated from 5 to 6 hearts. Data presented as box-and-whisker plots. $^*p < 0.05$ versus Aged by one-way ANOVA-test for multiple comparisons

3.7 | In vivo treatment with a PKG inhibitor or insulin provides cardioprotection via affecting altered parameters of the heart and isolated left ventricular cardiomyocytes from aged rats

To validate the contribution of depressed KCNQ1/KCNE1-channel current to the prolonged APD as well as the beneficial effect of insulin on these alterations in the aged rat heart, we treated one group of aged rats with insulin (2 IU/kg, intraperitoneally for 2 weeks) and the other group with a PKG inhibitor (1 mg/kg, intraperitoneally for 2 weeks). The third group of aged rats kept their controls treated with serum physiology intraperitoneally for 2 weeks for the same period. Either insulin or a PKG inhibitor treatment of the aged rats did not provide any effect on their body weights and OGTT values (obtained by measuring blood glucose levels before and after orogastric gavage of 1 g/kg glucose administration at 0, 15, 30, 60, and 120 min; Figure 6a,b). Furthermore, insulin but not the PKG inhibitor treatment induced significant recovery in the depressed heart rate of the aged rats (Figure 6c). More importantly, both treatments induced significant recoveries in the parameters of surface ECG parameters such as both long intervals of RR and QT (Figure 6d,e).

In another group of examinations, we recorded APs in freshly isolated left ventricular cardiomyocytes from treated aged rats in comparison to those of untreated aged and adult rats. The representative original AP traces are given in Figure 6f. As shown in Figure 6f, the insulin treatment of the aged rats provided significant recoveries in all phases of prolonged repolarizations of APs (Figure 6g), a PKG inhibitor treatment induced significant recoveries in the late phases of repolarizations such as 75% and 90% of repolarization of the APs. More importantly, the effect of this inhibitor treatment was much higher in 90% of repolarization than that of 75% of the repolarization (Figure 6h).

In the last part of these groups of experiments, we determined the status of ROS production in the aged cardiomyocytes in comparison to the adults as well as the effects of the treatment on the ROS production in those cells. The representative fluorescence dye DCFDA loaded cells are shown in Figure 6i. The quantification of the fluorescence intensity changes as a response to H_2O_2 exposure is given in Figure 6j. The ROS status in the aged cardiomyocytes was significantly high than those of the adults. Both treatments of the aged rats provided significant reverses in the high ROS status in those aged cardiomyocytes.

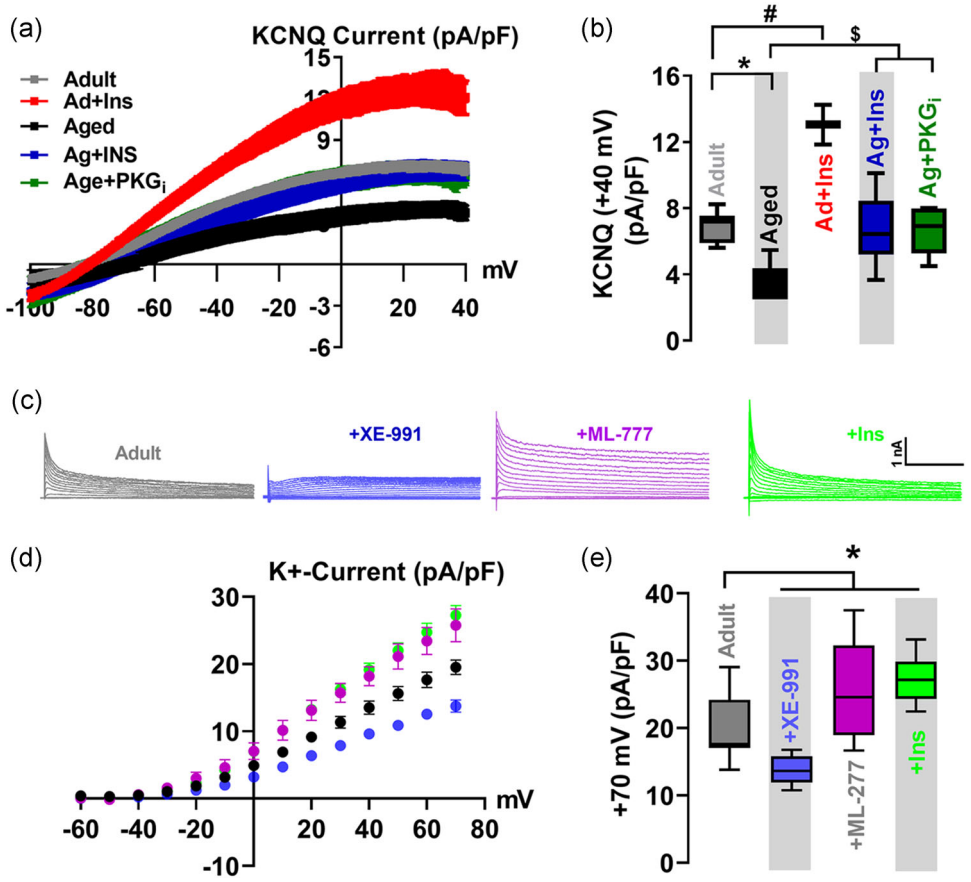

FIGURE 4 The effect of a PKG inhibitor on either KCNQ1/KCNE1 currents or total voltage-dependent K^+- currents comparison to insulin in left ventricular cardiomyocytes from aged rats. (a) The current density voltage characteristics of KCNQ1/KCNE1 currents in cardiomyocytes either from aged or adult rats at room temperature. The effect of a PKG inhibitor (100 nM, Cayman 159995) application (for 30 min) to the aged cardiomyocytes induced a marked recovery in the inhibited KCNQ1/KCNE1 currents with no effect in the adult cardiomyocyte current (data not shown here). Marked increasing effect of insulin (100 nM for 30 min, Sigma-Aldrich I0516) on the adult KCNQ1/KCNE1 currents as well as on the aged ones, recorded by whole-cell patch-clamping. (b) The peak current density values are at +40 mV. Every group has records from 15 to 20 cells isolated from 5 to 6 hearts. Data presented as box-and-whisker plots. $^*p < 0.05$ versus Adult; $^{\#}p < 0.05$ versus Adult; $^{\$}p < 0.05$ versus Aged by one-way ANOVA-test for multiple comparison. (c) Representative total voltage-dependent K^+-currents for each group of cells under either KCNQ1-channel blocker (5 μM, Sigma-Aldrich XE-991) or a KCNQ1-channel activator (1 μM, Sigma-Aldrich ML-277) comparison to the insulin (INS; 100 nM, Sigma-Aldrich I0516). (d) The current-voltage characteristics of K^+-channel current density in cardiomyocytes isolated from 8-month-old male rats (adult rats, Con group), elicited from −120 to +70 mV electrical stimulations (with square pulses of 200 ms duration and 5 mV amplitude) at −80 mV holding potential at room temperature. (e) The peak inward parts of the current density were calculated at +70 mV. Every group has records from 15 to 20 cells isolated from 5 to 6 hearts. Data presented as box-and-whisker plots. $^*p < 0.05$ versus Adult by one-way ANOVA-test for multiple comparisons

3.8 | Effects of in vivo treatment of aged rats with insulin on co-localization of KCNQ1 and KCNE1, and the protein levels of PKG and PIP2 as well as the protein levels of β-adrenergic receptor subtypes in isolated left ventricular cardiomyocytes

To test further the insulin effect on the co-localization of KCNQ1 and KCNE1, we examined their co-localization in the ventricular cardiomyocytes isolated from insulin (2 IU/kg/day for 2 weeks) treated rat heart by using confocal imaging and then quantified with Pearson correlation coefficients. As shown in Figure 7a, their co-localization is not significantly different from those of untreated aged rats. Furthermore, these parameters were not significantly different in the cardiomyocytes from a PKG inhibitor (1 mg/kg for 2 weeks) treated aged rats.

In another group of investigations, we used co-IP analysis to demonstrate whether the insulin treatment of the aged rats can affect the co-localization of KCNQ1 and KCNE1. As demonstrated in our previous IP examinations, the significantly less co-localization of these two proteins in the aged rat cardiomyocytes was found full recovery in their co-localization in the insulin-treated aged group (Figure 7b). However, the recovery in their localization was 40% in the PKG inhibitor-treated aged group.

We also determined the protein level of PKG and PIP2 in the cardiomyocytes isolated from the aged rats as well as insulin-treated aged rats. As shown in Figure 7c,d, PKG level was increased about twofold in the aged group compared to those of adults, while PIP2 level was decreased 50% compared to the adults. Two-week insulin treatment of the aged rats reversed these two parameters almost fully.

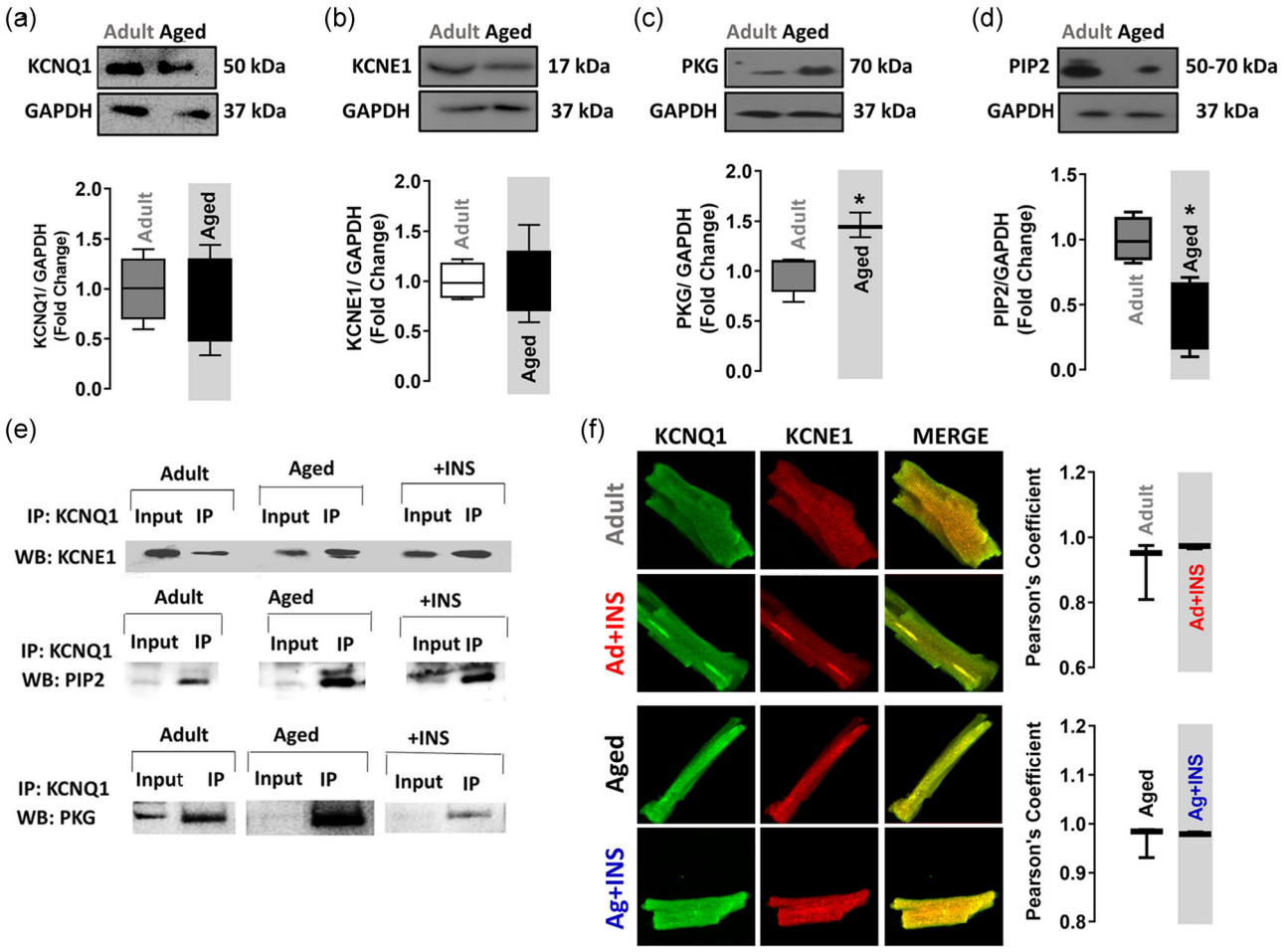

FIGURE 5 Analysis of KCNQ1 and KCNE1 in left ventricular cardiomyocytes from aged rats by using Western blot analysis and confocal imaging. The Western blot analysis of KCNQ1 (a) and KCNE1 (b) in freshly isolated left ventricular cardiomyocytes from aged rats compared to those of the adult rats. The protein levels of PKG and PIP2 are given in (c) and (d), respectively. The representative protein bands are given in the upper parts of the bars. (e) The co-immunoprecipitation (IP) determinations for the demonstration of co-localization of KCNQ1 with KCNE1 (upper part), PIP2 (middle part), and PKG (lower part) in the same cardiomyocytes with or without insulin incubation (100 nM for 3–4 h, Sigma-Aldrich I0516). (f) Confocal imaging of co-localization of KCNQ1 and KCNE1 in adult and aged rat cardiomyocytes with and without insulin incubation (100 nM for 3–4 h, Sigma-Aldrich I0516) at room temperature (left). All cells were fixed and permeabilized (and then incubated with specific primary antibodies and were followed by secondary antibodies). The cells were mounted in a medium containing DAPI (blue to stain nuclei). Scale bars = 10 mm. For quantification of co-localization of these proteins, Pearson correlation coefficients were calculated for them by using Huygens software (g). Data presented as box-and-whisker plots. Cardiomyocytes are isolated from 3 to 4 rats/groups. *$p < 0.05$ versus Adult

To explain the possible relationship between PKG activation and a subtype of β-adrenergic receptors (β-ARs), β_3-ARs, we determined the protein levels of three subtypes of β-ARs. The β_1-ARs are significantly downregulated (Figure 7e) with no change in β_2-ARs (data not shown) determined in the left ventricular cardiomyocytes isolated from the aged rats. Furthermore, the protein level of β_3-ARs is significantly increased in those cells (Figure 7f). Insulin treatment of the aged rats (2 IU/kg) for 2 weeks provided significant reverses in those parameters.

To validate the possibility of altered levels of β-ARs subtypes, particularly the increase in the protein level of β_3-ARs is associated with insulin resistance, we examined the protein levels of β-ARs subtypes in insulin-resistant MetS Similar to those of the aged rat cardiomyocytes. The protein level of β_3-ARs is significantly increased with no change in the level of β_2-ARs and a decrease in the level of β_1-ARs (Figure S4d–f).

3.9 | Confirmation of the activated β_3-ARs role on the depression of KCNQ1/KCNE1-channel current through PKG-PIP2 pathway

We further performed some more in vitro experiments to confirm the role of activated β_3-ARs in the aged rat heart on the decrease of KCNQ1/KCNE1-channel current through the PKG-PIP2 pathway. We first overexpressed the protein level of β_3-AR in embryonic rat heart ventricular myocytes (H9c2 cells) and confirmed with Western blot analysis (Figure 8a). We also determined the protein levels of KCNQ1 and KCNE1

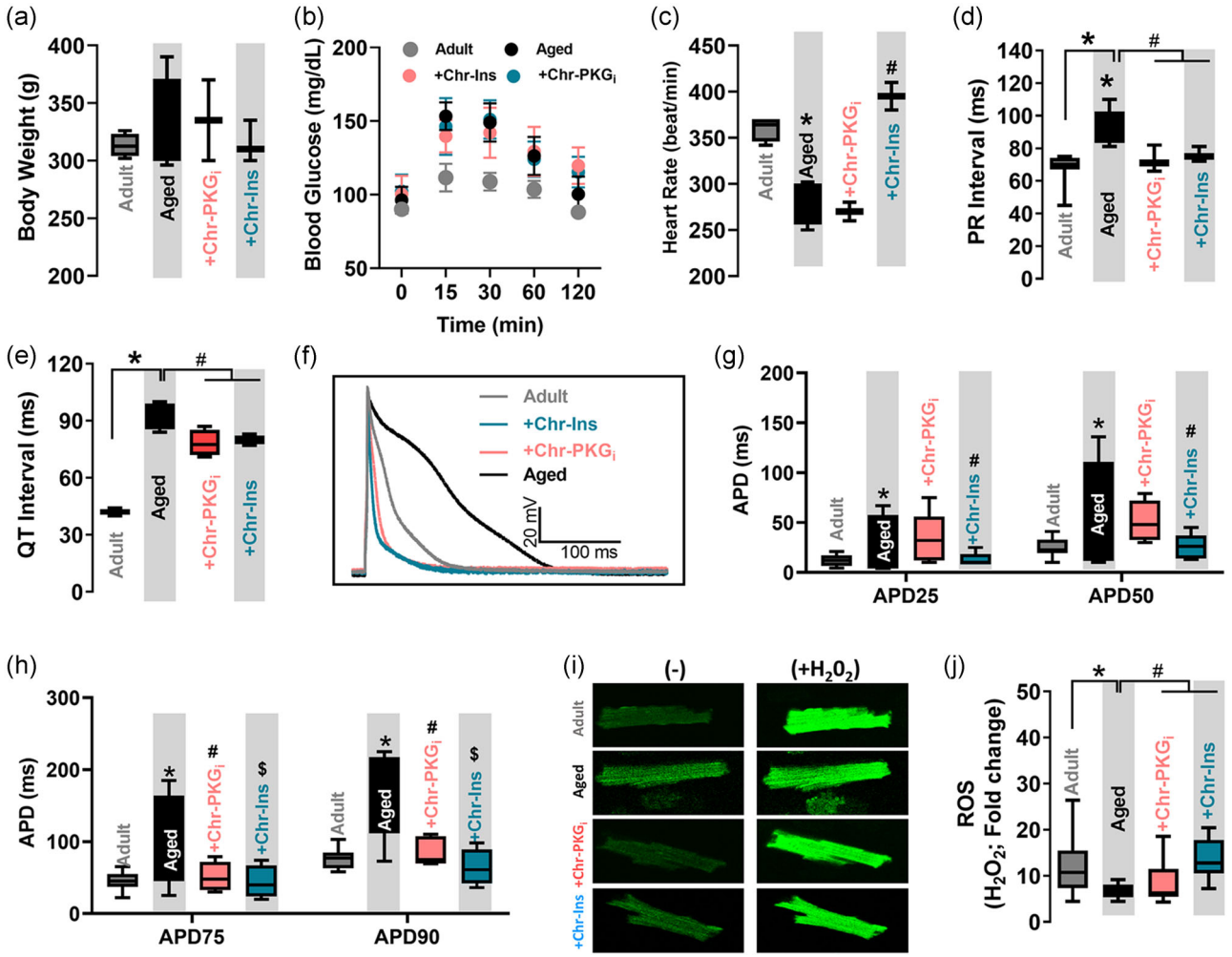

FIGURE 6 In vivotreatment with a PKG inhibitor or insulin provides cardioprotection via affecting altered parameters of the heart and isolated left ventricular cardiomyocytes from aged rats. The treatment of aged rats (24-month-old) with either insulin (2 IU/kg/day, for 2 weeks) or a PKG inhibitor (1 mg/kg/day, for 2 weeks) did not affect significantly the body weight (a) and the oral glucose tolerance test results obtained by measuring blood glucose levels before and after orogastric gavage of 1 g/kg glucose administration at 0, 15, 30, 60, and 120 min to the rats (b). (c) The effects of these treatments of the aged rats on the depressed heart rate (c), the PR-interval (d), and the QT-interval (e). The data for every protocol is from 5 to 6 hearts and presented as box-and-whisker plots. $^{*}p < 0.05$ versus Adult and $^{\#}p < 0.05$ versus Aged by one-way ANOVA-test for multiple comparison. Representative APs of every group in (f). The durations of APs at 25% and 50% of repolarizations (APD_{25} and APD_{50}, respectively) in (g) and 75% and 90% of repolarizations (APD_{75} and APD_{90}, respectively) in (h). Assessment of oxidative stress status in freshly isolated left ventricular cardiomyocytes in treated or untreated aged rats compared to adult rats. Intracellular ROS level ([ROS]i) imagined with confocal microscopy in the cells loaded with a specific dye DCFDA (i). Maximal fluorescence intensity was achieved by a HEPES-buffered solution supplemented with H_2O_2 (100 μM) in (j). The total number of cells/group/protocol; $n = 16–20$ from 3 to 4 rats. Significance level at $^{*}p < 0.05$ versus Adult, $^{\#}p < 0.05$versus Aged and $^{\$}p < 0.05$ versus Aged by one-way ANOVA-test for multiple comparison

in those β_3-AR overexpressed cells. These protein levels were not significantly different from those of their controls (Figure 8b,c). On further examinations, we determined the protein levels of PKG, pNOS3 and NOS3 (as their ratio), and PIP2 in those β_3-AR overexpressed cells. Similar to that of the aged rat cardiomyocytes, those parameters were changed in the same ways (Figure 8d–f, respectively).

For further confirmations of the hypothesis on the association between β_3-AR activation and KCNQ1/KCNE1-channel current inhibition through PKG activation, we performed some more experiments in freshly isolated left ventricular cardiomyocytes from adult rats by stimulating β_3-ARs with an β_3-AR agonist (0.1 μM CL316243). We first examined the co-localization of KCNQ1 and KCNE1 by using co-IP to test whether β_3-AR stimulation can have any effect on that localization. We observed a 70% loss in that co-localization of this β3-AR stimulated adult cardiomyocytes (Figure 8g).

In another group of investigations, we examined the status of KCNQ1/KCNE1-channel currents in these β_3-AR stimulated adult cardiomyocytes. As shown in Figure 8h (left), the current density versus voltage changes of these channels are changed in these β3-AR stimulated adult cardiomyocytes compared to those of unstimulated ones.

In a further investigation, to test the contribution of PKG activation on these currents under β_3-AR stimulation, we applied a

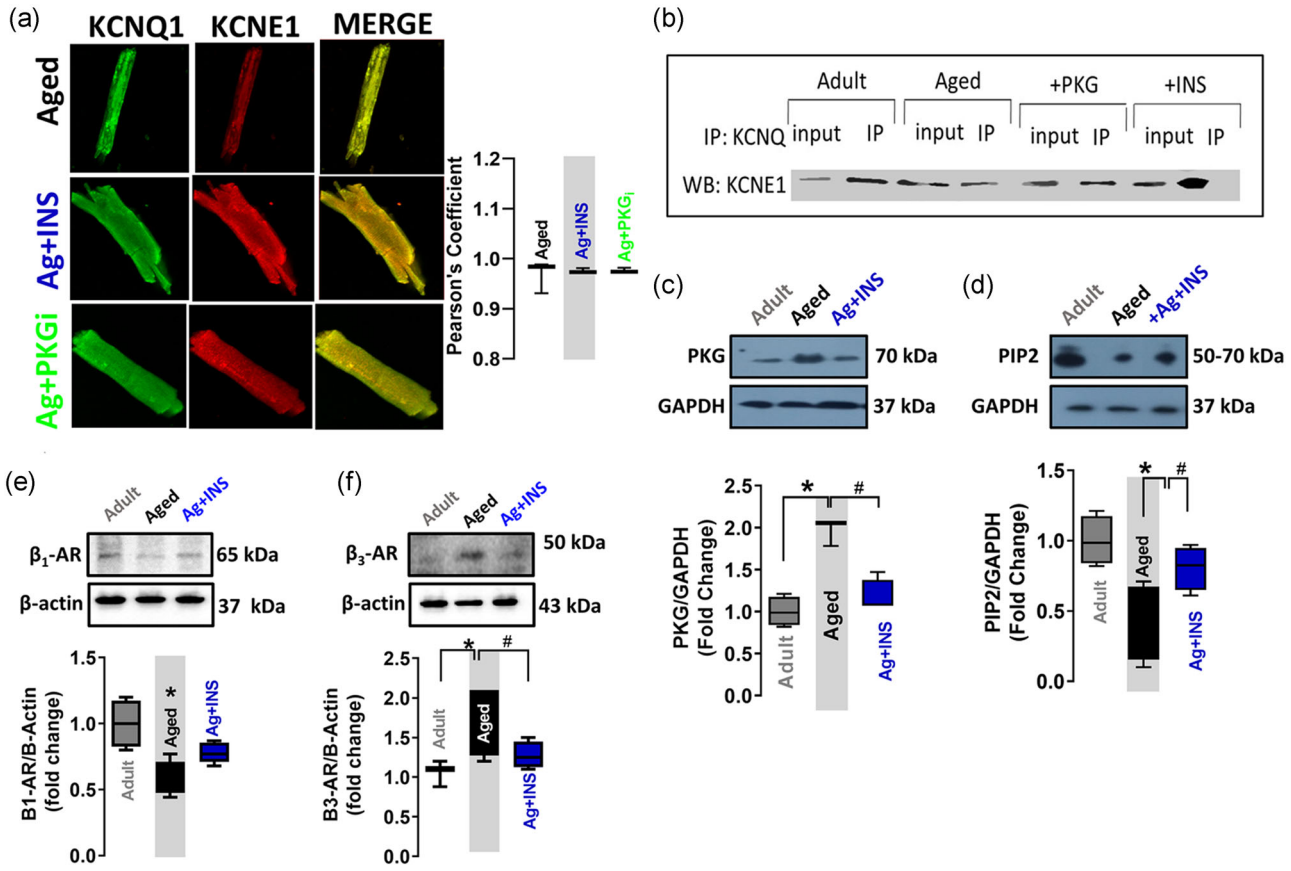

FIGURE 7 Effects of in vivotreatment with insulin on co-localization of KCNQ1 and KCNE1, and the protein levels of PKG and PIP2 as well as the protein levels of β-ARs subtypes in isolated left ventricular cardiomyocytes from aged rats. The co-localization of KCNQ1 and KCNE1 was investigated by either confocal imaging (a) and co-immunoprecipitation (IP) analysis (b) in the isolated left ventricular cardiomyocytes from insulin (2 IU/kg/day, for 2 weeks) treated or untreated 24-month-old aged rats. For quantification of co-localization of these proteins by confocal analysis, Pearson correlation coefficients were calculated for them by using Huygens software (lower part in (a)). The Western blot analysis of protein expression level of PKG (c) and PIP2 (d) in the insulin-treated rat cardiomyocytes comparison to those of untreated rat cells (previously used the same bands in Figure 5c,d) as well as the subtypes of β-adrenergic receptors (β-ARs) as β_1-ARs (e) and β_3-ARs (f). The cells/group/protocol are isolated from 3 to 4 rats and measurements with double assays in each sample from each group for each type of measurement. Significance level at $^{*}p < 0.05$ versus Adult and $^{\#}p < 0.05$ versus Aged by one-way ANOVA-test for multiple comparison

PKG inhibitor (100 nM 159995) on CL incubated cells and then determined the current density versus voltage changes. That application induced a significant reverse in those changes. In the last part of this group investigation, we determined the peak values of these current densities at +40 mV and presented them in the right part of this figure. Consequently, the last group of investigations can demonstrate that activation of β_3-ARs can lead to activation of PKG, which further can form a relationship with KCNQ1/KCNE1-channels and lead to the inhibition of that channel current.

4 | DISCUSSION

This study was designed to explore whether an outward current through dysfunctional KCNQ1/KCNE1-channels and the related potential molecular pathways can contribute to the long-QT abnormalities in the aged rat heart function. Furthermore, we sought to demonstrate whether insulin application provides cardioprotective benefits through affecting one or more parameters related to dysfunctional KCNQ1/KCNE1-channels in the insulin-resistant aged rat heart. By using in vitro and in vivo investigations with electrophysiological and biochemical analysis, we demonstrated that the observed inhibited KCNQ1/KCNE1-channel currents (I_{Ks}) and the long-QT could be reversed by insulin treatment via inhibition of activated PKG, mostly through prevention of β_3-AR overexpression. Consequently, overall our present results provided evidence for the impact of I_{Ks} as a novel target in aging-associated remodeling in the heart, and insulin can be a cardioprotective agent in the maintenance of normal heart function during the aging process. Moreover, our data also provided the important role of β_3-AR overexpression in the remodeling of the heart function during the aging process, as well as presenting further information for understanding the mechanism/pathway of aging-related cardiac dysfunction.

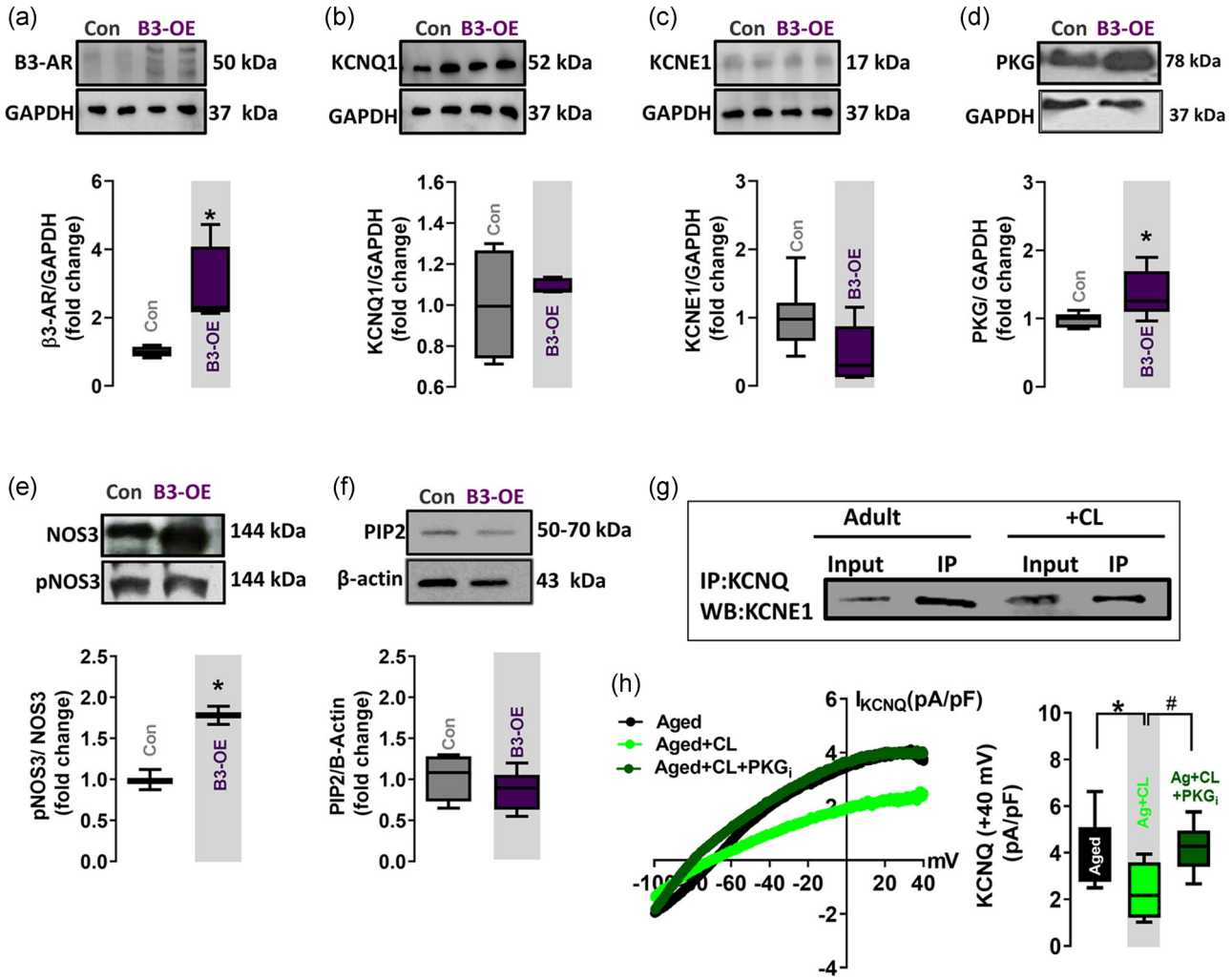

FIGURE 8 Validation of the activated β_3-ARs role on KCNQ1/KCNE1-channel current through PKG-PIP2 pathway. The confirmation of β_3-AR protein overexpression in embryonic rat heart ventricular myocytes, H9c2 cells (B3-OE) (a). The Western blot analysis of protein levels of KCNQ1 (bb), KCNE1 (c), PKG (d), phospho-NOS3 to NOS3 ratio (e), and PIP2 (f) in those B3-OE cells. The representative protein bands for a reference protein GAPDH or β-actin are given in the upper parts of the graphs. In (a–f) significance levels at $^{*}p < 0.05$ versus Con by Student's *t*-test for comparison. (g) Demonstration of co-localization of KCNQ1 and KCNE1 in β_3-AR activated adult left ventricular cardiomyocytes isolated from 8-month-old rats with an β_3-AR agonist stimulation (0.1 μM CL316243, for 3–4 h incubation) by using co-immunoprecipitation (IP). The measurements were performed for every protocol with double assays in each sample for each type of experiment. (h) Further confirmation of association between activation of β_3-ARs and depression of KCNQ1/KCNE1 current density as well as the role of PKG activation on that depressed current density by presenting the current density–voltage relation under either β_3-AR stimulation (0.1 μM, CL316243) or this stimulation plus PKG inhibition (100 nM; Cayman, 159995) (left). The peak current densities were determined at +40 mV for each protocol (right). The cells/group/protocol are isolated from 3 to 4 rats and measurements with double assays in each sample from each group for each type of measurement. Significance level at $^{*}p < 0.05$ versus Adult and $^{\#}p < 0.05$ versus Aged by one-way ANOVA-test for multiple comparison. The total number of cells/protocol; $n = 13–15$ from 3 to 4 rats. Significance level at $^{*}p < 0.05$ versus Aged and $^{\#}p < 0.05$ versus Aged+CL, by one-way ANOVA-test for multiple comparison

Aging is a physiological process of all living organisms and cardiac aging is defined as a progressive and intrinsic decline in heart function, which is also characterized by a long QT in ECGs, reduction in the maximum heart rate, and decline in contractile activity (Lakatta et al., 2001). The age-dependent alterations in cardiovascular function include not only structural and functional abnormalities but also altered responses to different stimuli such as reduced secretion of the protective myokines creating cardiometabolic disturbances, chronic inflammation, and oxidative stress (Sinclair & Abdelhafiz, 2020). In addition, the most important alterations exist in the sympathetic nervous system, its chronic stimulation becomes detrimental and causes decline cardiac function as well as reduced inotropic reserve, mostly due to β-ARs responsiveness together with changes in the distribution of their subtypes (Dincer et al., 2001).

In the present study, we used insulin-resistant aged rats and demonstrated that their cardiac functions are very similar to those of MetS parameters, such as long-QT, decreased heart rate, and structural changes including increases in left ventricular wall thickness and

collagen levels. These results are similar to those of our previously published data, from both MetS rats and aged rats (Durak et al., 2018, 2020; Olgar et al., 2019; Olgar, Billur, et al., 2020; Olgar, Tuncay, et al., 2020). Literature data have arrived at a consensus related to the contribution of several pathways to the aging-related cardiac changes. Cardiac aging has been characterized and its molecular mechanisms include mainly the contributions of high cellular levels of both ROS and RNS, abnormal mitochondrial ROS production, detoxification in mitochondrial dysfunction, and increased susceptibility to mitochondrial permeability transition pore opening (Dai et al., 2009; Olgar et al., 2019; Olgar, Billur, et al., 2020; Olgar, Tuncay, et al., 2020). Several clinical and experimental studies documented the impact of aging on the appearance of long-QT (Goldenberg et al., 2008; Guettler et al., 2019). In older patients, an association between aging and increases in the prevalence of atrial and ventricular arrhythmias has been shown, which is reflecting, at least, disruption of the normal functioning of ion channels (channelopathies) further generating the alterations in the propagated cardiac APs. Cardiac ion channel expression and properties are distinct in different regions of the heart, leading to unidirectional propagation of electrical activity. Changes in APs, synchronization, and/or propagation of electrical impulse predispose to potentially malignant arrhythmias. Our present and previous studies have shown that significant prolonged APD, at most, parallel to the depressed voltage-dependent total K^+-channel currents (I_K) existed in left ventricular cardiomyocytes from aged rats with long-QT and insulin resistance. Supporting these findings, authors in their early articles documented an important relationship between reduced repolarizing I_K and long-QT in the ECGs (Clancy et al., 2003; Fernández-Falgueras et al., 2017). Furthermore, the relationship between insulin sensitivity, altered heart rate, long-QT via longitudinal changes of cardiac repolarization heterogeneity in patients with diabetes and MetS as well as in aged mammals has been demonstrated (Matsumoto et al., 2019; Su et al., 2017). Under the light of these findings, our present data added important information and insight on the beneficial cardioprotective effect of insulin (either in vivo or in vitro) on long QT, prolonged APD, and inhibited I_K in aged mammalians, without affecting insulin resistance. Supporting data are provided in early studies by Shimoni et al. (1998) to demonstrate an insulin stimulation (100 nM) of rat ventricular I_K. In a later study, the beneficial effects of insulin on cardiac function have been also shown via supporting the hypothesis on the production of abnormal repolarization in cardiomyocytes with a lack of insulin signaling (Dubó et al., 2016). This study also is supporting nicely our present data in the aged rat heart. In addition, the authors comprehensively documented the cardioprotective effects of insulin by pointing how intensive insulin therapy can provide benefits in cardiac function such as in patients with cardiac surgery or diabetes (Naccache, 2020; Ng et al., 2012). All the above benefits with insulin application can be related, at least, with its stimulative action on membrane conductance (Kilic et al., 2001). However, Wu et al. (2014) investigated a direct inhibitory effect of insulin (0.1–10 μM) on I_{Ks} in KCNQ1 and KCNE1 co-expressed Xenopus oocytes (Wu & Sanguinetti, 2016). In that study, either the use of overexpressed KCNQ1 and KCNE1 protein including cells, high insulin application, or both could induce that inhibitory effect of insulin in their experimental condition. Interestingly, in a later study, the authors demonstrated an augmentation in KCNQ1/KCNE1 currents but not alone KCNQ1 current (Wu et al., 2017) via an effect on KCNE1 expression (Wu et al., 2017). Also, although it was not defined as I_{Ks}, in an early study, Zhang and Hancox (2003) have shown a novel, voltage-dependent nonselective and outwardly rectifying cation current activated by insulin in guinea pig isolated ventricular myocytes.

We also for the first time demonstrated that not only insulin but also either a PKG inhibitor or I_{Ks} activator could provide significant reverses in the prolonged APD in the aged rat ventricular cardiomyocytes. The important differences between insulin and either a PKG inhibitor or I_{Ks} activator are their actions on repolarization phases of APs. The insulin application can significantly shorten the prolonged APD calculated at all phases of the repolarization, whereas the above two agents affected significantly only the late phases of repolarization such as calculated at 75% and 90% of the repolarization. From these data, we sought to investigate the contribution of I_{Ks} status into aged cardiomyocyte APD changes. Our further investigation strongly supported the hypothesis on the contribution of depressed I_{Ks} on the prolonged APD in the aged cardiomyocytes. Our data are in line with previously published data: Indeed, studies have shown that the changes in a slow delayed rectifier channel can contribute to AP repolarization (Marx et al., 2002) and among them, a type of K^+-channel, carrying slow outward K^+-current, I_{KS}, can contribute to the induction of prolonged APD, further leading to further cardiac arrhythmias through long-QT (Abbott, 2020; Chan et al., 2020; Huang et al., 2018; Wu & Sanguinetti, 2016). Similar findings in vivo PKG inhibitor applied aged rat cardiomyocytes were determined, as well. Moreover, our confocal imaging and biochemical analysis of the proteins associated with this I_{KS}, such as KCNQ1 and KCNE1 have shown not their protein levels but their co-localization is affected by aging such as an important loss in that co-localization. Interestingly, that loss was reversed significantly with insulin or a PKG inhibitor. It has been previously shown how KCNQ1/KCNE1 function and localization in cardiomyocytes are controlled and regulated by several intracellular signaling pathways and factors in those pathways (Abbott, 2020; Chan et al., 2020; Huang et al., 2018; Wu & Sanguinetti, 2016). In even earlier studies, it has been documented that intracellular regulation of the KCNQ1/KCNE1 complex can shorten or lengthen the cardiac APD, enabling ventricular repolarization to be in rhythm with changes in heart rate and contractility (Jespersen et al., 2005; Marx et al., 2002). However, in this field, there are some contradictory results in the literature. For instance, activated PKC can phosphorylate a subunit of KCNE1, further leading to I_{Ks} density in humans, rats, and mice, in contrast, this current was not downregulated by PKC in guinea-pig (Kanda et al., 2011; Z. J. Zhang et al., 1994). In other studies, authors demonstrated inhibition and/or regulation of the I_{KS} by the AMP-activated protein kinase (Alesutan et al., 2011), calcium/calmodulin-dependent protein kinase II (Shugg et al., 2018), and

protein kinase A-dependent signaling complex (Marx et al., 2002). More importantly, the function of I_{Ks} is widely documented in many studies, which demonstrated multimodal regulation of KCNQ1 with several factors associated with or without the partner of KCNE1, including phosphodiesterases, adenylyl cyclase, PKA, calmodulin, and Yotiao (Abbott et al., 2014). In the present study, the protein level of PKA or calmodulin was not changed, significantly in the aged cardiomyocytes.

In aged cardiomyocytes, the co-localization but not protein levels of KCNQ1 and KCNE1 did not change while the I_{Ks} was depressed. However, Ramaker et al. (2003) demonstrated a coordinated down-regulation of KCNQ1 and KCNE1 expression contributes to the reduction of this current in canine hypertrophied hearts. Importantly, we determined a significantly decreased level of PIP2 in the aged cardiomyocytes, which was responding to insulin application. However, the co-localization of KCNQ1 with PIP2 was not significantly different in the aged group. Considering the role of PIP2 in the regulation of I_{Ks} through a non-KCNE1 subunit, as documented by Abbott et al. (2014), the decrease in PIP2 level can contribute to the decrease in the aged cardiomyocytes. PIP2 is a relatively minor, acidic phospholipid component of the plasma membrane, and is now considered to be an essential cofactor for many ion channels (Loussouarn et al., 2003; Taylor & Sanders, 2017; Zaydman & Cui, 2014). Further support is given by using a PIP2 substitute which revealed the structural basis of PIP2 regulation of KCNQ1 channels and indicates a potential approach for the development of antiarrhythmic therapy (Liu et al., 2020; Zaydman & Cui, 2014).

In addition, the protein level of PKG was about 50% increased in the aged cardiomyocytes compared to the adults, while the co-localization of KCNQ1 with PKG in the aged cardiomyocytes in comparison to those of adults was fourfold higher. Interestingly, insulin treatment of the aged rats, as well as cardiomyocytes from the aged rats, provided significant recoveries in these changes. These last two pieces of data provide a hypothesis that activation of PKG with decreases in PIP2 can lead to loss of function of KCNQ1/KCNE1-channels in aged cardiomyocytes, which further leads to long QT via prolongation of AP repolarization late phases. In addition, insulin application affecting any parameters of this pathway can preserve cardiac function in aged rats without affecting insulin resistance. Indeed, KCNQ1 participates in the regulation of cell volume, which is, in turn, critically important for the regulation of metabolism by insulin. Supporting that statement, it has been demonstrated that the influence of KCNQ1 on insulin-induced cellular K^+-uptake and glucose metabolism in isolated cells from KCNQ1-deficient mice and demonstrated influences K^+-dependent insulin signaling on glucose metabolism, emphasizing KCNQ1 is a novel molecule affecting insulin sensitivity of glucose metabolism (Boini et al., 2009).

Our further investigations are providing almost full support to our current hypothesis. Recent studies show that insulin can affect β-AR signaling in the heart, to modulate cardiac function in clinically important pathological conditions such as diabetes, MetS, and heart failure. Conversely, activation of β-AR regulates cardiac glucose uptake and promotes insulin resistance in heart failure, as well. With this knowledge and considering the insulin is a pleiotropic hormone with various effects on glucose metabolism, the central nervous system, and cardiovascular system, one can propose cross-talks in the heart in the concept of insulin and β-AR receptor signaling. In that concept, an association between KCNQ1 and β_2-ARsv has been shown, and further demonstrated modulation of I_{Ks} function under overexpressed β_2-ARs (Dilly et al., 2004; Fu et al., 2017). In our investigations, the protein expression level of β_2-ARs in the β_2-ARs from either the aged or MetS rats was similar to those of adults. On the other hand, Klein et al. (1999) presented the differential inhibition of insulin signaling by β_3-AR stimulation via the reduction in insulin-induced tyrosine phosphorylation of the insulin receptor and insulin receptor substrates, and also reduction of phosphatidylinositol 3-kinase (Klein et al., 1999). Besides the other two subtypes, the β_3-AR is found in cardiac myocytes and affects cardiac function and remodeling (Balligand, 2013; Moniotte et al., 2001; Tuncay et al., 2019). In particular, β_3-AR is upregulated in cardiac diseases and is less sensitive to desensitization (Balligand, 2013; Dincer et al., 2001; Moniotte et al., 2001; Tuncay et al., 2019; Zhao et al., 2007). Subsequently, β_3-AR was shown to signal through the eNOS/NO/cGMP pathway in the human ventricle and is directly coupled to PKG signaling (Brixius et al., 2004). In this regard, our in vitro studies with β_3-AR stimulation in the ventricular cardiomyocytes significantly decreased the I_{Ks} while that decrease could be prevented with a PKG inhibitor. From another insight, activation of β_3-AR can induce an increase in NO-dependent cGMP production, via PI3K-dependent mechanism (Bergandi et al., 2003). However, previous studies also have shown a relation between NO-pathway and PKG activity under hyperinsulinemia (Zhuang et al., 2005). Of note, it has to be mentioned an already known study demonstrates the important role of activated PKG on PIP2 synthesis (Taoufiq et al., 2013), while PKG has roles in insulin-activated glucose transport and also in insulin resistance (Piwkowska et al., 2014). Furthermore, an increased NO-dependent PKG activity can stimulate tachycardia and regulate the upstream degradation of wrong protein folding (Agnetti et al., 2014), which in turn induces cardiac dysfunction (McLendon & Robbins, 2015). More importantly, it is today a commonly accepted issue that ion channelopathies, induced by dysfunctional ion channels, are important diseases associated with even underlying the sudden death. Therefore, considering the role of activated PKG on the modulation of PIP2 in the inner side of membranes (Kirk et al., 2016), our present data imply that PKG can be activated through the activation of β_3-AR in insulin-resistant aged cardiomyocytes, which in turn, inactivate I_{Ks} also leading to long-QT, and therefore play important role in the remodeling of the aging heart. Furthermore, our present study provided an important additional issue associated with induction of cardioprotection by insulin treatment of aged rats via affecting either activated β_3-AR, activated PKG, or both, which further leads to recovery in the depressed I_{Ks} and thereby ECGs in the aged rat heart. One can propose that any type of PKG inhibition would be able to provide cardioprotection in the aged mammal heart.

Correspondingly, insulin is a key component of GIK-cocktail (glucose-insulin-potassium) through modulation of PI3K-Akt-eNOS-dependent signal mechanism, and the end product NO is important for a healthy heart (Alburquerque-Bejar et al., 2015; Boucher et al., 2014; Lopez-Izquierdo et al., 2014). Taking into consideration the role of cAMP and PKA activation on I_{Ks} (Kanda et al., 2011), one can hypothesize their contribution to this current. However, previous studies demonstrated that both cAMP and PKA were found to be depressed in insulin-resistant hearts, in a manner parallel to activation of β_3-AR with inhibition of β_1-AR (Dincer et al., 2001; Okatan et al., 2015). This type of benefit can further provide recovery in long-QT in the aged mammals. For validation of this hypothesis, we also used MetS rats parallel to the aged rats to investigate our hypothesis, and then validated our hypothesis once more with an investigation of the relationship between long QT, prolonged APD, and decreased I_{Ks} in the ventricular cardiomyocytes from the MetS rats. Overall, already known data up to now mentioned the role of β-AR signaling in the molecular mechanisms of cardiac aging. More importantly, some studies discussed a cross-talk between β-AR signaling and insulin signaling in heart dysfunction because they shared similar downstream signaling components (Fu et al., 2017). In this regard, the targeting of β_3-ARs could represent a novel potential strategy to improve cardiac metabolism, function, and remodeling during an aging heart (Gauthier et al., 2011; Michel et al., 2020).

Although the underlying mechanisms of alterations in I_{Ks} and EGCs in pathological conditions, as well as aging, are not yet known exactly, recent studies are pointing out the involvement of voltage sensor domain mutations as the mechanisms of KCNQ1 channel dysfunction in long QT syndrome (Huang et al., 2018). Additionally, the defective interactions of protein partners with ion channels are accepted as alternative mechanisms of membrane channelopathies (Kline & Mohler, 2014). Correspondingly, considering the clinical outcomes associated with the correlation among high serum insulin level and long QT interval in nondiabetic elderly (Van Noord, Eijgelsheim, et al., 2010), together with the association between anti-KCNQ1 autoantibodies found in a subgroup of dilated cardiomyopathy patients and QT interval shortening and increased I_{Ks} (Li et al., 2013; Maguy et al., 2020), here, we, for the first time, demonstrated that I_{Ks} can be a novel target in aging-associated remodeling in the heart and insulin may be a cardioprotective agent in the maintenance of normal heart function during the aging process, by accelerating the ventricular AP repolarization through reversing the depressed I_{Ks} via affecting the $9\beta_3$-ARs signaling pathway, particularly affecting activated PKG.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Belma Turan conceived the study and wrote the manuscript; Yusuf Olgar, Aysegul Durak, and Ceylan Verda Bitirim performed the majority of the experiments; Erkan Tuncay contributed to the materials and methods and analyzed the data; all the authors approved the submission of the manuscript.

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SUPPORTING INFORMATION

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