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ORIGINAL ARTICLE

Glucagon-like peptide-1 receptor agonist treatment of high carbohydrate intake-induced metabolic syndrome provides pleiotropic effects on cardiac dysfunction through alleviations in electrical and intracellular Ca^{2+} abnormalities and mitochondrial dysfunction

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Abstract

The pleiotropic effects of glucagon-like peptide-1 receptor (GLP-1R) agonists on the heart have been recognised in obese or diabetic patients. However, little is known regarding the molecular mechanisms of these agonists in cardioprotective actions under metabolic disturbances. We evaluated the effects of GLP-1R agonist liraglutide treatment on left ventricular cardiomyocytes from high-carbohydrate induced metabolic syndrome rats (MetS rats), characterised with insulin resistance and cardiac dysfunction with a long-QT. Liraglutide (0.3 mg/kg for 4 weeks) treatment of MetS rats significantly reversed long-QT, through a shortening the prolonged action potential duration and recovering inhibited K^+-currents. We also determined a significant recovery in the leaky sarcoplasmic reticulum (SR) and high cytosolic Ca^{2+}-level, which are confirmed with a full recovery in activated Na^+/Ca^{2+}-exchanger currents (I_{NCX}). Moreover, the liraglutide treatment significantly reversed the depolarised mitochondrial membrane potential (MMP), increased production of oxidant markers, and cellular acidification together with the depressed ATP production. Our light microscopy analysis of isolated cardiomyocytes showed marked recoveries in the liraglutide-treated MetS group such as marked reverses in highly dilated T-tubules and SR-mitochondria junctions. Moreover, we determined a significant increase in depressed GLUT4 protein level in liraglutide-treated MetS group, possibly associated with recovery in casein kinase 2α. Overall, the study demonstrated a molecular mechanism of liraglutide-induced cardioprotection in MetS rats, at most, via its pleiotropic effects, such as alleviation in the electrical abnormalities, Ca^{2+}-homeostasis, and mitochondrial dysfunction in ventricular cardiomyocytes.

KEYWORDS
arrhythmia, electrical activity, insulin resistance, ion-homeostasis, sodium-calcium exchanger

1 | INTRODUCTION

Metabolic syndrome (MetS) is a combination of metabolic risk factors for both diabetes and cardiovascular diseases and is associated with insulin dysregulation. Circulating concentrations of the incretin hormone, glucagon-like peptide-1 (GLP-1) correlate with increased insulin response to high carbohydrate intake in mammals.[1] Accordingly, GLP-1 receptor (GLP-1R) agonists improve cardiovascular outcomes in diabetic patients and obese patients. However, these agents cause sympathetic activation, and are therefore considered to be detrimental in cardiovascular disease.[2] GLP-1R agonists have positive actions on several abnormal parameters of diabetics, MetS, or obese patients such as high body weight, blood pressure and glucose metabolism.[3-5] Interestingly, these agonists have a wide range of pleiotropic effects on the cardiovascular system, independent of the above effects.[6-8] In that regard, it has been shown with experimental studies on GLP-1R benefits in cardiomyocytes,[2,6-9] and clinical outcomes on cardiovascular system abnormalities with their varied efficacy.[10-15]

Physiologically, GLP-1 in the heart can drive myocardial glucose uptake and thereby provide protection against pathological stimuli such as myocardial ischaemic-injury.[2,9,16-19] Both experimental and clinical studies emphasise that there are marked impaired myocardial GLP-1R agonist responses in obese, MetS, and diabetic individuals.[16-19] In these studies, it has been also demonstrated the important effects of GLP-1R agonists on key signal transduction associated with cardiomyocyte injury, and activation of post-conditioning protective pathways in the heart. Among GLP-1R agonist effects, one can summarise these effects such as stimulation of insulin secretion, decrease in the secretion of glucagon, decrease in gastric emptying, decrease in appetite, and a reduction in food intake by creating a sensation of a full stomach.[3,20,21] Despite significant research interest in the cardiovascular effects of GLP-1R, its role in mammalian ventricular cardiomyocytes remains the subject of much debate, and its effects on the electrical properties of intact ventricular myocardium remain unclear. Supporting these statements, there are some in vitro and in vivo studies performed with GLP-1R agonists, which are providing further novel information at cellular levels.[7,9,19,22] In that line, recent studies demonstrated that a GLP-1R agonist, liraglutide protects cardiomyocytes from metabolic disturbance and mitochondrial dysfunction.[6,23] This agonist also regulates Ca^{2+}-homeostasis, the electrophysiological activities of cardiomyocytes modulating Ca^{2+}-handling proteins,[2,7] reduces ventricular arrhythmic potential,[8] and activates ATP-sensitive K^{+}-channels.[9,17] Liraglutide further attenuates apoptosis, oxidative and nitrosative stress.[9,18] However, the liraglutide effect on myocardial function has conflicting results in clinical trials.[13-15,24-26] Thus, there is a need for mechanistic studies with animals and in vitro investigations to better understand the effect of liraglutide on myocyte function.

The pathophysiology of MetS includes very complex mechanisms, due to multiple interconnected mechanisms, including disturbances in insulin signalling.[27,28] Since MetS in mammals characterised by marked insulin resistance, and cardiac dysfunction, including an electrophysiological remodelling in left ventricular cardiomyocytes,[28,29] here, we sought to determine the underlying mechanisms of GLP-1R agonist liraglutide (in vivo) as a cardioprotective drug in MetS rats.

2 | RESULTS

2.1 | Systemic effects of liraglutide treatment of MetS rats

The experimental animals fed a high carbohydrate diet (33% sucrose; MetS group) had significantly different oral glucose tolerans test (OGTT) values compared to the controls, while the 4-week liraglutide (0.3 mg/kg) treatment of these MetS animals provided significant reverses in these values. As can be seen in Figure 1A, the area calculated from OGTT tests performed for 0, 15, 30, 60, and 120 min was significantly larger in the MetS group than the control group while it was normalised in the treated MetS group.

The MetS rats had significantly higher weight gain compared to the controls (498.6 ± 11.2 g *vs.* 405.6 ± 11.4 g; Figure 1B, left). The liraglutide treatment of MetS rats caused significant weight loss (416 ± 23.1 g). Their serum insulin levels were significantly high (8.01 ± 1.29 ng/mL) compared to the controls (3.63 ± 0.56 ng/mL) while liraglutide treatment provided a significant decrease in that level (5.17 ± 0.91 ng/mL; Figure 1B, right). The systolic and diastolic blood pressures were significantly high in the MetS group compared to those of controls (184.9 ± 5.10 mmHg and 118.2 ± 2.14 mmHg vs. 125.5 ± 2.10 mmHg and 88.5 ± 1.94 mmHg) while they were about normal levels in the liraglutide-treated MetS group (142.3 ± 4.3 mmHg and 108.1 ± 1.76 mmHg; Figure 1C).

2.2 | Liraglutide treatment affected the electrical activity of MetS rats

The original ECG recordings of the control, MetS, and liraglutide-treated MetS groups are provided in Figure 1D. The calculated values for the ST-interval and QRS-interval (Figure 1E, left and right) and RR-interval (Figure 1F, left) were significantly longer in the MetS group than the control group, whereas the mean heart rate of the MetS group was significantly lower than the control group (Figure 1F, right). Liraglutide treatment significantly preserved these altered parameters of ECGs in the MetS rats including recoveries in long QRS-interval and ST-interval with no significant effect on the low heart rate in MetS rats.

2.3 | Effect of liraglutide treatment on structural alterations of MetS rat cardiomyocytes

We examined the semi-thin sections of isolated ventricular cardiomyocytes by light microscopy. As can be seen in Figure 2, there were

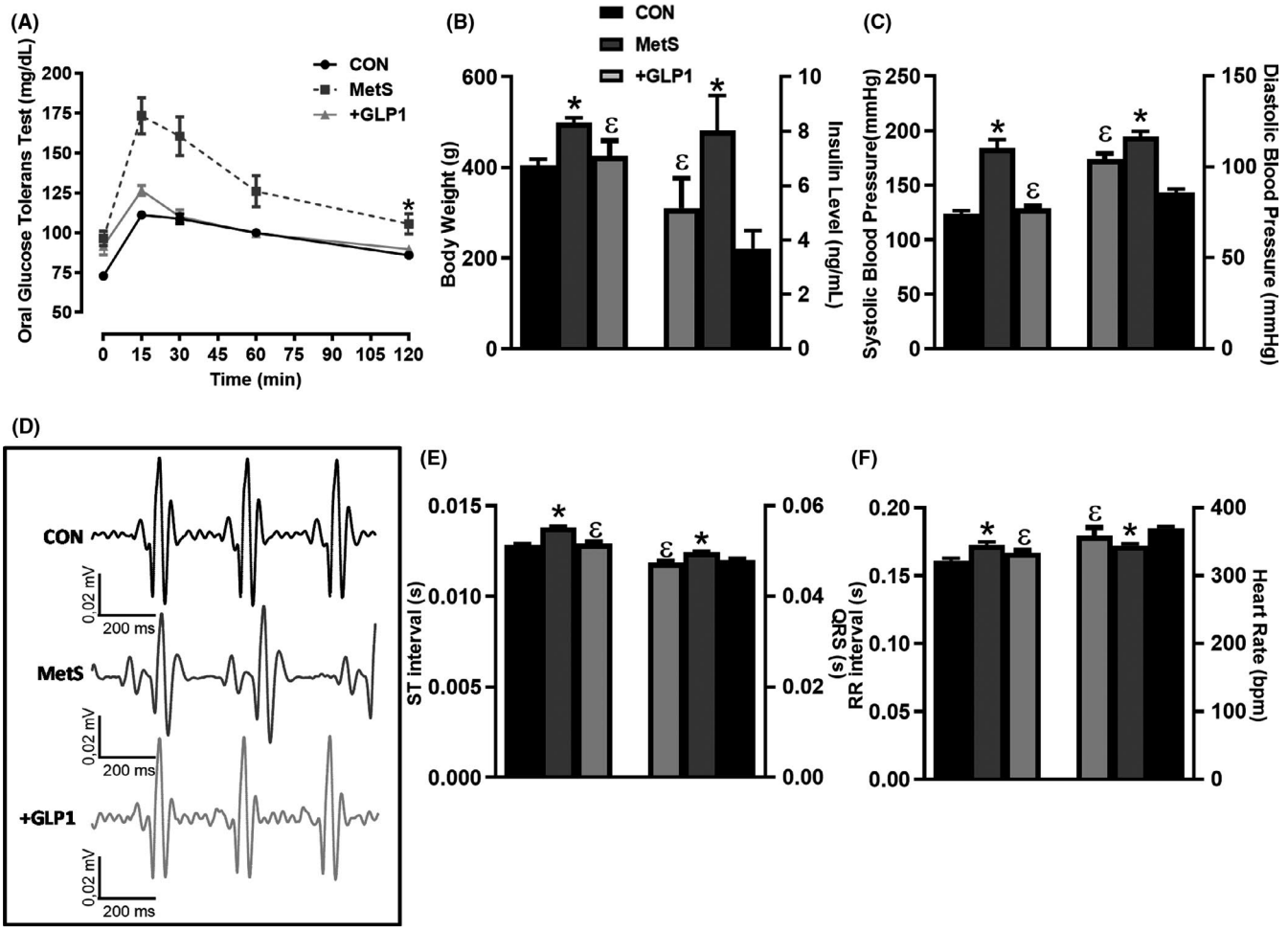

FIGURE 1 Glucagon-like peptide-1 agonist liraglutide ameliorates the parameters of electrocardiograms ε in rats with metabolic syndrome. (A) The area calculated from OGTT tests performed for 0, 15, 30, 60, and 120 min for control (CON), metabolic syndrome (MetS), and liraglutide-treated MetS (+GLP-1) rats. (B) The bodyweights of the groups (left) and the serum insulin levels (right). (C) Systolic (left) and diastolic blood pressures of the groups. (D) Original representative electrocardiograms (ECGs) of the rats. (E) The ST-interval (left) and QRS-interval (right) of the groups. (F) The RR-interval (left) and the heart rate (right) calculated from ECGs. All values are presented as mean (±SEM). The numbers of rats per group are 6–7. Significance levels at $^{*}p < 0.05$ *vs.* CON group and $^{\varepsilon}P < 0.05$ *vs.* MetS group

marked dilatations (red arrows) in the T-tubule and sarco(endo)plasmic reticulum junction of left ventricular cardiomyocytes isolated from the MetS rats compared to the controls. These alterations were slightly developed in the cardiomyocytes from liraglutide-treated MetS (lower part).

2.4 | Liraglutide treatment of MetS rats affected the electrical activity of cardiomyocytes

The original action potential recordings are given in Figure 3A. There were no significant changes in the resting membrane potentials calculated from single action potentials of the cardiomyocytes from the MetS group compared to the control group, while also no effect with liraglutide treatment of the MetS rats (Figure 3B). However, the action potential durations were shown to be significantly longer in the MetS group, determined at AP25, AP50, AP75, AP90, compared to the control group, while liraglutide treatment of the MetS rats significantly reduced these prolonged durations (Figure 3C). However, liraglutide treatment of the MetS rats did not affect the increases in the amplitudes of the action potentials (Figure 3D).

As seen in Figure 4A, we determined significantly increased peak amplitude of the I_{Na} in the MetS group compared to the control group. In addition, the maximum values increase significantly at −40 mV (Figure 4A bottom); however, liraglutide treatment of the MetS rats did not affect this increase in the I_{Na}.

The action potential repolarisation phase was found to be increased in the MetS group, significantly when compared to the control group. Taken into consideration the contribution of the I_K to the repolarisation phase of the action potentials, the peak values of I_K determined at −120 mV and +70 mV were found to be significantly decreased in the cardiomyocytes from the MetS compared to the control group. Figure 4B, shows the significant recoveries in these I_K in the liraglutide-treated MetS group. The maximum values of negative and positive potentials are given in the upper left part of Figure 4B in the inset.

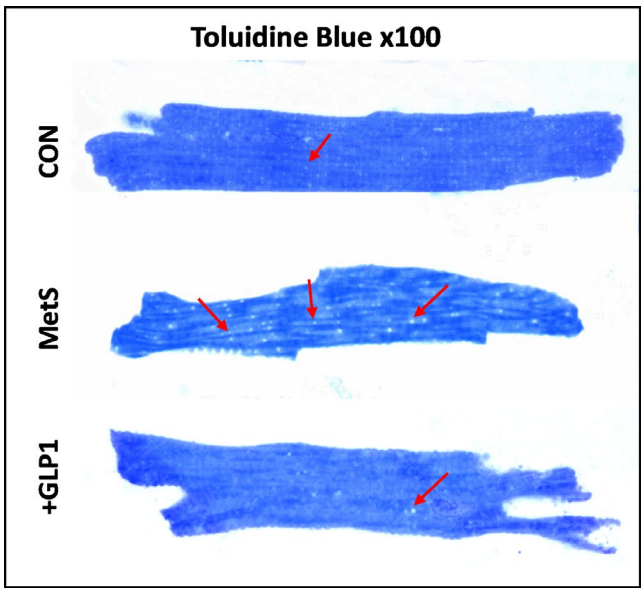

FIGURE 2 The light microscopy analysis of cardiomyocytes. The light micrographs show the semi-thin sections of isolated ventricular cardiomyocytes. In the metabolic syndrome (MetS) group, marked dilatations (red arrows) were noted in the regions corresponding to the T-tubule and the around of sarco(endo) plasmic reticulum junction (middle part). There are fewer amounts of dilations with normal appearances in the same locations in both control (upper part) and GLP-1 treated MetS group, MetS+GLP (lower part)

Considering the contribution of I_{NCX} to the formation of the shape of the action potential, we also examined I_{NCX} in the cardiomyocytes from the MetS rats. The peak amplitudes of I_{NCX} determined at −120 mv and +70 mV were found to increase significantly in the MetS group, while liraglutide treatment of the MetS rats provided significant recoveries in these currents.

We also examined the protein expression level of NCX by western blot analysis in the left ventricular tissues. As can be seen in Figure 4D, the NCX levels were found similar in the MetS and the control groups while no effect with liraglutide treatment of the MetS rats.

2.5 | Liraglutide treatment of MetS rats provided benefits on Ca^{2+}-handling machinery

Intracellular free Ca^{2+} levels ($[Ca^{+2}]_i$) were measured by loading FURA 2-AM in the control, MetS, and GLP1 groups. Figure 5A shows the original $[Ca^{+2}]_i$ measurements. The $[Ca^{+2}]_i$ was found to be increased significantly in the MetS group compared to the control group. Moreover, liraglutide treatment provided significant recovery in that level (Figure 5B left). When the $[Ca^{+2}]_i$ changes were examined under electrical stimulation, the averaged peak amplitudes of Ca^{2+}-transients were lower in the MetS group than the control group while liraglutide treatment of the MetS rats induced significant recoveries in these parameters (Figure 5B, right).

Since ryanodine receptors (RyR2s) have critical roles in the regulation of the contractile function of cardiomyocytes such as releasing Ca^{2+} from sarcoplasmic reticulum (SR),[30,31] we examined the function of SR by using an indirect approach such as determine Ca^{2+} release under tetracine application (Figure 5A). As can be seen in Figure 5B, the resting level of $[Ca^{2+}]_i$ was increased significantly in the MetS group (left), while transient changes of $[Ca^{2+}]_i$ under electrical stimulation was significantly decreased (right). In addition, the SR Ca^{2+} leak was significantly high in the MetS group compared to the control group with significant low caffeine response (Figure 5C). The liraglutide treatment of the MetS rats provided significant recoveries in these parameters.

In the last part of these group examinations, we determined the phosphorylation and protein levels of RyR2 (pRyR2 and RyR2) in the left ventricular tissues by western blot analysis. The pRyR2 level but not RyR2 protein level was 2-fold higher in the MetS group than the control group (Figure 5D, left and right, respectively), while pRyR2 to RyR2 ratio was also high in the MetS group, as well (Figure 5E). The liraglutide treatment of the MetS rats provided significant recoveries in these parameters.

2.6 | Effect of liraglutide treatment on mitochondrial function of the cardiomyocytes from MetS rats

The redox status of the cardiomyocytes by determination of cellular reactive oxygen species (ROS) level in DCDFA loaded cardiomyocytes. A significantly high ROS level determined in the MetS group was reversed with liraglutide treatment of the MetS rats (Figure 6B).

We also determined the reactive nitrogen species (RNS) level in DAF-AM loaded cardiomyocytes. Similar to the ROS level, the RNS level was significantly high in the MetS group compared to the control group, while this level was recovered in the liraglutide-treated MetS group, significantly (Figure 6D).

Also, determined mitochondrial membrane potential (MMP) determination was performed by using JC1-loaded cardiomyocytes as response to FCCP. The MMP values were found to be depolarised significantly in the MetS group compared to the controls, while they were recovered significantly in liraglutide-treated MetS group (Figure 6E).

In another set of experiments, we determined the intracellular pH level ($[pH_i]$) in the cardiomyocytes from Mets rats compared to the control rats. As can be seen in Figure 6F, the $[pH_i]$ in the MetS group was significantly lower that the control group and that level was fully reversed with liraglutide treatment of the MetS rats.

Since a depolarisation in MMP induces dysfunctional mitochondria, we examined the ATP production level in the cardiomyocytes. We determined the ATP levels in the cardiomyocytes by using a commercial kit and found a significantly lower ATP production in the MetS group than the control group, while that level was found to be reversed slightly but significantly in liraglutide-treated MetS group (Figure 6G).

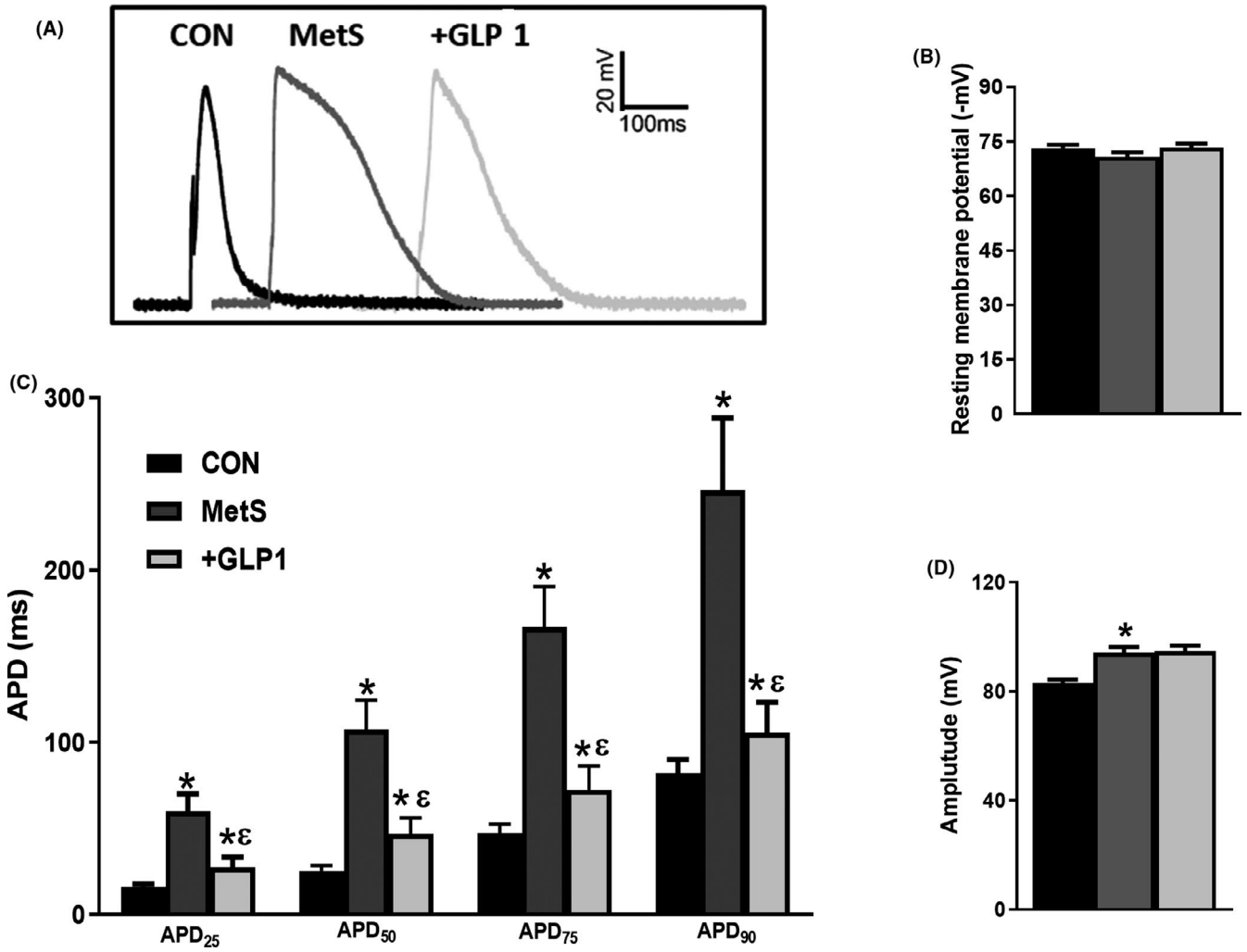

FIGURE 3 Effects of liraglutide treatment on action potential parameters in freshly isolated cardiomyocytes from metabolic syndrome rats. (A) Original action potential recordings in left ventricular cardiomyocytes from control (CON), metabolic syndrome (MetS), and liraglutide-treated MetS (+GLP-1) rats. The maximum amplitude of action potential (B), and the repolarisation phases of action potentials calculated at 25%, 50%, 75% and 90% (APD_{25}, APD_{50}, APD_{75}, APD_{90}) of repolarisations (C). (D) The resting membrane potentials of the groups. Values are presented as mean (±SEM) from at least 20 recordings from 20 cells/group. Significance levels at $^{*}p < 0.05$ *vs*. CON group and $^{\varepsilon}P < 0.05$ *vs*. MetS group

2.7 | Effect of liraglutide treatment of MetS rat on glucose regulation

We also examined the levels of insulin receptor substrate-1 (IRS1) and insulin-responsive glucose transporter (GLUT4) in the hearts of the MetS rats by using western blot analysis. As can be seen in Figure 7A,B, both protein levels were found to be significantly decreased in the MetS group compared to the control group, while the GLUT4 level, but not the IRS1 level, was reversed significantly with liraglutide treatment of the MetS rats.

In another set of experiments, we determined the casein kinase 2α (CK2α) protein level in the hearts of the rats. The CK2 level was determined to be significantly higher in the MetS group than the control group. Liraglutide treatment of the MetS rats induced a full recovery in the protein level of CK2α (Figure 7C).

3 | DISCUSSION

It is widely acknowledged that cardiovascular disease is the number one cause of death in humans worldwide who have either metabolic syndrome (MetS), obesity, or type 2 diabetes,. These pathological conditions are accepted as high risk factors for cardiometabolic disorders in the mammals. For this reason, it is important to implement optimal prevention and treatment strategies for patients with comorbidities. There is now an emerging interest to determine the potential cardiovascular benefits of novel drugs and/or chemical agents, as well as discover the novel actions of already established ones. Among them, GLP-1R stimulation is attracting a great deal of interest as a means by which to prevent the induction of cardiovascular diseases in patients with MetS, obesity, or diabetes.[32-36] Although several studies are focused on developing new strategies

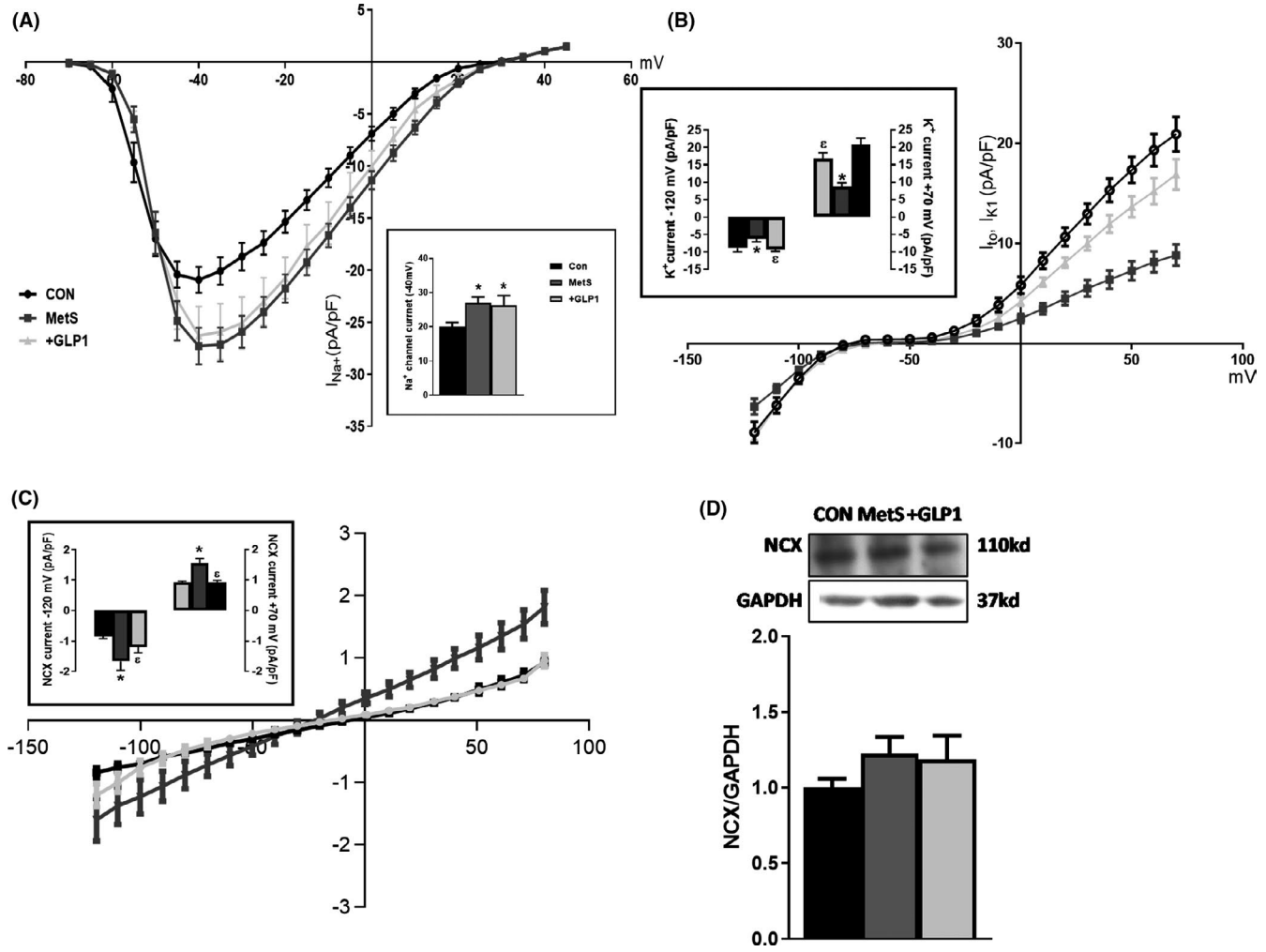

FIGURE 4 Effects of liraglutide treatment on ionic currents in freshly isolated left ventricular cardiomyocytes from metabolic syndrome rats. (A) The current–voltage characteristics (I–V) of the Na^+-channel currents were obtained by applying voltage steps and the maximum values of the currents at −40 mV depolarisation potential (inset). (B) The I–V characteristics of the K^+-channel currents by applying different voltage steps and the maximum responses obtained at −120 mV (maximum inward) and +70 mV (maximum outward) depolarisation potentials (inset). (C) The I–V characteristics of Na^+/Ca^{2+}-exchanger (NCX) current obtained from the ramp descending from +80 mV to −120 mV. The maximum responses at positive potentials obtained for NCX currents at +80 mV (right) and maximum responses at negative potentials obtained for NCX currents at −120 mV (left) are given in the inset. (D) The western-blot analysis of NCX. Electrophysiological studies were performed on at least 15 cells for each group. The groups: control (CON), metabolic syndrome (MetS), and liraglutide-treated MetS (+GLP-1). Significance level at $^*p < 0.05$ *vs*. Con group

in order to help people for the protection and/or treatment of their hearts under above pathological conditions, the most outcomes of them could not be expected levels and failed to induce health-heart function. Among these studies, GLP-1R agonists are the suggested strategy for the treatment, not only for patients with diabetes but also obese or insulin-resistant patients. Furthermore, documented data suggest that these agonists may benefit patients with heart disease without these above syndromes. Even though the positive effects of GLP1 analogue therapies on metabolic conditions could theoretically improve cardiovascular disease outcomes, little is known about the cardiovascular effects at cellular levels. Here, we, for the first time, demonstrated an important beneficial pleiotropic effect of GLP-1R agonist, liraglutide, on abnormal electrical activity of the MetS rat heart such as recovery in the long-QT and low heart rate. Our investigations at cellular levels demonstrated that these above recoveries at system level are, at most, due to the alleviations in both abnormal electrical activities (such as recoveries in action potentials and ionic currents) and intracellular Ca^{2+} of left ventricular cardiomyocytes as well as recoveries in mitochondrial dysfunction. Our present data demonstrate that reverses in both the depressed voltage-dependent K^+-channel currents and the increased Na^+/Ca^{2+}-exchanger currents in liraglutide-treated MetS rats can contribute to the recovery in action potentials of the cardiomyocytes, which, most probably, in turn, lead to shorting of the long-QT and then recovery in the low heart rate. Supporting data from beta-cells demonstrate that GLP-1R signalling can antagonise long repolarisation in β-cells by affecting the K^+-channel currents, through contributing to GLP-1 associated glucose-dependent insulinotropic effect.[37] Furthermore,

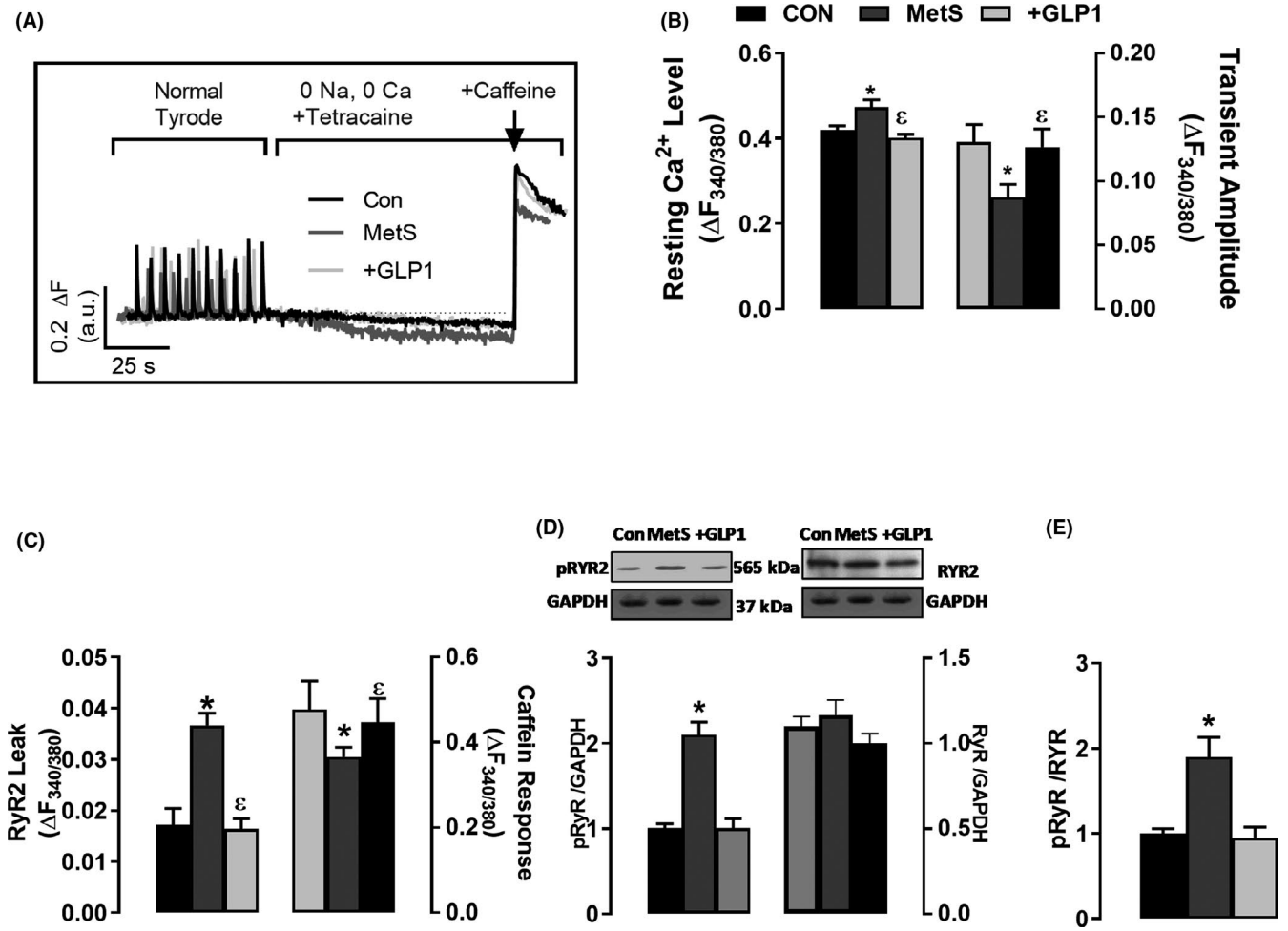

FIGURE 5 Effects of liraglutide treatment on sarcoplasmic reticulum Ca^{2+}-leakage in left ventricular cardiomyocytes from metabolic syndrome rats. (A) Representative protocols used to measure sarcoplasmic reticulum (SR) Ca^{2+}-leakage in groups of control (CON), metabolic syndrome (MetS), and liraglutide-treated MetS (+GLP1). (B) The resting level of cellular Ca^{2+} (left) and the maximum amplitude of transient Ca^{2+} releases from SR under electrical stimulation (right). (C) The calculated level of the Ca^{2+}-leakage (calculated with the application of 1 mmol/L tetracaine in 0 Na^{+}-0 Ca^{2+} solution via proportional to corresponding caffeine response; left) and the caffeine-induced Ca^{2+}-transients (right). (D) The western-blot analysis of phospho-ryanodine receptor levels (pRyR2; left) and protein level of RyR2 (right) for reference protein GAPDH. All data are presented as mean (±SEM). $^{*}p < 0.05$ vs. Con and $^{\varepsilon}P < 0.05$ *vs*. MetS group (number of cells in experiments as $n_{Con} = 8$, $n_{MetS} = 11$, $n_{+GLP1} = 13$)

in another study,[38] it has been shown that GLP-1R activation results in vagal afferent excitation, at least in part, due to an effect on the inactive K^{+}-channel currents. In studies on human patients, it has been demonstrated that those GLP-1R agonists (such as liraglutide) reduced blood pressure, being largely independent of weight loss, and increased heart rate.[39,40] In the present study, we demonstrated that 4-week liraglutide treatment of the MetS rats provided significant recoveries in not only high bodyweight and serum insulin level but also marked recoveries in high systolic and diastolic blood pressures. Furthermore, liraglutide treatment of the MetS rats provided slight but significant recoveries in the abnormal ECG parameters and low heart rate. In these concepts, there are some studies focused on to strength the beneficial effects of GLP-1R agonists, particularly, for the improvement of cardiovascular outcomes in patients with diabetes.[8] In these studies, the authors investigated the ventricular monophasic action potentials in anesthetised (urethane) rats in vivo and isolated perfused rat hearts during sinus rhythm and ventricular pacing with either systemic or acute administration of exendin-4. Their data demonstrated that despite causing sympathetic activation, that administration increased significantly action potential duration at 90% repolarisation during ventricular pacing with no significant effect on heart rate.

We also have demonstrated that the depressed contractile activity observed in the MetS rats[29] can be related to the increased level of basal Ca^{2+}, at least, through the hyperphosphorylation of cardiac ryanodine receptors and leaky sarcoplasmic reticulum (SR). This statement is strongly supported by our previous study performed in diabetic rat ventricular cardiomyocytes[41-45] and also other studies.[46-48] Indeed, as reviewed several recent and early articles, it has been shown that hyperglycaemia increases both cytosolic and mitochondrial reactive oxygen species in mammalian ventricular myocytes, which further leads to increases in both cytosolic and

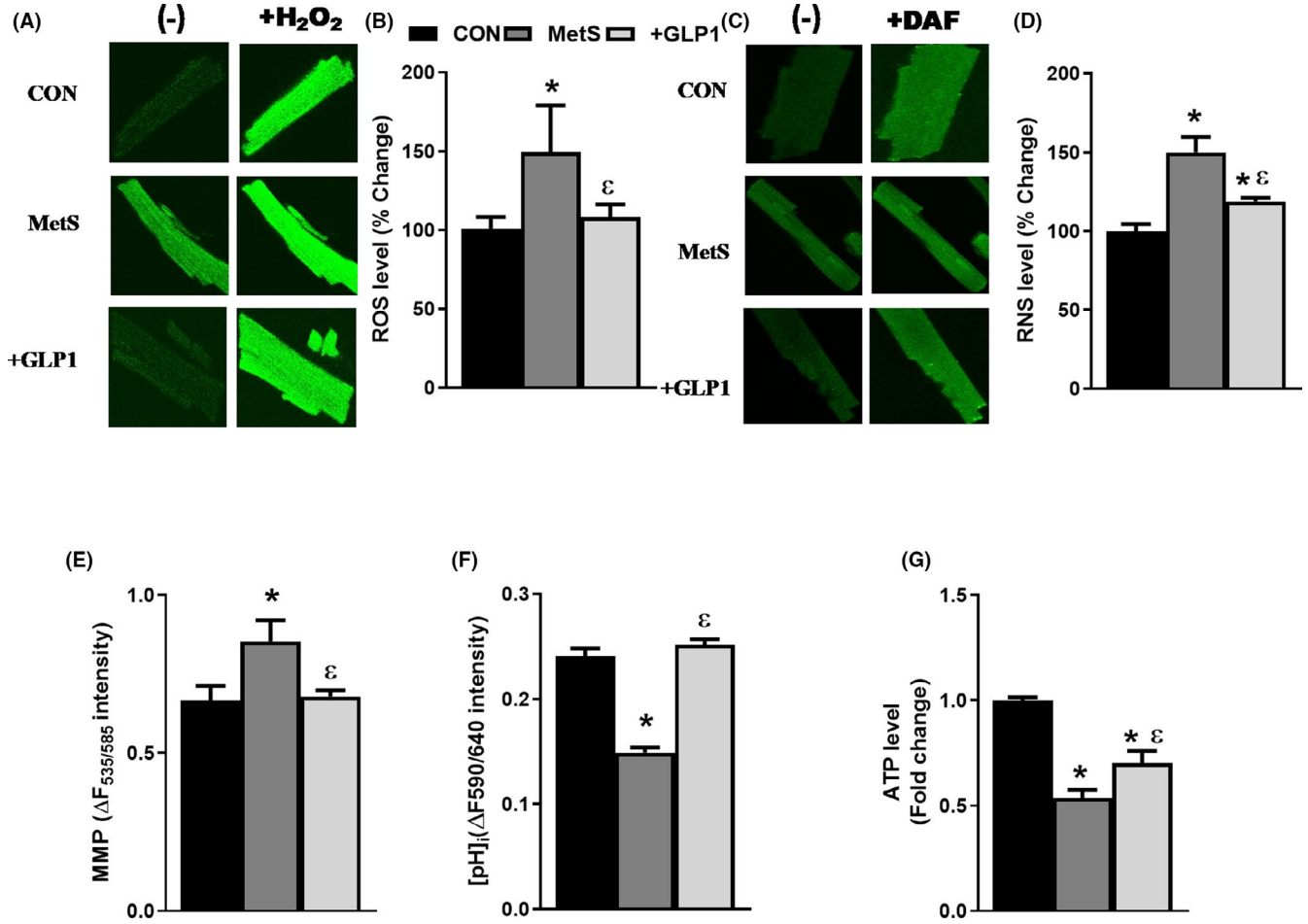

FIGURE 6 Effects of liraglutide treatment on mitochondria function in the cardiomyocytes from rats with metabolic syndrome. (A) Representative fluorescence intensity in isolated cardiomyocytes loaded with the ROS-sensitive dye DCDFA in the CON, MetS and +GLP1 group. (B) The calculated fluorescence intensity changes to present ROS level in isolated cardiomyocytes loaded with an oxidant-sensitive fluorescence dye DCDFA. (C) Representative fluorescence intensity in isolated cardiomyocytes loaded with the NO sensitive dye DAF in the CON, MetS and +GLP1 group. (D) The calculated fluorescence intensity changes to present RNS level in isolated cardiomyocytes loaded with NO-sensitive dye DAF. (E) The mitochondrial membrane potential (MMP) measurement was performed in isolated cardiomyocytes loaded with JC-1, by using a confocal microscope as the ratio of fluorescence intensity changes. (F) The presentation of cellular free H+-level or $[pH]_i$ in the isolated cardiomyocytes loaded with SNARF-1AM. (G) Determination of ATP level in isolated cardiomyocytes using a commercial kit. Significance levels at $^{*}p < 0.05$ *vs.* Con group and $^{\varepsilon}P < 0.05$ *vs.* MetS group

mitochondrial Ca^{2+} and increased oxidative stress at the cellular level.[42,49] These abnormalities then induce mitochondrial dysfunction, further leading to less ATP production. It has been also demonstrated a similar relation in diabetic rat ventricular cardiomyocytes, as well.[50] As discussed by Rowlands et al[51] GLP-1 analogues have important effects on cell signalling, metabolism, and function as well as cardiac tissue through direct receptor-mediated responses. Indeed, it is recognised that the classical response initiated by GLP-1R activation leads to facilitation of cardiac function through enhanced glucose uptake, and also overall cardiac function.[22,52-54] However, the mechanism through which GLP-1R agonists directly influence cardiac function under insulin resistance and metabolic disturbances has yet to be determined. In the light of previously published data, our present study provides valuable additional new data on the unanswered questions underlying the beneficial effects of GLP-1R agonists via their pleiotropic actions in the MetS rats. Supporting our present data, in another study performed in mice cardiomyocytes (GLP-1RCM−/−), pre-treatment with liraglutide could promote cardioprotection via reduction in the infarct size following ischaemia–reperfusion injury and increase the survival of these animals.[55] Thus, although there is an ongoing debate regarding whether the direct and/or indirect mechanisms underpinned the beneficial effects of GLP-1R agonism on cardiac dysfunction under hyperglycaemia and/or insulin resistance remain to be clarified, our present data may provide critical information in this field. Indeed, the literature reports that GLP-1R activation leads to the re-establishment of SR homeostasis, cytoprotection, and the restoration of signalling pathways disrupted by several pathological stimuli.[56,57] Furthermore, due to the direct actions of the GLP-1R agonists in the heart, it has been shown that liraglutide also prevents palmitate-induced lipotoxicity in isolated mice cardiomyocytes[56] and attenuate glucose toxicity-induced cardiac injury through the induction of mTOR-dependent

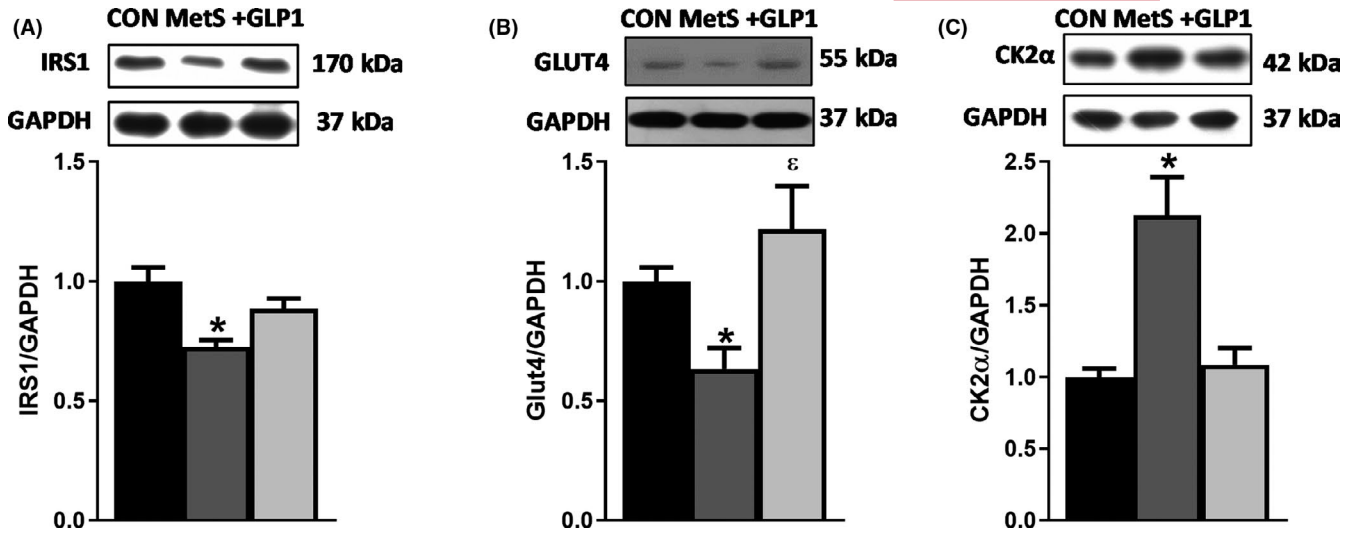

FIGURE 7 Effects of liraglutide treatment on cardiometabolic parameters in the rats with metabolic syndrome. Western blot analysis of protein expression levels of IRS-1 (A), GLUT4 (B), and CK2α (C) in the cardiomyocytes determined for a reference protein GAPDH. Significance level at $^{*}p < 0.05$ *vs*. Con group and $^{\varepsilon}P < 0.05$ *vs*. MetS group

autophagy.[33] These findings are further supported by other animal studies trials in which GLP-1R agonists improved the left ventricular function and altered the heart rate and blood pressure.[58,59]

Furthermore, it has been previously shown that hyperglycaemia can induce a depolarisation in mitochondrial membrane potential and opening of PTP channels, which further lead to increased production of ROS. Increased cytosolic ROS also leads to a decrease in GLUT4 production and lack of its plasma membrane localisation.[60] The data in these studies have shown important interactions between mitochondrial ROS and cellular glucose metabolism. In addition, ROS can affect the regulation of glucose uptake, such as transcriptional regulation of GLUT4 expression, regulation of GLUT4-vesicle translocation to the plasma membrane.[61] On the other hand, authors have demonstrated that RNS can be induced by hyperglycaemia in ventricular cardiomyocytes[29,42,60] and this increase seems to be associated with activation of β_3-adrenergic receptors in the hyperglycaemic heart.[62,63] Taking into consideration the relationship between ROS and RNS in the cytosol, there are interconnected pathways among these two parameters, which are affected by hyperglycaemia, and altogether they can affect the status of intracellular Ca^{2+}, and glucose metabolism by GLUT4 in ventricular cardiomyocytes from insulin-resistant hyperglycaemic MetS rats. Furthermore, there are more studies to show that not only liraglitude but also other GLP-1R receptors reduce ROS and RNS species under pathological conditions.[18,24,64]

In line with our data, studies showed that the GLP-1 analogue exenatide exerted cardioprotective effects in an in vitro model of heart failure and conclude that this cardioprotection may be due to the improvement of the mitochondrial function.[23] In another study, it has been also demonstrated an attenuation of cardiac remodelling and improvement of heart function after abdominal aortic constriction through blocking angiotensin II type 1 receptor in liraglutide-treated rats.[19] Other studies are also in line with our present data demonstrating the benefits of exendin-4 and liraglutide in alleviating oxidative stress in diabetic mice,[18] as well as other cellular signalling pathways.[65] In addition, it has been also mentioned that GLP-1R agonism stimulates mitochondrial ATP synthesis in pancreatic MIN6 β-cells,[66] reverses cardiac remodelling by normalising cardiac steatosis and oxidative stress in diabetes,[18,67] and prevents arterial stiffness, left ventricular myocardial deformation, and oxidative stress in subjects with newly diagnosed diabetes.[18,24] Furthermore, GLP-1R agonists have cardioprotective effects by improving mitochondrial membrane potential and reducing ROS.[33] Moreover, it has been reported that the GLP-1R agonist exendin-4 improves both systemic and cardiac insulin resistance, reducing the amount of ROS while also preventing mitochondrial dysfunction.[67]

Our study observed a significant increase in the protein level of GLUT4. This may be associated with decreases in the activity of protein kinase CK2α, which is highly activated under hyperglycemia.[42] Disturbances in Ca^{2+}-homeostasis are in part related to alterations in NCX, which is further associated with cardiovascular complications under pathological conditions, including MetS.[7,68] In hypoxia/reoxygenated H9C2 cells, liraglutide has been shown to prevent Ca^{2+}-overload and increase Ca^{2+} release under electrical stimulation.[69] It is widely acknowledged that NCX plays an important role in Ca^{2+}-homeostasis, particularly in Ca^{2+}-overload of ventricular cardiomyocytes.[70,71] In this regard, which, an increase of NCX gene expression level was observed in left ventricle of MetS humans with liraglutide treatment in parallel to the improvement in the cardiac function.[72] It has been shown that GLP-1 activates both the insulin signalling pathway including increases in GLUT4 level in insulin-resistant skeletal muscle cells.[73] Our present results demonstrate that liraglutide treatment of the MetS rats could significantly increase the depressed GLUT4 in the heart tissue. We also observed that liraglutide had a reducing effect on insulin resistance, both at the systemic and

heart level, similar to those of previously published studies in skeletal muscle cells.[74]

In conclusion, we demonstrate a molecular mechanism of how a GLP-1R agonist liraglutide treatment provides cardioprotection in MetS rats, providing data related to alleviations in electrical abnormalities, Ca^{2+}-homeostasis, and mitochondrial dysfunction in ventricular cardiomyocytes besides alleviation in the systemic parameters such as ECGs.

4 | MATERIALS AND METHODS

4.1 | Metabolic syndrome induction in rats

This study used 2-month-old Wistar rats (180–200 g) fed on standard rat diet and tap water. The experimental group (metabolic syndrome, MetS group) was prepared by adding 32% (935 mmol/L) sucrose in their tap water for 24 weeks. The rat diet was a standard local commercial diet and its composition contained (as a percentage) torula yeast 30.0%, corn oil 2.0%, sucrose 59.0%, DL-methionine 0.3% together with AIN-76 mineral, and vitamin mixture as 5.0% and 1.0%, respectively. The MetS model in rats was confirmed by parameters such as fasting blood glucose, body weight, serum insulin level, systolic–diastolic pressure and oral glucose tolerance test, as described previously.[28] After 24 weeks, the MetS group was randomly divided into two groups and GLP-1R agonist (liraglutide; 0.3 mg/kg) was administered to one group for an additional 4 weeks. Therefore, we had two MetS groups: liraglutide-treated MetS group and untreated MetS group.

4.2 | In situ electrocardiogram recordings

Electrograms (ECGs) were recorded under mild anaesthesia before the scarification of all rats. The ECG recordings are performed as described previously.[28] Briefly, three electrodes were placed on the paws of the animals and the signal was recorded for 10 min. The recordings were filtered with a bandpass (50–500 Hz) filter. Afterwards, the RR, QRS, and ST segments were analysed, and the heart rate was calculated from ECGs.

4.3 | Cardiomyocyte isolation

The hearts were removed from rats under mild anaesthesia (30 mg/kg sodium pentobarbital), then placed in a Ca^{2+}-free physiological solution, as described previously with some slight modifications.[75,76] Briefly, the heart was first perfused at 37°C with a HEPES-buffered solution containing (in mmol/L): NaCl 123, KC1 5.4, $NaHCO_3$, 5, NaH_2PO_4, 2, $MgCl_2$, 1.6, glucose 10, taurine 20, HEPES 20 and bubbled with 100% 0_2. The pH was adjusted to 7.1 with NaOH. After 5 min perfusion of the hearts, a fresh buffer supplemented with 1.2 mg/mL collagenase (Worthington collagenase type 2) was recirculated for 30–40 min with a speed of 8 mL/min. Following the collagenase perfusion, the ventricles were cut off and stirred slowly into the Ca^{2+}-free medium to disperse the myocytes. The cells were then suspended at 37°C in the HEPES-buffered solution while the Ca^{2+} level was increased in a graded manner to a concentration of 1 mmol/L. The isolated cardiomyocytes were kept in the HEPES-buffered solution at 37°C until used in the experiments. Six to seven billion cells were generally obtained with a 70%–80% yield of well-elongated cells following 1 mmol/L Ca^{2+} exposes. Dissociations with lower cell amounts and yields were not in the experiments. Examples of the isolated cardiomyocytes just after isolation and their viability following incubation with different time points are presented in the supplementary document.

4.4 | Electrophysiological measurements in isolated cardiomyocytes

The eletrophysiological parameters of the cardiomyocytes were recorded by using the Axoclamp patch-clamp amplifier (Axopatch 200B amplifier, Axon Instruments) at room temperature (23 ± 2°C). The cardiomyocytes were sampled and digitised at 5 kHz using an analogue-to-digital converter and software (Digidata 1200A and pCLAMP 10.0; Axon Instruments).

Action potential recordings were performed in freshly isolated left ventricular cardiomyocytes as described previously.[28] Briefly, the action potential recordings were performed at a frequency of 0.5 Hz by using 1.5–2.5 MΩ electrode resistance. In the current-clamping configuration, membrane potential changes were obtained by stimulating the cells by injecting small depolarising pulses (current in 5 nA with 4 ms duration). Resting membrane potentials, maximum depolarisation potentials, and repolarisation times of 25, 50, 75, 90 ($APD_{25,\ 50,\ 75,\ 90}$) were calculated from the original action potential traces.

Voltage-dependent Na^+-channel currents (I_{Na}) recordings were performed in the whole-cell configuration of the voltage clamping. These recordings were made using 1.5–2.5 MΩ patch-electrodes. After the cells were kept as a membrane potential at −80 mV level and the Ca^{2+}-channels were blocked with Co^{2+}, they were recorded with depolarising increases of 5 mV and 150 ms from −70 mV potential.

Voltage-dependent K^+-channel currents (I_K) recordings were performed by using 1.5–2.5 MΩ patch-electrodes in a whole-cell voltage clamping configuration. After clamping and applying pre-pulse protocol and Co^{2+} to block Na^+-and Ca^{2+}-currents, 500 ms pulses were applied 20 times at 5 s intervals to the cells whose membrane potential was kept at −70 mV.

Voltage-dependent Na^+/Ca^{2+}-exchanger (NCX) currents (I_{NCX}) were performed as described previously.[77] I_{NCX} was recorded using an electrode with an internal solution containing (in mmol/L): CsCl 65, $CaCl_2$ 10.92, EGTA 20, HEPES 10, MgATP 5, $MgCl_2$ 0.5, TEA-Cl 20 at pH 7.2. The extracellular bathing solution contained in mmol/L: NaCl 130, TEA-Cl 10, Na-HEPES 11.8, $MgCl_2$ 0.5, $CaCl_2$ 1.8, ryanodine 0.005, nifedipine 0.02, glucose 10 at pH 7.4. The total NCX

current (both inward and outward), I_{NCX}, was obtained from protocol composed of descending ramp from +80 to −120 mV at 0.1 mV/ms while the holding potential was −40 mV.

4.5 | Intracellular Ca^{2+}-measurements

Isolated cardiomyocytes were incubated with Fura-2AM (4-μmol/L, ratiometric fluorescence dye) for 45 min at room temperature (20–25°C) in Tyrode's solution (content in mM; 137 NaCl, 4 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 10 HEPES and 10 glucose and pH 7.4). Intracellular basal and Ca^{2+} transients (under electric-field stimulation) were measured by applying a 25 V square pulse at room temperature, stimulating at a frequency of 0.2 Hz for 10 ms. Fluorescence measurements were performed using a microspectrofluometer and FELIX software (Photon Technology International, Inc) with an emission of 520 nm by stimulating at wavelengths of 340–380 nm. Sarcoplasmic reticulum leakage was measured in the presence of tetracaine by using Na^+ and Ca^{2+} free solution.[78]

4.6 | Measurement of intracellular free H^+ level

The level of intracellular free H^+ (or $[pH]_i$) in cells was determined using acetoxymethyl ester (AM) dye of seminafarhodaflor (SNARF-1) dye. Cardiomyocytes were loaded with 3 μmol/L SNARF1 for 30 min and then stimulated with a He/Ne laser (543 nmol/L). Relative $[pH]_i$ values were examined by calculating the ratio of the two excitation intensities (580/690).

4.7 | Intracellular ROS and RNS

In isolated cardiomyocytes, reactive oxygen species (ROS) were measured using a fluorescent dye DCDFA (10 μmol/L) under a confocal microscope (Leica TCS SP5). Cells were incubated at room temperature for 45 min, then stimulated at 490-nm wavelengths after 520-nm emission was recorded. After the basal response was obtained from the cells, the maximum ROS formation was observed by giving 100 μmol/L H_2O_2 and the value was calculated, indirectly.

DAF-AM (10 μmol/L) dye was used for measurements of reactive nitrogen species (RNS) in cells. After the cells were incubated for 60 min at 37°C, the basal response was obtained with a confocal microscope (Leica TCS SP5). DAF-FM was excited at 488-nm and emission was collected at 520 nm. Then, the maximum state was observed by giving ZipNONO (100-μmol/L) a special NO-donor.

4.8 | Mitochondrial membrane potential

Isolated cells were incubated at room temperature for 30-min using JC-1 dye (cell persistent, 5 μmol/L). The prepared cells were stimulated at 488-nm using a laser scanning confocal microscope (Leica TCS SP5) and the red fluorescence image was detected at both 535 nm and 585 nm. Carbonyl cyanide 4 (trifluoromethoxy) phenylhydrazone (FCCP; 5-μmol/L) was used for calibration.

4.9 | Cellular ATP level

The cellular ATP level of the cardiomyocytes was measured using a Luminescent cell viability assay kit (Promega CellTiter-Glo 2.0, G9241). Equal amounts of cardiomyocytes were added to 96 well plates together with the CellTiter-Glo 2.0 Reagent and incubated for 10-min at room temperature. Luminescence signals were measured using a microplate reader.

4.10 | Western blot analysis

Western blotting experiments were performed using isolated cardiomyocytes. Isolated cells were first homogenised using RIPA buffer and the supernatant was centrifuged at 10,000 × g for 15 min. Following this, protein concentration was determined by using the Bradford method. Equal quantities of proteins were run using SDS-polyacrylamide gel and transferred to the polyvinylidene difluoride membrane. The membrane was incubated with bovine serum albumin for 3 h and then incubated with primary antibody pRYR (A010-32 1:1,000 Badrilla), RYR (sc-8170 1:200; Santa Cruz), NCX (sc-32881 1:500; Santa Cruz), IRS1 (sc-8038 1:400; Santa Cruz), GLUT4 (sc-1607 1:400; Santa Cruz), CK2α (sc-12738 1:400; Santa Cruz) in 3% BSA. For quantitative purposes, GAPDH (sc-365062 1:1,000; Santa Cruz) was used. Then incubated with secondary antibodies (anti-mouse 1:2,000, anti-goat 1:7,500, anti-rabbit 1:7,500) and visualised with chemiluminescent reaction using ECL (Amersham Pharmacia). ImageJ software was used to determine band density.

4.11 | Statistical analysis and chemical reagents

Chemicals were obtained from Sigma-Aldrich unless otherwise stated. Data were presented as mean ± SEM with GraphPad Prism 6.0 (GraphPad Software, Inc). Comparisons between quantitative variables were assessed using either the Student's *t*-test or one-way ANOVA at a 0.05 level of significance.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that influenced the work reported in this paper.

AUTHOR CONTRIBUTIONS

Aysegul Durak: Investigation, Methodology, Validation, Data curation, Formal analysis, Resources. Erman Akkus: Methodology, Data curation, Formal analysis, Resources. Asena Gokcay Canpolat: Resources, Validation, Data curation. Erkan Tuncay: Investigation, Methodology, Validation, Data curation. Demet Corapcioglu: Resources, Supervision, Writing - review & editing. Belma Turan: Conceptualisation, Methodology, Project administration, Supervision, Validation, Visualisation, Funding acquisition, Writing - original draft, Writing - review & editing.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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