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Acute GLP-1 Agonism Induces Arrhythmogenic Electrical Activity in Aged Mice Heart Through Impaired Cellular Na^+ and Ca^{2+} Handlings: The Role of CK2 Hyperphosphorylation

ABSTRACT

Background: Glucagon-like peptide-1 receptor agonists (GLP-1RAs) are known for their benefits in conditions like cardiovascular diseases in type 2 diabetes and obesity. They also show promise for aging-related conditions with minimal side effects. However, their impact on cardiovascular risk is still debated. Notably, some long-acting GLP-1RAs cause a sustained increase in heart rate on the first day of use without a clear mechanism. To understand their short-term effects, we examined acute GLP-1R agonism on the electrical activity of elderly hearts.

Methods: In this study, we utilized *in vivo* electrocardiography, *in vitro* cellular electrophysiology experiments, and biochemical measurements on heart preparations from 6-month-old (Adult) and 24-month-old (aged) BALB/c mice.

Results: A single liraglutide injection (0.3 mg/kg) induced repetitive, self-sustained arrhythmogenic electrocardiograms in aged mice (24 months old) but had no effect on adults (6 months old) within the first 10 minutes. Acute application of liraglutide to isolated ventricular cardiomyocytes from aged mice significantly prolonged the late phase of action potential repolarization (APR_{90}). This was due to suppressed K^+ currents and increased persistent Na^+currents (Late-I_{Na}), primarily through delayed recovery from inactivation of Na^+ currents. Additionally, liraglutide increased Ca^{2+} spark frequency and wave formation by enhancing Ca^{2+} release from the sarcoplasmic reticulum, affecting both Na^+ and Ca^{2+} regulation in aging cells. Liraglutide also induced casein kinase 2 (CK2) hyperphosphorylation in aged cardiomyocytes, which a CK2 inhibitor could reverse, normalizing APR_{90} by reducing Late-I_{Na} and enhancing K^+ currents.

Conclusion: These findings reveal that acute GLP-1R agonism can disrupt electrical signaling and induce arrhythmia in aged mice through CK2 hyperphosphorylation, providing new insights into the cardiovascular effects of GLP-1RAs in the elderly.

Keywords: Aging, arrhythmia, glucose-lowering therapy, casein kinase 2, persistent Na^+-currents

INTRODUCTION

Epidemiological studies mention that aging is accompanied by downhill structural and functional changes, further leading to comorbidity and mortality worldwide.[1,2] The latest projections related to the rate of increase of the elderly population emphasize the arising serious problems within the next 25 years, particularly associated with cardiometabolic disorders among them as marked risk factors.[3] Notably, impairment of insulin signaling induces alterations in neurohumoral environments, especially further contributing to adverse left ventricular remodeling in the elderly heart.[4-7] Mammalian aging is a physiological process that accompanies significant negative changes in various organ functions, such as cardiovascular diseases, which include structural and functional remodeling in the heart. These changes are most commonly characterized by cardiometabolic disturbances, including cardiac insulin resistance.[5,6,8-10]

ORIGINAL INVESTIGATION

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In addition, experimental studies have strongly demonstrated increases in oxidative stress, metabolic flexibility, and mitochondrial dynamics in cardiomyocytes from elderly mammalian hearts.[11-14] Correspondingly, experimental animal studies further support these statements with findings associated with either cardiac-specific insulin receptor deletion or insulin application to animals.[5]

Intrinsic glucose-lowering therapy is mainly regulated by 2 hormones, such as glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), which are secreted from the gastrointestinal tract into the circulation in response to nutrient intake. These hormones enhance glucose-stimulated insulin secretion.[15] Incretins regulate glucose concentrations and also exert multiple nonglycemic actions in the cardiovascular system.[16-18] From this perspective, the use of incretins during physiological aging is receiving significant attention for their potential benefits on the aging-associated insufficient function of the cardiovascular system.[19-21] Furthermore, these drugs could exert some pleiotropic effects, providing a favorable impact on cardiac dysfunction besides GLP-1R agonism.[22] Furthermore, we, recently, have shown the important beneficial effects of chronic GLP-1R agonism with liraglutide on the abnormal electrical activity of the elderly rat heart, such as augmentation of prolonged QRS duration, increased systolic and diastolic pressures, and prolonged action potential duration in ventricular cardiomyocytes.[8,21]

Several studies are underway to investigate the potential adverse effects of GLP-1 receptor agonist drugs on mammalian organ structure and function. In addition to established side effects like nausea, vomiting, and diarrhea, ongoing discussions are focused on elucidating the impact of GLP-1 receptor agonists on cardiovascular risk and outcomes.[23,24] Indeed, in this regard, Lorenz et al[25] published that short-acting GLP-1RAs are associated with a modest and transient heart rate (HR) increase before returning to baseline levels, which further points out the possible adverse clinical outcomes in those with advanced heart failure.

Despite significant improvements underpinned by the chronic GLP-1R agonist application in aging-related organ/tissue/cell dysfunction, the acute effects of this peptide on the electrical properties of either intact ventricular myocardium or cardiomyocytes in elderly are not known yet. On the other hand, there are no solid data associated with heart dysfunction during physiological aging. Among already-known events, increased oxidative stress is one of the mechanisms that contributes to cardiometabolic remodeling in elderly mammalians.[26-30] In these studies, authors demonstrated that mitochondrial dysfunction impairs oxidative phosphorylation, reducing adenosine triphosphate (ATP) production and increasing production of reactive oxygen species (ROS) in cardiomyocytes. Increased ROS further affects the availability of heart substrates, causing both metabolic remodeling and contractile dysfunction. Supporting studies have shown the association between high glucose levels and stimulation of ROS production through the activation of protein kinase C (PKC) in cardiovascular system cells.[31,32] Correspondingly, it is accepted that increases in ROS and nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase-associated cellular factors are the main facilitators of cardiovascular complications in diabetes. Supporting these statements, we, in our early studies, have shown a significant increase in total PKC content in the isolated membrane fractions of the cardiomyocytes from streptozotocin (STZ)-diabetic rat hearts, with a decreased level in cytosolic fractions being parallel to an increased level of ROS production. These alterations could be reversed by the administration of a nonspecific PKC inhibitor, bisindolylmaleimide I. In addition, in the same study, this PKC inhibitor application could restore the altered parameters of Ca^{2+} sparks and resting Ca^{2+} levels with no change in L-type Ca^{2+}-channel currents, parallel to reverses in the ratio of the protein content of total PKC in the membrane to that in the cytosolic fraction.[33,34] Furthermore, we also demonstrated that there was a close relationship between elevated levels of endoplasmic reticulum (ER) stress markers and increases in the total PKC and PKCα expression and PKCα-phosphorylation in the samples from mammalian heart failure, while a PKC inhibition induced a significant decrease in expressions of these ER stress markers compared to controls.[35] Although there is some experimental data to demonstrate the status of the PKC superfamily in elderly mammalian hearts,[36,37] however, studies demonstrating the role and status of PKC in aging-related cardiac dysfunction need further clarification. Interestingly, we demonstrated a direct role of casein kinase 2 (CK2) phosphorylation not only in physiological aging (characterized by insulin resistance) but also in insulin deficiency-associated alterations in ionic currents and both intracellular Ca^{2+} and Zn^{2+} homeostasis in isolated cardiomyocytes.[5,21,38]

Taking into consideration the increases in the Na^+-channel currents and intracellular free Na^+ levels as well as Ca^{2+} without any significant change in Ca^{2+}-channel currents, being parallel to increased levels of ROS production and activation of CK2 in isolated cardiomyocytes from insulin-resistant elderly rat hearts,[5,8] here, we aimed to examine the acute effect of GLP-1R agonism in elderly mice and cardiomyocytes freshly isolated from these elderly mice hearts. We focused on investigating the electrophysiological parameters of heart and cardiomyocytes with some biochemical analysis in these elderly animals compared to those of adult mice. Our overall data have shown the possible adverse effects of acute GLP-1R agonism in insulin-resistant elderly mammals, such as induction of arrhythmias, at least, through

HIGHLIGHTS

- A single liraglutide injection induced arrhythmogenic ECGs in aged mice but not in adults.
- Liraglutide prolonged action potential repolarization in aged cardiomyocytes by suppressing K^+ currents and augmenting persistent Na^+ currents.
- Liraglutide-induced casein kinase-2 hyperphosphorylation disrupted electrical signaling in aged cardiomyocytes, which a casein kinase-2 inhibitor could reverse.

augmentation in Late-Na^+-channel currents being associated with the phosphorylation of CK2 at the cellular level.

METHODS

Experimental Animals

We used 6-month-old male BALB/c mice to represent adults, as they are considered mature and exhibit stable cardiovascular function. In contrast, 24-month-old male mice represent the aged group, roughly equivalent to 70-75 human years. This age model is well-supported in the literature, as studies show that aged BALB/c mice exhibit cardiovascular changes similar to those seen in elderly humans, such as increased fibrosis and altered ion handling, making them suitable for our investigation of age-related arrhythmias.[39,40]

All animals were exposed to a 12-hour light–dark cycle and were given free access to tap water. They were fed standard chow ad libitum daily and were housed in standard mouse cages at 5 animals per cage.

In Situ Surface Electrocardiogram Recording

In situ surface electrocardiography and HR monitoring in mice were performed as described previously.[41] Experimental animals intraperitoneally received ketamine/xylazine (150/5 mg/kg) before the measurements. Bipolar limb leads (lead I, II, and III) were carried with carefully injecting subcutaneously 20-Gauge needles close to the forearms and hind limbs. Electrocardiograms (ECG) data were acquired using an analog-to-digital converter BIOPAC MP35 (Goleta, California) and processed with the band-pass filter at 50-500 Hz, eventually analyzed by BIOPAC Student Lab Pro. All measurements were performed in a Faraday cage to reduce any external signals. Liraglutide (0.3 mg/kg) was intraperitoneally given to the experimental animals after obtaining stable recordings, and acute effects were evaluated 10 minutes following the injection.[42] The duration of the recordings, such as PR-, RR-, and QT-intervals, was calculated from processed ECG traces for each animal.

Cardiomyocyte Isolation

The hearts were quickly removed after the mouse stopped responding to tail/toe pinches following ketamine/xylazine (150/5 mg/kg) anesthesia. Afterward, the heart was transferred into ice-cold, Ca^{2+}-free N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-buffered solution [(in mmol/L) NaCl 143, KCl 5.4, $MgCl_2$ 0.5, HEPES-NaOH 5, and glucose 5.5, (pH = 7.4)]. The heart was then cannulated to a temperature-controlled Langendorff-perfusion system with calcium-free HEPES buffer at a flow rate of 5 mL/min for about 4-5 minutes. Enzymatic digestion was performed with 1 mg/mL collagenase type 2 (Worthington Cat: LS004176) for approximately 25 minutes until the heart became slightly pale and flaccid. The left ventricles were removed, minced into small pieces, and dissociated through a nylon mesh. Reintroducing the Ca^{2+} for adaptation was carried out in a graded manner to a final concentration of 1 mmol/L. Cell suspensions that contained an adequate fraction of elongated, non-granulated, rod-shaped, and quiescent cardiomyocytes with clear cross-striations were selected for experiments.

Voltage-Clamp Recordings

Electrophysiological parameters of cardiomyocytes were recorded using Axoclamp patch-clamp amplifier (Axopatch 200B amplifier, Axon Instruments, USA) at room temperature (22 ± 1°C). Data were sampled and digitized at 5 kHz using an analog-to-digital converter and software (Digidata 1200A and pCLAMP 10.3; Axon Instruments, USA). Borosilicate glass capillary tubes were used for electrode preparation (3-5 MΩ). Liquid junction potential was compensated before establishing the Giga seal. No leak or capacitance subtractions were performed during the current and voltage recordings.

Action potential (AP) and K^+-channel current recordings in freshly isolated ventricular cardiomyocytes were performed in a HEPES-buffered bathing solution containing (in mmol/L): NaCl 137, KCl 4, $MgCl_2$ 1, $CaCl_2$ 1.8, Na-HEPES 10, and glucose 10 at pH = 7.40. The pipette solution consisted of the following (in mmol/L): KCl 140, HEPES 10, Mg-ATP 3, EGTA [Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid] 1, and $CaCl_2$ 0.00037 (100 nM free Ca^{2+}), at pH = 7.2. Specifically, $CdCl_2$ (0.25 mmol/L) was added to the bath solution to block L-type Ca^{2+}-channel currents during K^+-channel recordings. The APs were elicited under small depolarizing rectangular pulses (4 ms duration and 10 mV amplitude) at 0.5 Hz frequency by using the whole-cell configuration of patch-clamping in the current-clamping mode, while whole-cell voltage-dependent total K^+-channel currents were recorded in the voltage-clamping mode in cardiomyocytes, as described previously.[13] Cells were evoked with a prepulse from the holding potential (−80 mV) to −50 mV to inactivate Na^+-currents then total K^+-currents were recorded by using stepwise pulses ranging from −100 mV to 60 mV with 20 mV increments for 3 seconds.

Whole-cell patch clamp recordings of Late-Na^+-channel currents (Late-I_{Na}) and the peak I_{Na} were recorded using an internal solution containing the following (mmol/L): 10 NaCl, 20 tetraethylammonium chloride (TEACl), 123 CsCl, 1 $MgCl_2$, 5 MgATP, 10 HEPES, and 1 EGTA, while free Ca^{2+} was maintained at 100 nmol/L with $CaCl_2$ at pH 7.2. The extracellular bathing solution for Late-I_{Na} recordings contained (mmol/L): 140 NaCl, 4 CsCl, 1.8 $CaCl_2$, 2 $MgCl_2$, 0.1 $CdCl_2$, 10 HEPES, 0.2 $NiCl_2$, and 10 glucose, at pH 7.4 adjusted with CsOH. For peak I_{Na} recordings, the extracellular bathing solution was altered by reducing NaCl to 40 mM and adding 100 mM N-methyl-D-glucamine sodium salt (NMDG) to establish ionic replacement.

Late-I_{Na} was elicited by applying a step pulse to −30 mV from a −120 mV holding potential. The charge carried by channels was calculated as the current integral from 30 to 330 ms from the beginning of the pulse.

Current–voltage relationship of the Na^+-channels was assessed by applying a protocol ranging from −80 mV to 30 mV with 5 mV increments. The steady-state inactivation was evoked by 2 pulse protocols, as described previously.[43] Conditioning pulses ranged from −140 mV to −55 mV while the test pulse was −40 mV. Inactivation curves were acquired by plotting the current amplitude obtained from the test pulse as a function of the voltage command of the

conditioning pulses and fitted to the Boltzmann function equation $y = [1+\exp\{(V-V_{1/2}/k\}]^{-1}$, where $V_{1/2}$ is the half-maximal voltage of inactivation and k is the slope factor. The recovery from inactivation was assessed using 2 protocols. A step conditioning pulse was applied to −40 mV from a −120 mV holding potential by a repetitive test pulse with 1 ms delays. Reactivation curves were analyzed by the peak current of the test pulse to conditional peak current and fitted to the following equation, $I = I_{max}[1-\exp(-t/\tau)]$.

For comparison among the groups, all recorded currents were divided by the cell membrane capacitance and presented as current density (pA/pF).

Western Blotting

Western blot analysis was performed to determine the relative phosphorylation status of protein kinase C (PKC) and CK2 under GLP1-RA activation in cell homogenates. After 10 minutes of GLP1-RA incubation cells were snap-frozen and kept at −80°C. On the day of the experiments, cells were homogenized in ice-cold radioimmunoprecipitation assay (RIPA) buffer as described previously.[44] Electrophoresis was performed after calculating the protein concentration levels (mg/mL) with the Pierce BCA Protein Assay Kit (cat. number: 23225). Equal amount of the proteins were run on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with p-CK2 (Invitrogen, PA5 37540), p-PKC (Cell Signaling, T514), and alpha-tubulin (Cell Signaling, DM1A) to detect any changes among the groups. Band densities were visualized with the Azure-300 Chemiluminescent Imaging System and analyzed with Image J program.

Confocal Imaging

Reactive Oxygen Species Measurements

Confocal imaging of ROS levels in adult and aged cardiomyocytes was determined as described previously.[13] Briefly, cells were loaded with a ROS indicator, chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (DCFDA, 10 μM for 60-minutes) and then examined with a laser scanning microscope (Leica TCS SP5). After GLP1-RA challenge, records were monitored for 10 minutes. The HEPES-buffered solution was supplemented with H_2O_2 (100 μM) added to the bathing solution to confirm the fluorescence quality for each recording (data not shown). All signals were normalized and presented as fold changes for all groups.

Measurements of Intracellular Ca^{+2} Changes

The measurement of Ca^{2+}-sparks in freshly isolated ventricular cardiomyocytes was performed as described elsewhere.[44-46] Briefly, cardiomyocytes were incubated with Fluo-3 AM (5 μM for 60-minutes) calcium dye (Fluo-3, AM, Calcium Indicator, Invitrogen cat. no.: F1241) in HEPES-buffered Tyrode's solution. The linear scanning mode (x-t mode) was applied in a Leica TCS SP5 laser scanning microscope. The scanning line (512 px) was vertically positioned on the long axis, water-immersed with 63× (NA = 1.3) objective, at 800 Hz speed at room temperature (20-25°C). The spark frequencies were calculated as 100 $\mu m^{-1}s^{-1}$. Spark Master (an ImageJ plugin) and ImageJ programs were used for further analysis.

Thapsigargin-sensitive Ca-release was conducted to evaluate sarcoplasmic reticulum (SR) Ca^{+2} content ($[Ca^{2+}]_{SER}$) and ryanodine (RyR2) stability, a methodology consistent with prior studies. [47] Cells loaded with Fluo-3 AM underwent a thapsigargin challenge (10 μM) and were observed using confocal microscopy. Changes in fluorescence were analyzed to determine RyR2 stability and expressed as fold change. This chemical was prepared in stock solution with dimethylsulfoxide (DMSO) and then diluted to a 1/1000 concentration to minimize potential solvent effects.

Ratio-metric cytosolic free Ca^{2+} level ($[Ca^{2+}]_i$) measurements were performed in Ca^{2+}-sensitive fluorescence dye (4-μM Fura-2 AM) loaded isolated ventricular cardiomyocytes by using the changes in fluorescence intensities. Resting fluorescence recordings were collected with a micro-spectrofl uorometer (PTI Ratiomaster and FELIX software; Photon Technology International, Inc., NJ, USA) as previously described.[6]

Data Analysis

Statistical analysis is presented as mean ± standard error of measurement (SEM) for normally distributed data, and as median (interquartile range: IQR) when Kruskal–Wallis analysis is performed, unless otherwise stated. The normality of the data was assessed using the Shapiro–Wilk test to determine whether to apply one-way analysis of variance (ANOVA) or the Kruskal–Wallis test, followed by Tukey's and Dunn's tests for post hoc analyses. For multiple comparisons of categorical data, Fisher's correction was applied. Descriptive statistics and probability values are provided in the tables, and original probability values, rather than symbol representations, are displayed in graphs. GraphPad Prism (GraphPad Prism for Windows 8.0.1, GraphPad Software, San Diego, CA, USA) statistical software was used for the analyses and $P < .05$ was considered significant.

The research and content in this manuscript were created without using artificial intelligence.

RESULTS

Acute GLP1-R Agonist Application Induces Spontaneous Non-Sustained Arrhythmic ECGs in Aged Mice

First, we examined the overall effects of GLP-1R agonism by using liraglutide (LG) on the in vivo electrical activity of the heart in aged mice compared to adults following a single LG injection (0.3 mg/kg i.p.). To analyze the appearance of spontaneous ECGs, we used the in situ surface ECG traces recorded during the first 10 minutes following LG injection in animals. As can be seen in Figure 1, there were 3-4 non-sustained spontaneous ECGs (arrhythmias) during the first 10 minutes of recording following LG injection in the aged (elderly) mice (B), while none of the adult mice showed any spontaneous arrhythmias under similar injection during the same period (A).

In situ recorded surface ECGs from experimental mice in light anesthesia revealed that aged mice had prolonged QRS duration and QT intervals compared to adult mice (Figure 1C and D; $P = .05$), while the average durations of P-waves (PW) and RR were not significantly different between these 2

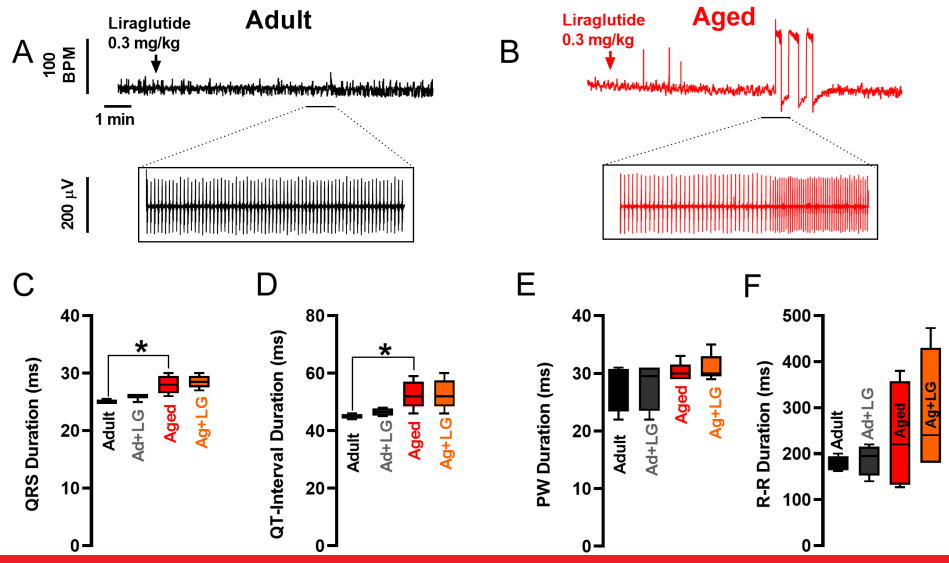

Figure 1. Monitoring *in situ* simultaneous surface ECGs and heart rates in experimental mice. (A, B) Representative heartbeats (above) and ECGs (below) for the adult and aged groups. Acute effects of the GLP1-RA (0.3 mg/kg liraglutide, i.p.) were assessed 10 minutes following the injection. Note that GLP1-RA causes self-sustaining arrhythmic events in aged mice. The summary data for the duration of QRS time (C), QT interval (D), P-wave (PW) (E), and R–R intervals (F) were elicited from the original ECG traces. All data were presented as mean ± SEM. The number of animals; N_{Adult}: 4, N_{Aged}: 5. $P < .05$ vs. adult, One-way ANOVA or Kruskal–Wallis test.

groups (Figure 1E and F). We also assessed the age-related changes in HR and their association with spontaneous cardiac arrhythmias. Parallel to the previously shown data,[48] here, there were no significant differences in the average HR values between these 2 groups, demonstrating no increased arrhythmic risk with advancing age under physiological conditions (data not shown).

Acute LG Application Further Enhances the Increased Late-I_{Na} Responsible for Prolongation in the Late Phase of Action Potential Duration of Aged Cardiomyocytes

We examined the effects of GLP1-R agonism on the electrophysiological properties of isolated cardiomyocytes. The average duration of APs in the aged cardiomyocytes was slightly but significantly longer than that of the adult cardiomyocytes, mainly through prolongation of the 90% repolarization of APs, APD_{90} (Figure 2A, lower part). Interestingly, an acute LG (1-µM) application during AP recordings produced a more significant prolongation in APD_{90} in the aged cardiomyocytes compared to non-treated aged cardiomyocytes. The original AP traces are given in the upper part of Figure 2A.

Since APD_{90} is constituted by the balance of the Late-I_{Na} currents (inward) and K^+ (outward) currents, we investigated these currents in response to the acute LG application. Interestingly, the average value of Late-I_{Na} in the aged cardiomyocytes was significantly higher than that of the adult mice cardiomyocytes. In addition, the acute LG application induced a further significant increase in Late-I_{Na} while a slight but not significant change was observed in the adult cardiomyocytes (Figure 2B, lower part). The original current traces are given in the upper part of Figure 2B. Furthermore, we examined the effect of acute LG application on total K^+-channel currents in these 2 groups of cardiomyocytes. As can be seen in Figure 2C, the LG application did induce a marked reduction in the K^+-channel currents in the aged group without any effect in the adults (lower part). The upper part of Figure 2C shows the original current traces in each group of cardiomyocytes.

When one performs a further comparison between the currents contributing to the prolongation in the APDs via an acute LG application, the increase in the inward Late-I_{Na} is higher than 100% while the decrease in outward K^+-currents is about 17%. These data can indicate the important contribution of upregulated inward Na^+-influx rather than the downregulated K^+-efflux to the prolonged APDs under acute LG exposure in the aged cardiomyocytes.

Acute LG Application Induces Alterations in the Na^+-Channels of the Aged Cardiomyocytes

In further investigations, we examined the modulation of the Na^+-channels in the presence of the acute LG application. As can be seen from Figure 3A, voltage-dependent Na^+-channels exhibited a significantly activated gain of function in the aged group compared to the adult group with no further significant change in these cardiomyocytes under acute LG application. Similarly, there was a depolarizing shift in voltage-dependent steady-state Na^+-channel inactivation in the aged group in comparison to the adult while no significant changes in these parameters following the acute LG application in both groups of cells (Figure 3B). Interestingly, an acute LG application elicited a decelerated time-dependent recovery from the inactivation of the Na^+-channels only in the aged group (Figure 3C).

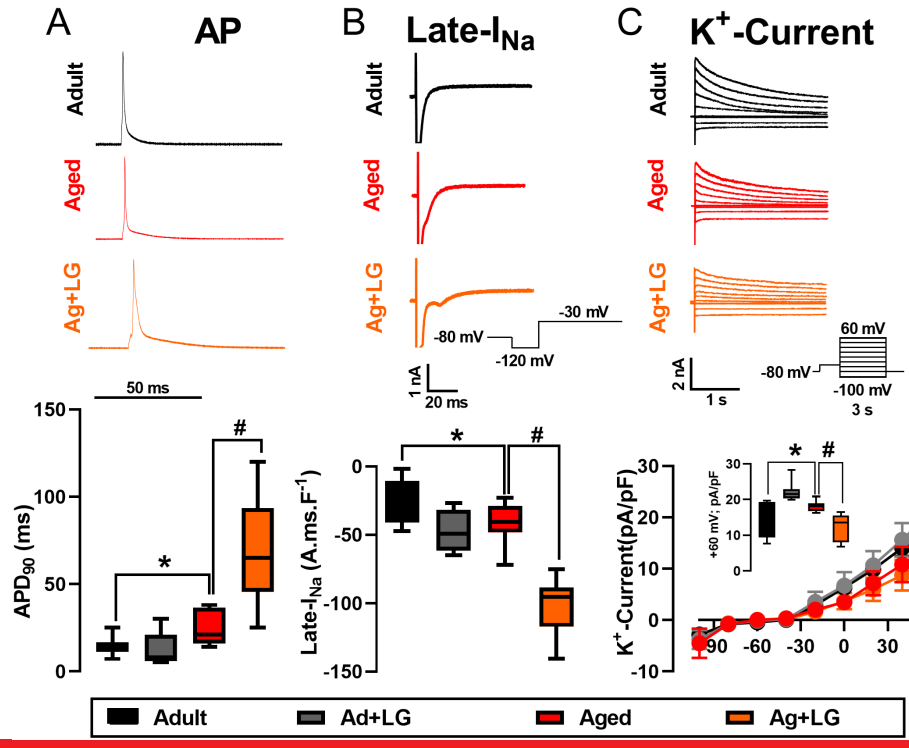

Figure 2. Glucagon-like peptide-1 receptor agonist elicited prolonged electrical activity with abnormal repolarizations in freshly isolated cardiomyocytes from aged mice. The sarcolemmal electrical activity was determined with AP recordings, while repolarizations were assessed with Late-I_{Na} (inward) and total K^+-currents (outward) in cardiomyocytes. (A) Representative AP traces (*top-right*) and the summary data (*bottom-right*) are given for the corresponding groups. (B) Representative persistent Late-I_{Na} traces for the groups, elicited using the voltage protocol shown in the figure (*middle top*). In comparison to the adult group, Late-I_{Na} GLP1-RA (1 μM) exerted an unequal and remarkable increase in aged cardiomyocytes (*middle bottom*). (C) Representative K^+-current traces using the applied protocol are shown in the figure (*top-left*). Interestingly, the GLP1-RA challenge causes a slight increase in outward K^+-current in adults and a significant decrease in aged cardiomyocytes (*bottom-left*). All data were presented as mean ± SEM. The number of cardiomyocytes for each group: N_{Cells}: 9-13. *$P < .05$ vs. adult, #$P < .05$ vs. aged, one-way ANOVA or Kruskal–Wallis test.

Effect of the LG Application on Cellular Redox State in the Aged Cardiomyocytes Under In Vitro Conditions

Figure 4A (left) shows the original representative cardiomyocytes loaded with the fluorescent dye DCFDA to monitor the fluorescence intensity changes associated with ROS production in the cardiomyocytes. The acutely monitored fluorescence intensity-level analysis demonstrated that ROS production was significantly higher in the aged rat cardiomyocytes in comparison to the adult ones, while there was no significant further increase in the LG incubated aged cells (Figure 4A, right).

Considering the association between high glucose levels and stimulation of ROS production through the activation of PKC[31,32] and phosphorylation of CK2 in cardiomyocytes ,[5,8,21,38] we determined the phosphorylation levels of PKCα and CK2 in the LG-treated and untreated aged cardiomyocytes in comparison to the adults. As can be seen in Figure 4B, the phosphorylated PKC levels (p-PKC) were not significantly different between aged and adult cardiomyocytes while the LG incubation could not induce any further phosphorylation in the aged cardiomyocytes (Figure 4B). However, the phosphorylation levels of CK2 (p-CK2) were significantly higher between aged and adult cardiomyocytes while the LG incubation could induce further significant phosphorylation in the aged cardiomyocytes (Figure 4C).

Inhibition of Phosphorylated CK2 Restores Abnormal Electrical Activity in Aged Cardiomyocytes Under Acute GLP-1R Agonism

To examine the role of CK2 phosphorylation on the abnormal electrical activity of the aged cardiomyocytes under acute GLP-1R agonism, we used a specific CK2 inhibitor (Silmitasertib, 5 μM),[49] and then we determined first the Late-I_{Na} under the LG application. The representative Late-I_{Na} traces are given in Figure 4D. As can be seen in Figure 4E, the average value of activated Late-I_{Na} in the aged cells under the LG application was significantly reversed by the application of this CK2 inhibitor. Furthermore, under this inhibition, the prolonged APD_{90} was significantly reversed to that without LG level (Figure 4F). We also determined the effect of a CK2 inhibitor on GLP-1R agonism-induced inhibited K^+-channel currents. As can be seen in Figure 4G, the maximum values of K^+-channel currents calculated at +60 mV in the LG-applied aged cardiomyocytes were significantly lower than those of untreated aged cardiomyocytes, while there was significant recovery with the application of the CK2 inhibitor. Furthermore, there was a full reversal in

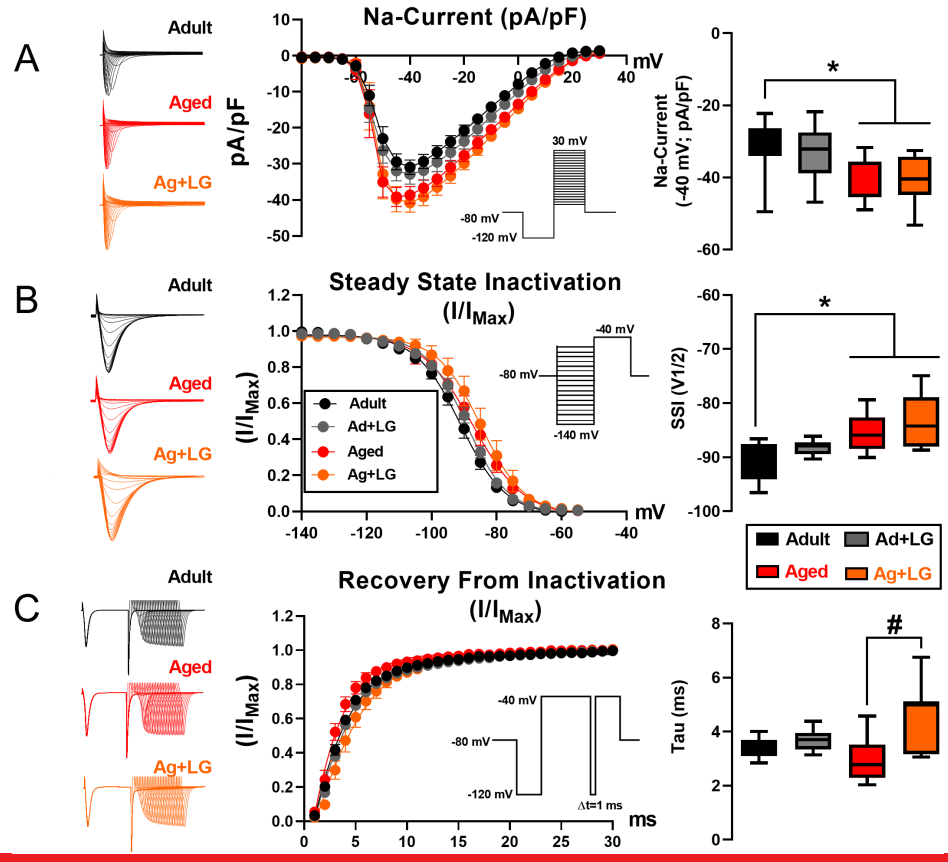

Figure 3. Modulation of the Na^+-channels in GLP1-RA activation in adult and aged cardiomyocytes. (A) Representative traces of the Na^+-channels for the groups (*left*). Voltage-dependent of the Na^+-currents and used voltage protocol in the presence of the GLP1-RA activation (*middle*). The summary data elicited at maximum Na^+-current activation revealed a gain of function in aged cardiomyocytes (*right*). (B) Experimental traces of the steady-state inactivation of the Na^+-channels are shown (*left*). Voltage-dependent inactivation of the Na^+-channels with the elicited protocol (*middle*) and calculated summary data (*right*) of the half maximum ($V_{1/2}$) values for the groups. (C) Representative traces for the time-dependent recovery from inactivation of the Na^+-channels (*left*). Overall time-dependent recovery from inactivation and applied protocol are shown (*middle*). Notably, GLP1-RA activation causes a remarkable delay in aged cardiomyocytes (*right*). All data were presented as mean ± SEM. The number of cardiomyocytes for each group N_{Cells}: 7-11. **P* < .05 vs. adult, #*P* < .05 vs. aged, one-way ANOVA or Kruskal–Wallis test.

the altered current–voltage relationships of these channels under GLP-1R agonism by using a CK2 inhibitor in these aged cardiomyocytes (data not given).

An Acute LG Application Induces Increases in the Occurrence of Ca^{2+} Waves in the Quiescent-Aged Cardiomyocytes

To also assess the effect of acute LG application on cellular Ca^{2+} regulation, we initially determined the status of Ca^{2+} sparks in cardiomyocytes both with and without acute LG applications. Representative confocal imaging of the Fluo-3 AM loaded cells is given in Figure 5A. The average frequency of Ca^{2+}-sparks was significantly higher in the aged group than in the adult group, whereas the acute LG application could not induce further change in the aged group (Figure 5B). Interestingly, the acute LG application induced higher extent production of the Ca^{2+}-waves (as the spatial and temporal summation of the Ca^{2+}-sparks) in the aged cardiomyocytes than in the adult cardiomyocytes without any further under acute LG application (Figure 5C). Of note, this acute LG application induced Ca^{2+}-waves incidences were higher in the aged group (85 waves) than in the adult group (20 waves) during 10 minutes following the LG applications. We, here, did not examine the effect of acute LG application on L-type Ca^{2+}-channel currents because we previously have shown neither change in this current under physiological aging nor the application of LG in the aged cardiomyocytes.[8]

In further investigations, we determined transient Ca^{2+} releases from the sarcoplasmic reticulum (Ca^{2+} transients) in Fluo3-loaded cardiomyocytes under electrical stimulation by using a ratiometric micro-spectrofluorometric system as relative fluorescence intensity changes as described previously.[50] As can be seen in Figure 5 (D and E), an acute LG application elicited aberrant transient Ca^{2+} changes (as fluorescent intensity change) in the aged cardiomyocytes together with increases in the fluorescence intensity changes associated with changes in the resting level of free Ca^{2+} ($[Ca^{2+}]_i$) in the quiescent cells.

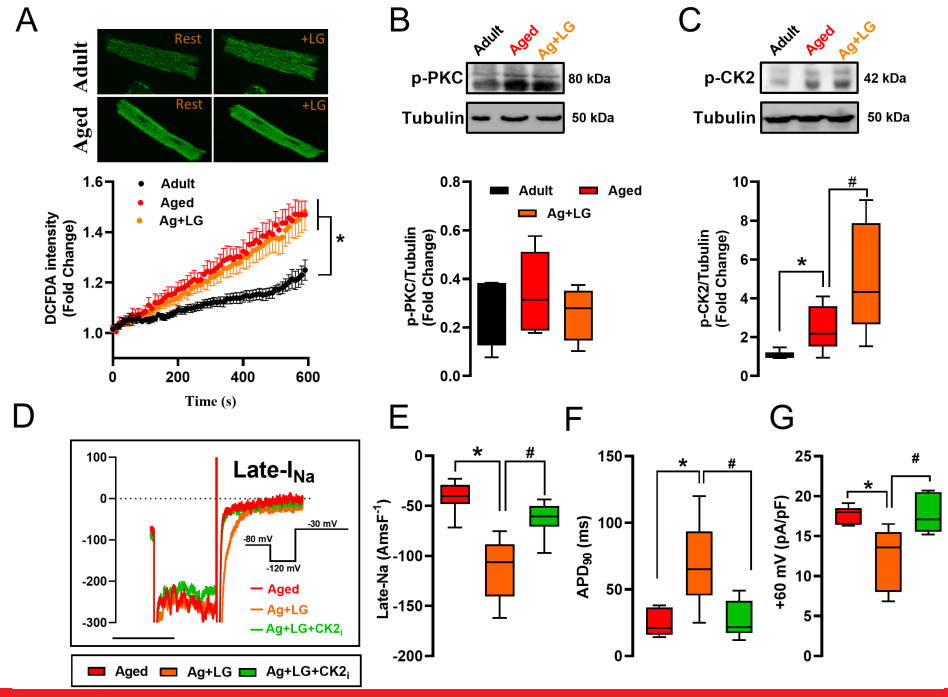

Figure 4. Casein kinase 2 inhibition provides substantial recovery in repolarization in the presence of GLP1-RA in sustained higher redox imbalance. (A) Time-dependent changes in DCFDA fluorescence intensity were plotted beneath the experimental images for the respective experimental groups. Immunoblotting images and summary data for p-PKC (B) and p-CK2 (C) are shown. Notably, CK2 but not PKC was hyperphosphorylated following acute GLP1-RA activation in aged homogenates. (D,E) Casein kinase 2 inhibitor (Silmitasertib, 5 μM) markedly reduces Late-I_{Na} in aged cardiomyocytes during the GLP1-RA challenge. Casein kinase 2 inhibitor restores APD prolongation (F) and restores outward K^+-currents (G) during GLP1-RA activation in aged cardiomyocytes. All data are presented as mean ± SEM. The number of cardiomyocytes for each group N_{Cells}: 5-13. Immunoblotting experiments were repeated 5 times for all the groups. *$P < .05$ vs. adult, #$P < .05$ vs. aged, one-way ANOVA or Kruskal–Wallis test.

In the last part of this group examination, to demonstrate sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA) pump function and Ca^{2+} leak from ryanodine receptors (RyR2s), we used thapsigargin application protocol as described elsewhere.[47] The stabilities of RyR2s are represented by changes in fluorescent intensity (with high intensity associated with high RyR2 stability), initially comparing aged cardiomyocytes to those of adults As can be seen in Figure 5F (representative fluorescent-loaded cells) and G (the average intensity levels under different conditions in different cell groups), the RyR2 stability in the aged cardiomyocytes was significantly lower (indicating more leaky status) than those of the adults. Furthermore, an acute LG application induced further leaky status in both aged and adult cardiomyocytes, significantly.

DISCUSSION

The present study aimed to examine whether there is an acute effect of GLP-1R agonism in elderly mouse heart function, although it has demonstrated its important cardioprotective effects, particularly in diabetic hearts.[8,22] Eventually, there are some important studies focusing on the impact of GLP-1 receptor agonists on cardiovascular risk and outcomes.[23,24] On the other hand, some studies also mention the side effects of GLP-1R agonism in humans during daily administrations. In this regard, it has been shown how an acute GLP-1R agonist application has an unexpected side effect on the heart, characterized by a modest and transient HR increase before returning to physiological levels, while some long-acting GLP-1 RAs are associated with a more pronounced and sustained increase in HR during the day and night. These findings can further indicate the possible adverse clinical outcomes in individuals who have advanced heart failure associated with an increase in HR by these drugs, particularly in patients with insulin resistance.[25]

The GLP-1R agonists, beyond their beneficial effects on several pathologies, including cardiovascular diseases in type 2 diabetes and obesity, clinical trials, and preclinical data suggest that GLP-1R agonism can improve outcomes in aging-related pathology by acting directly on multiple organs with minimal unwanted side effects. Epidemiological data emphasize the important relationship between age-related changes in cardiometabolic alterations and cardiovascular risk, which in turn accelerate dysregulation of physiological pathways in aging. Correspondingly, an acute GLP-1R agonism can induce unexpected side effects in elderly hearts, such as self-sustaining arrhythmias. Our present data obtained in both in vivo and in vitro conditions, at the levels of system, organ, and isolated cardiomyocyte levels, strongly imply that these acute side effects can arise through augmentation in Late-Na^+-channel currents being mediated by phosphorylation of CK2 in insulin-resistant cardiomyocytes.

Supporting data for these actions are also presented with the monitoring of repetitive Ca^{2+} waves under acute GLP-1R

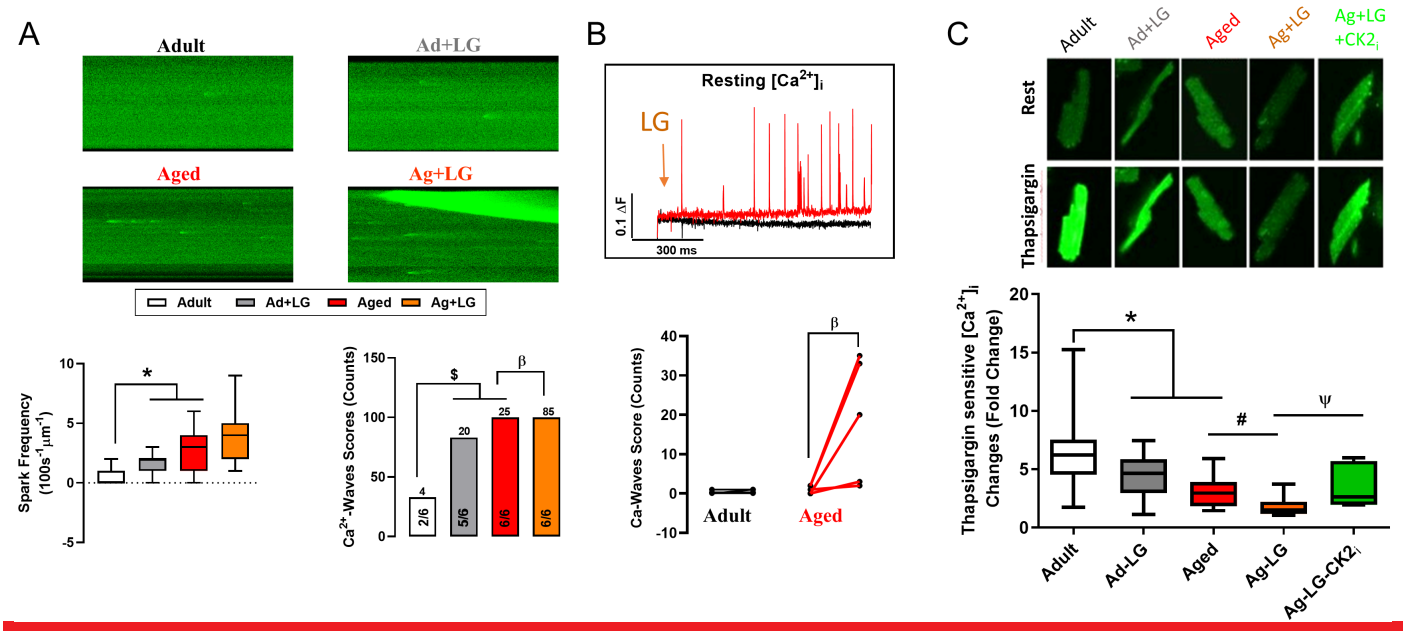

Figure 5. Casein kinase 2 inhibition substantially restores GLP1-RA mediated impaired Ca^{2+}-release. (A) Representative confocal line-scanned images loaded with Fluo-3 AM for the groups. Of note, GLP1-RA increased Ca^{2+} spark frequency in all groups, but it also turned to activate wave formation in aged cardiomyocytes. Summary data for spark frequency (*left*) and wave events *(right)* for the groups. (B) Cells loaded with Fura-2 AM elicited spontaneous Ca^{2+} transients during GLP1-RA in aged cardiomyocytes. Counted wave events exerted aberrant self-sustaining Ca^{2+} transient in aged cardiomyocytes. (C) Summary data for Thapsigargin-sensitive Ca^{2+} release were plotted beneath the original images. The number of cells N_{Cells}: 6-7 for each group. *P < .05 vs. adult, $^{\#}P$ < .05 vs. aged, $^{\Psi}P$ < .05 vs. Ag-LG, one-way ANOVA or Kruskal–Wallis test. $^{\$}P$ < .001 vs. adult, $^{\beta}P$ < .0001 vs. aged, Fisher's exact test.

agonism by liraglutide application in isolated cardiomyocytes either from elderly hearts or adult ones, with a significantly higher effect in the elderly group. Interestingly, being independent of possible changes in cellular redox balance, CK2 is hyperphosphorylated under this application, which plays a role in the downstream signaling cascade during the GLP-1R agonism challenge. Our data, for the first time, provided new insight into the acute effect of a glucose-lowering drug GLP-1R agonist application. Indeed, here, our data demonstrated hyperphosphorylation of CK2, which in turn subsequently impairs Na^+ and Ca^{2+} handling and thereby eventually precipitates the occurrence of arrhythmia in the aged myocardium.

Insulin resistance elicits detrimental outcomes by altering cellular signal transduction, changing substrate metabolism, and also affecting electrical propagation in the elderly heart, including increased oxidative stress.[5,6,8,13,21,51] Recent literature addressed the importance of glucose-lowering therapy associated with reducing hyperglycemia and insulin resistance and provided substantial pleiotropic improvement in cardiovascular changes in physiologically aging hearts.[15,17,21,52,53] However, in our recent study, we used the same amount of liraglutide injection into aged rats for 4 weeks and demonstrated significant recoveries in the altered parameters of heart preparations, such as recoveries in prolonged APD duration via improving the inhibited repolarizing outward K^+-currents, altered cytosolic Ca^{2+}-handling, and mitochondrial dysfunction in both aged rats and high carbohydrate-induced metabolic syndrome adult rats.[8] On the other hand, meta-analyses elicited some adverse cardiovascular effects of incretin-based therapies in humans,[54] and also prolonged APD in animal models.[53] Heterogeneous results between the studies arise due to the observations of only the long-acting action rather than short-acting (fast 8-10 minutes) effects of GLP-1R agonism, as well as lack of knowledge of the age, sex, and other cardiovascular risk factors.[55]

Taking into consideration the lack of information related to the underlying mechanisms of the increased susceptibility to arrhythmia, especially in aging mammalians,[56,57] our present data can bring new insight on this topic. Correspondingly, our present study can point out the importance and role of acute agonism of this receptor on the induction of a new acute electrical remodeling in the myocardium in response to the GLP-1R agonist application, particularly in the aging heart in mammals.

Glucagon-like peptide-1 receptor agonism impaired the repolarization phase of APs by enhancing Late-I_{Na} and reducing total K^+-currents as if mimicking Long QT-3 in aged cardiomyocytes.[58-61] In this scenario, Na^+-channels result in a gain of function due to the impaired inactivation and generate sustained Na^+-permeation.[62] Also, altered recovery from the inactivation of the Na^+-channels increases the probability of the channel "reopening" during AP repolarization.[43,63] Our present data strongly support these statements. We have overall observed elicitation of a depolarizing shift in steady-state inactivation in aged cardiomyocytes which is

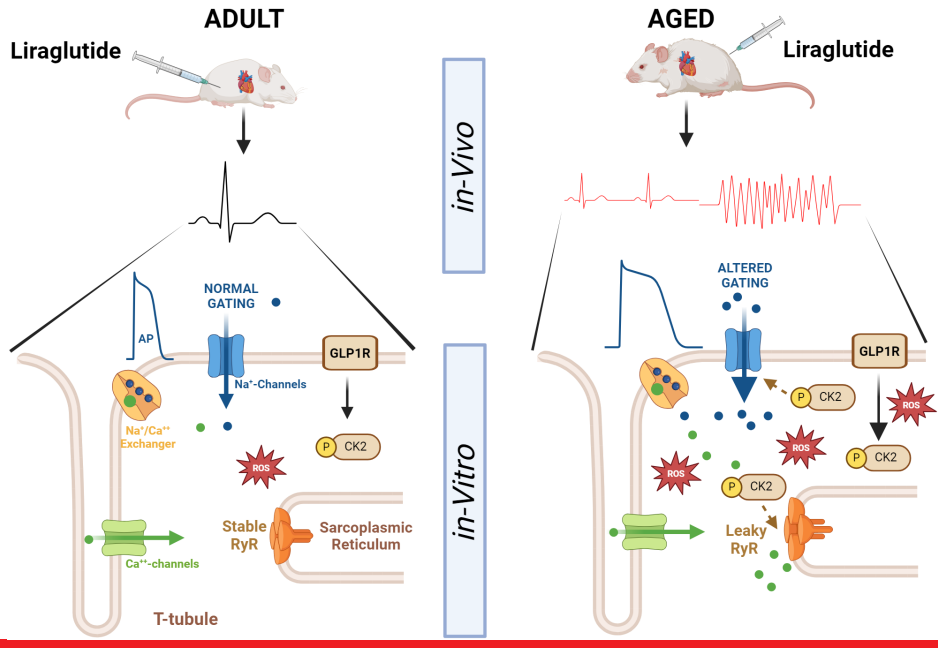

Figure 6. A summarization of possible molecular mechanisms of the acute GLP-1R agonist effect on the electrophysiological and biochemical properties of the insulin-resistant aged rat heart compared to the adult rat heart. Acute administration of LG elicited sustained and repetitive arrhythmia in the aged mice hearts. Electrophysiological analyses demonstrated prolonged action potential duration along with a notable increase in CK2 phosphorylation in the context of redox imbalance within aged cardiomyocytes. These modifications led to altered Na^+-channel gating and destabilized RyR, leading to disrupted calcium handling during the short-term phase of LG treatment. The figure was created with BioRender.com.

associated with GLP-1R agonism-mediated deceleration in recovery from inactivation of Na^+-channels. A GLP-1R agonism can also enhance Ca^{2+}-spark frequency and marked Ca^{2+}-wave formation in both aged and adult groups, with a significantly higher percentage in the quiescent-aged cardiomyocytes compared to the adults. It is widely established that sustained Na^+ permeation is also a substrate for arrhythmia, subsequently inducing Ca^{+2}-sparks by disrupting proper Na^+ and Ca^{2+} handling in the myocardium.[64-67] Our results highlight that augmented Late-I_{Na} may increase the reverse mode of the Na^+-Ca^{2+} exchanger further leading to the accumulation of Ca^{+2} in the vicinity of the ryanodine receptors. In this regard, our previously published data associated with the activation of Na^+–Ca^{2+} exchanger in the aged cardiomyocytes strongly support the above interpretation associated with Late-I_{Na} related to the accumulation of Ca^{+2} in the vicinity of the ryanodine receptors.[6,8]

Our biochemical results revealed activated CK2 mediation of the electrical remodeling under acute GLP-1R agonist application in the aging mice hearts. We also observed that oxidative stress and PKC do not play a role during this remodeling. Beyond a variety of different regulatory processes, such as Akt signaling, splicing, and DNA repair in mammalian cells, CK2 directly phosphorylates a variety of channel proteins to regulate the gating properties,[68,69] while CK2 can be hyperphosphorylated by increased oxidative stress under pathological conditions, including hyperglycemia.[70] It also phosphorylates scaffold proteins such as ankyrin G, which bind to channel proteins for physiological transport to and positioning into the membrane.[69,71,72] Hsu et al[73] demonstrated that CK2 interacts with fibroblast growth factor 14 and regulates Na(V) channels and neuronal excitability. In addition, Bachhuber et al[74] also demonstrated the regulation of the epithelial Na^+-channels by the CK2. Consequently, we summarized our present overall data in Figure 6. As can be seen from this figure, our findings suggest that CK2 hyperphosphorylation and further alterations in Na^+ and Ca^{2+} handling play important roles in GLP-1R agonism-associated fast electrical remodeling in the heart.

Data availability: Data are available from the authors upon request.

Ethics Committee Approval: All animal procedures and experiments were approved by the Ankara University (Reference number: 2023-8-69) Ethics Committee on 26/04/2023, according to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and in accordance with the principles of the Declaration of Helsinki.

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Author Contributions: Concept – Y.O, B.T.; Design – Y.O., B.T.; Supervision – Y.O., B.T.; Resources – Y.O.; Materials – Y.O.; Data Collection and/or Processing – Y.O., A.D.; Analysis and/or Interpretation – Y.O., A.D.; Literature Search – Y.O.; Writing – Y.O., B.T.; Critical Review – Y.O., B.T.

The authors declare that all data were generated in-house and that no paper mill was used. Yusuf Olgar wrote the manuscript and, together with Ayşegül Durak, performed the experiments. Belma Turan conceived of the study.

Declaration ofInterests: The authors have no conflicts of interest to declare.

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