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Ryanodine Receptor Phosphorylation by CaMKII Promotes Spontaneous Ca^{2+} Release Events in a Rodent Model of Early Stage Diabetes: the Arrhythmogenic Substrate

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Abstract

Background—Heart failure and arrhythmias occur more frequently in patients with type 2 diabetes (T2DM) than in the general population. T2DM is preceded by a prediabetic condition marked by elevated reactive oxygen species (ROS) and subclinical cardiovascular defects. Although multifunctional Ca^{2+} calmodulin-dependent protein kinase II (CaMKII) is ROS-activated and CaMKII hyperactivity promotes cardiac diseases, a link between prediabetes and CaMKII in the heart is unprecedented.

Objectives—to prove the hypothesis that increased ROS and CaMKII activity contribute to heart failure and arrhythmogenic mechanisms in early stage diabetes.

Methods-Results—Echocardiography, electrocardiography, biochemical and intracellular Ca^{2+} (Ca^{2+}_i) determinations were performed in fructose-rich diet -induced impaired glucose tolerance, a prediabetes model, in rodents. Fructose-rich diet rats showed decreased contractility and hypertrophy associated with increased CaMKII activity, ROS production, oxidized CaMKII and enhanced CaMKII-dependent ryanodine receptor (RyR2) phosphorylation compared to rats fed with control diet. Isolated cardiomyocytes from fructose-rich diet showed increased spontaneous Ca^{2+}_i release events associated with spontaneous contractions, which were prevented by KN-93, a CaMKII inhibitor, or addition of Tempol, a ROS scavenger, to the diet. Moreover, fructose-rich

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Disclosure Statement

None

All authors have approved the final article

diet myocytes showed increased diastolic Ca^{2+} during the burst of spontaneous $Ca^{2+}{}_i$ release events. Micetreated with Tempol or with sarcoplasmic reticulum-targeted CaMKII-inhibition by transgenic expression of the CaMKII inhibitory peptide AIP, were protected from fructose-rich diet-induced spontaneous $Ca^{2+}{}_i$ release events, spontaneous contractions and arrhythmogenes is *in vivo*, despite ROS increases.

Conclusions—RyR2 phosphorylation by ROS-activated CaMKII, contributes to impaired glucose tolerance-induced arrhythmogenic mechanisms, suggesting that CaMKII inhibition could prevent prediabetic cardiovascular complications and/or evolution.

Keywords

arrhythmias; prediabetes; impaired glucose tolerance; CaMKII; ryanodine receptor

1. Introduction

Type 2 diabetes mellitus (T2DM) is a chronic and progressive disease that represents a serious Public Health problem worldwide due to its high mortality, morbidity and cost[1–3]. The rapid growth of T2DM is associated with high calorie diets and sedentary habits[4] of modern societies. In the USA the consumption of fructose rich syrups exceeds the changes in intake of any other food contributing to nearly 40% of the caloric intake[5]. Based on these records, some authors suggested that this increase in fructose consumption has contributed to the current epidemic of obesity and T2DM[6]. Furthermore, there is evidence that fructose-rich diet promotes glucooxidative stress[7]. In most cases of T2DM, metabolic defects precede overt clinical disease. These metabolic derangements include impaired glucose tolerance and impaired fasting glucose. The presence of these alterations defines the prediabetic state[3, 8]. Importantly, cardiovascular disease constitutes the leading cause of death in patients with diabetes and prediabetes[9–11]. Although the published data are limited, there is evidence that prediabetes and borderline diabetes associate with an increased risk of sudden cardiac death possibly due to cardiac arrhythmias[11, 12]. Moreover, increased arrhythmogenic risk is present in young people with diabetes even in the absence of detectable systolic dysfunction[13], suggesting that there is an early increased propensity to arrhythmias in T2DM. Thus, improved understanding of pro-arrhythmogenic mechanisms in prediabetes could lead to new approaches for preventing cardiovascular injury and sudden death.

Arrhythmias can be triggered by early (EADs) or delayed after depolarizations (DADs). EADs are membrane depolarizations that appear before action potential (AP) repolarization. It is generally accepted that EADs arise from current flowing through L-type Ca^{2+} channels[14]. In contrast, DADs occur following AP repolarization and have been associated to the higher frequency of intracellular Ca^{2+} Sparks (CaSp). CaSp can trigger a Ca^{2+}-activated transient-inward current, which is produced by the electrogenic Na^{+}-Ca^{2+} exchanger (NCX) working in the forward mode[15].

The frequency of CaSp depends on two main factors: the sarcoplasmic reticulum (SR) Ca^{2+} load and the sensitivity of ryanodine receptor (RyR2) to Ca^{2+} activation[16]. RyR2 dysregulation could result from different factors, including increases in the phosphorylation

at Ser^{2808}, a validated protein kinase A (PKA) site and/or Ser^{2814}, a validated Ca^{2+}-calmodulin dependent kinese II (CaMKII) site[17], redox modifications[18] and/or site receptor mutations[19]. In diabetes there is an increase in CaMKII activity, which has been associated with increased mortality after myocardial infarction[20]. However, the activity and potential role of CaMKII in impaired glucose tolerance has never been explored.

Fructose-rich diet is a well characterized model of impaired glucose tolerance[21]. High fructose diet disturbs metabolic homeostasis, increases plasma fructose, and induces systemic insulin resistance (IR). In the liver, fructose is metabolized into glyceraldehyde and dihydroxyacetone phosphate. These particular fructose end products can then readily converge with the glycolytic pathway. Of keyimportance is the ability of fructose to by-pass the main regulatory step of glycolysis, the conversion of glucose-6-phosphate to fructose 1,6-bisphosphate, controlled by phosphofructokinase. Thus, while glucose metabolism is *negatively* regulated by phosphofructokinase, fructose can *continuously* enter the glycolytic pathway. Therefore, fructose can uncontrollably produce glucose, glycogen, lactate, and pyruvate, providing both the glycerol and acyl portions of acyl-glycerol molecules. These particular substrates, and the resultant excess energy flux due to unregulated fructose metabolism, will promote the overproduction of triglycerides (reviewed in [22]), which in turn contributes to reduced insulin sensitivity and hepatic insulin resistance/glucose intolerance.

Here we used the fructose rich diet model to test the hypothesis that reactive oxygen species (ROS) and CaMKII contribute to proarrhythmic mechanisms in prediabetes by RyR2 hyperphoshporylation and disturbed intracellular Ca^{2+} homeostasis. Detection and recognition of the initial underling pathways that determine the arrhythmogenic substrates in prediabetes could enable the development of new strategies to either preventor diminish heart failure and arrhythmias associated with T2DM.

2. Methods

An expanded Methods section is available in the online Data Supplement.

2.1. Animals and protocols

All experiments were performed in accordance with the Guide for Care and Use of Laboratory Animal (NIH Publication No.85-23, revised 1996) and approved by the Ethics committee of the Faculty of Medicine, La Plata, Argentina). Experiments were performed in male Wistar rats (200 to 300g). Animals were divided into two groups: control diet and fructose-rich diet group which received the same diet plus 10% (W/V) fructose.

Wild type (WT) and transgenic male mice with cardiomyocyte-delimited transgenic expression of SR-targeted CaMKII inhibitor AIP (SR-AIP)[23], were divided into three groups: 1- control group; 2- fructose group (10% w/v fructose); and 3-Fructose+Tempol (10% w/v fructose plus 0.8 mM 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl). Tempol, was used as antioxidant. All treatments were performed for three weeks in the drinking water.

2.2. Plasma determinations

Blood samples were used to assess plasma glucose level and intraperitoneal tolerance test to glucose. Triglyceride (commercial kit) and insulin plasma (RIA) levels were also assessed. Insulin resistance was assessed with the HOMA-IR index, calculated as insulin (μU/ml) x glucose (mmol/L)/22.5[24].

A significant difference on the area under the curve of the intraperitoneal tolerance test to glucose between control diet and fructose rich diet animals was considered an impaired glucose tolerance status.

2.3. Echocardiographic Examination

Echocardiogram was performed in rats under light anesthesia (35 mg/kg sodium pentobarbital ip).

2.4. Electrocardiogram

Surface ECG were recorded in mice without anesthesia, for 30 min, using standard ECG electrodes (lead I) and a Power Lab 4ST data acquisition system. A catecholamine challenge [caffeine and epinephrine, 120 mg/kg and 1.6 mg/Kg,], was applied to unmask the presence of arrhythmias under stress conditions. Ventricular ectopic beats (premature or ventricular escape beats), ventricular fibrillation and ventricular tachycardia were quantifying during the recording period.

2.5. Myocyte Isolation

Before the sacrifice, the anesthetic and analgesic drugs used were 75mg/Kg of ketamine and 5mg/Kg of diazepam, respectively. The plane three of phase III of anesthesia was verified by loss of corneal reflex and the appearance of slow deep diaphragmatic breathing. Myocytes were isolated by enzymatic digestion as previously described[25].

2.6. Ca^{2+}_i and Cell Shortening

Rat cardiac isolated myocytes were loaded with Fura-2/AM and Ca^{2+}_i fluorescence, was measured in an IonOptix setup (Milton, MA). SR Ca^{2+} load was assessed with a caffeine pulse (25 mM). Fluorescence data were stored for an off-line analysis (ION WIZARD fluorescence analysis software).

2.7. Confocal imaging of intact cardiac myocytes

Confocal images of Ca^{2+} in cells loaded with Fluo-3AM were taken in line scan mode to assess Ca^{2+} transients, sparks, waves and spontaneous contractile activity. Sparks and waves were analyzed in quiescent cells and after 5 min stimulation at 0.5 Hz.

2.8. ROS Determinations

Superoxide Anion ($\cdot O_2^-$) Production—Anion superoxide ($\cdot O_2^-$) production was measured in cardiac tissue slices from the left ventricle after 20 min incubation with lucigenin (Bis-N-metilacridinium) 5 μmol/L- enhanced chemiluminescence method, in a luminometer (Chameleon, Hidex).

Lipid Peroxidation—Lipid peroxidation was determined by measuring the rate of production of thiobarbituric acid reactive substances (TBARS), expressed as nmol/mg protein.

2.9. Western Blot

Hearts were freeze-clamped, pulverized and homogenized. Protein was measured by the Bradford method using BSA as standard. Lysates were subjected to SDS-PAGE[26]. After western-blotting, membranes were probed overnight with specific antibodies.

2.10. Statistics

Continuous variables were expressed as mean ± SEM and were evaluated with either unpaired Student t test or ANOVA followed by Tukey's Post Hoc test, when comparison among different groups was performed. Spontaneous Ca^{2+} release events were expressed as nonparametric continuous data (individual data in values plots). Whenever the distribution was skewed, the Mann-Whitney test was used to compare these data. A p value <0.05 was considered significant.

3. Results

3.1. Characterization of fructose-rich diet model

Fructose-rich diet animals showed increased serum levels of insulin, triglycerides, non-esterified fatty acids (NEFA) levels, the area under the curve of serum glucose after the intraperitoneal glucose administration and insulin resistance, as shown by the higher values of the homeostatic model assessment(HOMA-IR[24]), compared to control diet rats (Table S1). These data are consistent with an insulin-resistance pattern. In contrast, fasting glucose levels and body weights were similar in control diet and fructose-rich diet rats, indicating a prediabetic state rather than an established T2DM. Table S2 depicts the food, water and caloric intake in both groups of rats.

Prediabetic fructose-rich diet rats have cardiac hypertrophy and systolic dysfunction as shown in Table S3 and Fig 1. Systolic blood pressure was similar (p=0.70) in control diet(118±2 mmHg) and fructose-rich diet rats (115±4 mm Hg), indicating that alterations of cardiac structure and function in fructose-rich diet rats were independent of blood pressure.

3.2. Fructose-rich diet increases spontaneous Ca^{2+}_i release events

The findings observed at the whole animal level, prompted us to study whether Ca^{2+} handling was altered at the cellular level. The protocol to study myocardial Ca^{2+}_i handling is depicted in Fig 2A.

Under non-stressed conditions (without Isoproterenol, Iso), cardiomyocytes isolated from fructose-rich diet rats showed a significantly higher number of spontaneous Ca^{2+}_i release events (Fig 2A-D) that correlated with spontaneous contractions (Fig. S1) associated with a shorter latency period (Fig 2E) compared to control dietmyocytes. Of note, fructose-rich diet but not control diet myocytes showed dyssynchronous Ca^{2+} release events between paced beats (Fig 2B-C). I so enhanced the number of spontaneous Ca^{2+}_i release events (Fig 2D)

and decreased the latency period (Fig 2E). These findings suggest that fructose-rich diet rats have defective, proarrhythmic Ca^{2+}_i handling patterns.

We next used confocal microscopy to measure local SR Ca^{2+} release events (CaSp) and Ca^{2+} waves in intact isolated rat cardiomyocytes under the same experimental protocol conditions of Fig 2A. At baseline, CaSp frequency, width (FWHM) and amplitude were not different between cardiomyocytes isolated from fructose-rich diet or control diet rats. In contrast, fructose-rich diet cardiomyocytes showed significantly increased Ca^{2+} wave frequency and spontaneous contractions compared to control diet myocytes (Fig 3A-B, and Table S3). I so did not affect CaSp frequency in control diet or fructose-rich diet cells, but significantly increased wave frequency and spontaneous contractions in fructose-rich diet cardiomyocytes (Fig 3A-B). These results suggest that the increase in Ca^{2+}_iwave frequency in fructose-rich diet hearts was sufficient to trigger spontaneous contractile activity, and that myocardial defects in rats with fructose-rich diet-induced prediabetic state are a consequence of a loss of Ca^{2+}_i homeostasis.

3.3. CaMKII mediates spontaneous Ca^{2+}_i release events in prediabetic cardiomyocytes

CaMKII over-expression and hyperactivation enhances SR Ca^{2+} leak, which can serve as a molecular trigger for arrhythmias[27–29]. To investigate a possible role of CaMKII in fructose-rich diet-induced spontaneous activity, we first asked if CaMKII was activated in hearts from fructose-rich diet animals. Fig 4A, shows that a constitutively active, auto phosphorylated form of CaMKII (P-CaMKII)[30] and phosphorylation of a CaMKII-catalyzed target site on phospholamban (PLN), Thr^{17}[26], were increased in fructose-rich diet compared to control rat hearts, with no changes in total proteins expression. In a second group of experiments, we aimed to determine whether spontaneous Ca^{2+}_i release events in myocytes from fructose-rich diet rat hearts could be prevented or reduced in the presence of an experimental CaMKII inhibitor, KN-93. Fig 4B-C shows that KN-93preventedthe increase in spontaneous Ca^{2+}_i release events and spontaneous contractions in cardiomyocytes from fructose-rich diet rats under basal and I so-stimulated conditions. In contrast, KN-92, an inactive an a log of KN-93, failed to prevent spontaneous Ca^{2+}_i release events in these cardiomyocytes.

3.4. Mechanisms of CaMKII-induced spontaneous Ca^{2+}_i release events and contractile activity in prediabetic hearts

Because CaSp and Ca^{2+}_i waves can occur due to SR Ca^{2+} overload[16] or as a consequence of an alteration in the function of RyR2[31], we performed studies to determine how these mechanisms could contribute to the generation of increased Ca^{2+}_i waves. It was found that caffeine induced Ca^{2+}_i transients (Ca_iT) were significantly decreased in fructose-rich diet with respect to control diet cells (Fig 5), which is in line with the increase in SR Ca^{2+} leak in LV cardiomyocytes (Fig 3). Of note, the decrease in SR Ca^{2+} content occurs in fructose-rich diet myocytes in spite of the increase in SERCA2a activity (Fig 5B, lower panel), which would tend to increase SR Ca^{2+} load. This increase, which may be due to the CaMKII-dependent increase in Thr^{17} phosphorylation of PLN, would contribute to avoid a further decrease in SR Ca^{2+} load. Moreover, although, diastolic Ca^{2+} did not change in steady state conditions (Fig 5B, middle panel), it greatly increases when spontaneous Ca^{2+}_i release

events occur (Fig S2 and see below). Thus, diastolic Ca^{2+} during spontaneous Ca^{2+}_i release events was greatly increased in fructose-rich diet than in control diet rats(Fig 5B middle panel, stripped bars). Moreover, the amplitude of stimulated Ca_iT was similar between control diet and fructose-rich diet animals. This indicates that fractional Ca^{2+} release (ratio of twitch/caffeine-induced Ca^{2+} transient), was significantly increased in fructose-rich diet with respect to control diet cells, as shown in Fig 5A-B. Together the results suggest that the increase in spontaneous activity- as well as the enhanced fractional Ca^{2+} release-, is associated to an alteration of RyR2 rather than to SR Ca^{2+} overload.

Notably, cell shortening showed a significant increase in fructose-rich diet vs. control diet myocytes, in spite of the similar Ca_iT, a finding that is in line with an increase in myofilament Ca^{2+} sensitivity, as previously described[32]. This finding is in contrast to the ones obtained in the echocardiographic studies, where a systolic dysfunction was observed (Fig 1 and Table S1). The cause for the discrepancy is not apparent to us and requires further investigation. Possible explanations to these findings are alterations at the tissue and whole organ level (i.e, interstitial fibrosis, apoptosis, vascular compromise), which we did not assess in this study and are not present in isolated myocytes. For instance, sustained hyperglycemia may increase glycation of interstitial proteins such as collagen, which results in myocardial stiffness and impaired contractility[33, 34].

Since it has been shown that CaMKII-dependent phosphorylation of RyR2 increases SR Ca^{2+} leak, CaSpF and arrhythmias[35], we next sought to examine the phosphorylation status of RyR2. Fig 5C shows that there is an increase in RyR2 Ser^{2814} phosphorylation in fructose-rich diet relative to control diet rats without changes in RyR2 expression. In contrast, phosphorylation of a PKA-catalyzed phosphorylation site, Ser^{2808}, was not altered in prediabetic compared with control hearts. Interestingly, the enhanced fractional SR Ca^{2+} release observed in fructose-rich diet cardiomyocytes supports the notion that RyR2 phosphorylation at Ser^{2814} site activates both diastolic and systolic RyR2 Ca^{2+} release[36, 37].

Taken together, our results are consistent with a concept where the presence of a proarrhythmogenic substrate is underlying by CaMKII phosphorylation of RyR2 in fructose-rich diet rats.

3.5. Mechanism of CaMKII activation and RyR2 destabilization

CaMKII is activated by an increase in Ca^{2+}/calmodulin. As mentioned above, there was a significant increase in spontaneous Ca^{2+}_i release events in fructose-rich diet rats with respects to control animals, both with or without electrical stimulation. Spontaneous Ca^{2+}_i release events usually appear as a burst of spontaneous events associated with an increase in diastolic Ca^{2+} (See Fig 5B, middle panel, stripped bar). Fig S1 shows a typical example of a burst of spontaneous Ca^{2+}_i release events in fructose-rich diet rats associated with the increase in diastolic and systolic Ca^{2+}. In contrast, spontaneous Ca^{2+}_i release events are sporadic in control diet animals and failed to significantly increase diastolic Ca^{2+}. Moreover, the increase in cytosolic Ca^{2+} that occurred during spontaneous Ca^{2+}_i release events, -estimated by the integral of these events during the 3 min period prior to electrical stimulation-, was also significantly increased by 3.14 ± 1.4 % ($p< 0.05$). Taken together the

results reveal a temporary increase in intracellular Ca^{2+} in fructose-rich diet rat myocytes with respect to cells of non-treated animals. Moreover, previous experiments have demonstrated that CaMKII can maintain its activity by ROS[38], even at sub diastolic Ca^{2+} levels[25] and that ROS-induced arrhythmias were linked to the ability of ROS to activate CaMKII[39]. Thus, we tested the possibility that an increase in ROS production may contribute to the increased in CaMKII sustained activity of fructose-rich diet myocytes. Fig 6A-B shows that lipid peroxidation (determined by TBARS) and ROS production (O_2^-) were significantly enhanced in fructose-rich diet animals. We next measured oxidized CaMKII (ox-CaMKII) in fructose-rich and control diet rats. Fig 6C depicts typical western blots and overall results showing that the hypothesized increase in ox-CaMKII, indeed occurred. These results indicate that ROS production and ox-CaMKII are enhanced in fructose treated animals, confirming the possibility that ROS contributes to CaMKII activity in fructose-rich diet myocytes.

To further investigate the possible role of ROS and CaMKII in the arrhythmogenic pattern observed in the fructose-rich diet model, we next examined fructose-rich and control diet WT and SR-AIP transgenic mice, as a genetic tool to confirm the results obtained in rat. SR-AIP mice have myocardial-delimited transgenic expression of the CaMKII inhibitory peptide AIP targeted to SR membranes by fusion with the PLN transmembrane domain[23]. As shown in the Table S1, fructose-rich diet WT and SR-AIP mice present significant increases in the area under the curve of the intraperitoneal tolerance test to glucose, indicating a prediabetic condition similar to fructose-rich diet rats. Fig 7A shows that fructose-rich diet WT mice had significantly (p=0.02) increased oxidative stress (TBARS) compared to control diet mice. To explore the possible participation of ROS in the spontaneous Ca^{2+}_i release activity evoked by fructose treatment in WT mice we used WT fructose-rich diet mice treated with the intracellular ROS scavenger Tempol. Co-treatment of WT fructose-rich diet mice with Tempol prevented the increase in TBARS and in CaMKII phosphorylation of RyR2 (Fig 7A-B). Moreover, the spontaneous events produced by fructose treatment were also prevented by Tempol (Fig 7C-D). Taking together the results suggest that ROS are involved in the spontaneous activity of fructose-rich diet animals through CaMKII-dependent phosphorylation of RyR2. To further explore this point, we performed experiments in mice with CaMKII inhibition at the SR level (SR-AIP mice). Fig 7E shows that SR-AIP fructose-rich diet mice had also a significant increase in oxidative stress compared to SR-AIP control diet mice (p<0.05), which was prevented by co-treatment with Tempol. Tempol treatment also significantly decreased ox-CaMKII in SR-AIP mice treated with fructose-rich diet to 75.04±7.34 %. However, these mice did not exhibit neither CaMKII-RyR2 phosphorylation nor spontaneous Ca^{2+} release events, either in the absence or presence of Tempol (Fig 7F-H). Lastly, we performed an additional series of experiments in isolated myocytes previously treated with H_2O_2 in the presence and absence of KN-93. It was found that the CaMKII inhibitor was able to prevent the arrhythmic events observed in the presence of H_2O_2 (Fig S3). Taken together, these results indicate that the increase in oxidative stress is an upstream event in a pathway that leads to oxidative CaMKII activation and proarrhythmic changes in Ca^{2+}_i handing induced by fructose treatment.

Finally, we performed electrocardiography (ECG) in the conscious intact animal for 30 min and measured spontaneous Ca^{2+}_i release events and spontaneous contractions in

cardiomyocytes by confocal microscopy. WT littermate control diet mice showed normal sinus rhythm, evidenced from continuous *in vivo* ECG measurements, opposite to fructose-rich diet WT mice which showed significant bradycardia (Table S4A and Fig 8A*i-ii*). Moreover, fructose-rich diet WT mice transiently exhibited at least one of the following ECG alterations, AF, AV block, bidirectional ventricular tachycardia, ventricular ectopic beats, sustained ventricular tachycardia and ventricular fibrillation after caffeine + epinephrine challenge (Table S4B and Fig 8A*iii-v*). All these arrhythmic events, were absent in fructose-rich diet WT co-treated with Tempol (Table S4B) and in fructose-rich diet-SR-AIP mice (Fig 8B). Fig 8C-E and Table S3 show that fructose-rich diet WT mice presented a significantly higher CaSpF, $Ca^{2+}{}_i$ waves and spontaneous contraction activity than control diet WT, SR-AIP and fructose-rich diet SR-AIP mice. Taken together, these results indicate that CaMKII actions at the SR are likely to contribute to the arrhythmic pattern observed in prediabetic mice.

4. Discussion

The prevalence of impaired glucose tolerance is growing worldwide[40] with the consequent increase in the risk of developing T2DM and cardiovascular disease. Impaired glucose tolerance has been shown to be a precursor of diabetes and usually coexist with an insulin resistance state, with higher risk for cardiovascular morbidity and mortality. The present results showed that ventricular myocytes from fructose-rich diet animals develop cardiac arrhythmogenic events. Our results indicate that CaMKII-dependent phosphorylation of RyR2 is a mechanism underlying the proarrhythmogenic pattern observed at the cellular level in fructose-rich diet rats and mice. Moreover, the enhanced activity of CaMKII observed in fructose-rich diet animals is mainly reliant on an increase on the oxidative stress produced by fructose-rich diet. To the best of our knowledge, this is the first report showing the presence of CaMKII-dependent proarrhythmic mechanisms in a prediabetic model.

4.1. Fructose-rich diet: the prediabetes model

The American Diabetes Association indicates that T2DM is usually preceded by a metabolic disorder, known as prediabetes[3]. This stage is characterized by elevated plasma glucose, which does not reach levels observed in diabetic subjects. Prediabetes can be diagnosed by simple blood tests that reveal an impaired glucose tolerance and/or impaired fasting glucose and/or elevated glycated haemoglobin (HbA1c), and it is thought to constitute a major risk factor for developing diabetes and/or other adverse outcomes like retinopathy or cardiovascular illness[8, 41]. Indeed, the increased risk of cardiovascular disease in people with impaired glucose tolerance was early recognized by the World Health Organization (WHO)[42].

Administration of a fructose-rich diet to normal rats for 3 weeks induces multiple abnormal changes in glucose (impaired glucose tolerance) and lipid metabolism, endocrine dysfunction, insulin resistance and increased in oxidative stress markers[21, 43, 44]. All these changes resemble those recorded in people with T2DM/prediabetes, supporting the view that fructose-rich diet is a useful model to test disease mechanisms relevant to

prediabetes. Indeed, fructose-rich diet model has been previously used by several authors to asses myocardial function[32, 45].

We successfully extended the fructose-rich diet model from rats to mice, which reproduce the abnormal intraperitoneal tolerance test to glucose and the frequency of spontaneous Ca^{2+}_i release events observed in rats. The use of fructose-rich diet in mice has the potential to capture the strengths inherent in genetically modified animals for mapping pathways and understanding disease mechanisms

4.2. Fructose-rich diet promoted spontaneous Ca^{2+}_i release events and spontaneous contractions

Increased risk of sudden death has been described in prediabetes and borderline diabetes[11, 12], and enhanced arrhythmogenic risk has been detected in young people with diabetes[13]. Nordin *et al*.[46], further showed an increase in DADs propensity in streptozotocin-diabetic rats and it is known that diabetic patients show several ECG alterations[47, 48]. Moreover, abnormal glucose metabolism and impaired fasting glucose were associated with an increased incidence of atrial fibrillation (AF)[49]. However and as far as we know, the possibility that this prediabetic condition may also predispose to ventricular arrhythmias has never been explored. In our model of impaired glucose tolerance we have shown an increase in the occurrence of spontaneous Ca^{2+}_i release events, including SR CaSp and waves which culminates in spontaneous contractions in ventricular myocytes. In addition, we have found ECG abnormalities, including episodes of AF, spontaneous ventricular beats and ventricular arrhythmias *in vivo*. Therefore the current results revealed that in fructose-rich diet animals, the presence of an arrhythmogenic substrate is evident well before the development of T2DM. Moreover, catecholamine stress produced an increase in spontaneous Ca^{2+}_i release events in LV cardiomyocytes that appears to be substantially dependent on the elevated CaMKII activity.

4.3. The arrhythmogenic pattern of fructose-rich diet animals is due to an increase in CaMKII-dependent phosphorylation of RyR2

Multiple studies have shown that abnormal SR Ca^{2+} handling plays a central role in the initiation and/or maintenance of atrial and ventricular arrhythmias in animals and humans[28, 50]. Defective Ca^{2+}_i handling predisposes to spontaneous, non-synchronized SR Ca^{2+} release, which in turn activates a transient inward current that is largely carried by the NCX[15] and is the dominant inward current triggering DADs[51]. Moreover, CaMKII-dependent phosphorylation of RyR2 increases SR Ca^{2+} leak and the susceptibility to cardiac arrhythmias has been described in a number of models[28, 52], suggesting that CaMKII inhibition could shield against proarrhythmic conditions in a variety of diseases marked by elevated ROS and disturbed Ca^{2+}_i. The present results showed an increase in Ca^{2+}_i sparks, waves and spontaneous contractions in fructose-rich diet animals. This increase occurred in the absence of an increment in SR Ca^{2+} content and was inhibited by CaMKII inhibition genetically targeted to the SR. These results suggest that CaMKII-dependent RyR2 phosphorylation is a critical mediator of the increased proarrhythmogenic events in the fructose-rich diet-induced prediabetic-model. Of note, CaMKII-dependent phosphorylation of Thr^{17} site of PLN is increased in fructose-rich diet hearts. This should at least partially

explain the increase in SERCA2a activity that we detected in our experiments and could have stimulatory effects on SR Ca^{2+} leak by increasing SR Ca^{2+} load. Although SR Ca^{2+} load is actually lower in fructose-rich diet vs. control diet rats, our results cannot rule out the possibility that an increase in CaMKII-dependent PLN phosphorylation may contribute to maintain SR Ca^{2+} leak.

In conflict with the present results, Nishio *et al.*[53] suggested that CaMKII is upstream ROS generation in a model of streptozotozin diabetic rats. Whether the different models studied or the different stages at which the illness was appraised can explain this discrepancy, is not apparent from the present results and further studies are required to explain these differences. We demonstrated that prediabetic SR-AIP mice, which showed an increase in lipid peroxidation, reflecting the increase oxidative stress, are protected against spontaneous $Ca^{2+}{}_i$ release events and ECG alterations. These results clearly indicate that ROS generation is upstream CaMKII activation.

4.4. Possible Mechanisms Underlying CaMKII Activation

CaMKII is initially activated by Ca^{2+}/CaM binding. Sustained increases in Ca^{2+}/CaM produce Thr^{287} autophosphorylation. Phosphorylated CaMKII increases the binding affinity for Ca^{2+}/CaM and is able to retain residual Ca^{2+}/CaM-independent activity. Our results demonstrated that the steady state diastolic Ca^{2+} and Ca_iT did not increase in fructose-rich diet animals. Diastolic Ca^{2+} only raised during the brief spontaneous $Ca^{2+}{}_i$ release events which represents a rather temporary change. It therefore seems that raised Ca^{2+} is a less likely source/cause of chronically activated CaMKII in fructose-rich diet rats. CaMKII is also oxidized at $Met^{281/282}$. Oxidation of CaMKII induces a similar Ca^{2+}/CaM-independent form of the enzyme[38]. Interestingly, ox-CaMKII resets the Ca^{2+} sensitivity of CaMKII in such a way that very low levels of $Ca^{2+}{}_i$ are sufficient for activation[25], suggesting that conditions of high ROS production may lead to increased CaMKII activity even without important changes in the levels of Ca^{2+}/CaM. Thus, our findings strongly suggest that the main source of chronic CaMKII activation in fructose-rich diet rats is the enhancement of ROS production. Although previous studies from Nishio *et al.*[53] showed that CaMKII activation was responsible for ROS generation in overt diabetes, the present work does not support a CaMKII-induced ROS generation, at least in the prediabetic state. The recently described O-GlcNAcylation or nitric oxide-dependent activation of CaMKII[54, 55] were out of the scope of this study. Further investigation is needed to establish whether these pathways also contribute to the fructose-rich diet phenotype. Although these pathways of CaMKII activation would be present in our model, preventing ROS increases with Tempol was sufficient to avoid Ca^{2+} sparks and waves, which potentially culminates in cardiac arrhythmias. Thus in our study O-GlcNAcylation and nitric oxide-dependent activation of CaMKII, if present, seem to be a minor source of activation for CaMKII vs. that provided by oxidation.

4.5. Cardiac hypertrophy in prediabetic rats

It has been previously described that diabetic animals and humans may develop cardiomyopathy and hypertrophy[56–60]. The pathogenesis of these cardiac alterations is far from being clear and multifactorial mechanisms were proposed, including autonomic

dysfunction, metabolic derangements and decreased of the energetic state, abnormalities in ion homeostasis, alteration in structural proteins and interstitial fibrosis. To the best of our knowledge, this is the first report showing that fructose treatment produces hypertrophy in rats. Although it was not the goal of the present study to investigate the mechanisms of hypertrophy in these animals, it is known that in cardiac hypertrophy and heart failure, disturbed intracellular Ca^{2+} handling is involved in contractile dysfunction and contributes to DAD-related arrhythmias[61–63]. An important question that therefore arises from the present study is whether the ECG alterations of fructose-rich diet rat hearts are the consequence of the hypertrophy observed in these hearts. The present results cannot either deny or support this possibility. Our study showed that Ca^{2+} handling was altered and intracellular Na^{+} was increased in fructose-rich diet rats, similar to what has been described in different models of hypertrophy[62, 64, 65]. It is therefore possible that alterations that are at the basis of arrhythmias in fructose-rich diet hearts, are also involved in the development of hypertrophy. Moreover, although assessment of energetic metabolism of fructose-rich diet hearts was out of the scope of this work, it has been shown that diabetes-induced metabolic changes decrease cardiac efficiency and energetics in both humans and animal models of diabetes[66]. Notably, a decrease of the energetic state of the fructose-rich diet hearts could also contribute to the development of cardiac hypertrophy in this model.

In summary, we have described for the first time a proarrhythmic pathway activated in the prediabetic condition. Here we presented new evidence showing the vulnerability of prediabetes to induce a ROS-dependent increase in CaMKII activity, which in turn produces enhanced RyR2 phosphorylation and SR Ca^{2+} leak capable of induces contractile activity. Thus, therapeutic strategies to decrease CaMKII activation may contribute to prevent or reduce the arrhymogenic pattern of prediabetes.

4.6. Clinical Relevance and Potential Limitations

A major challenge of T2DM is the recognition of the initial prediabetic stage to prevent its evolution and associated complications. The insidious beginning of T2DM includes a prolonged, subclinical prediabetic phase. In an animal model of prediabetes, we showed a striking propensity to ECG alterations. The finding that CaMKII is hyperactive in diabetic patients and the well-known increase in RyR2 Ca^{2+}-leak in clinical arrhythmias[20, 29, 37] argue for the possible clinical relevance of our work. These findings further suggest that prediabetes should motivate a careful search for arrhythmias. A possible limitation of our model is that the caloric intake in fructose-rich diet rats is slightly but significantly higher when compared to control diet rats. Whether this increased caloric intake has some modulatory role on CaMKII activity, myocardial remodelling and arrhythmogenes is described here, cannot be ascertain from the present results and deserves further investigation. However, the fact that similar results were obtained in mice in which the caloric intake was similar between fructose-rich diet and control diet animals, argues against this possibility. Moreover, it has been previously shown that phosphorylation of CaMKII was not affected by changes in caloric intake[67].

We are aware however, of the limitations of any animal model to mimicking prediabetes in humans and therefore extrapolation of our findings to the clinical setting should be very

cautious. Moreover, the mechanistic link between prediabetes, cardiac arrhythmias and RyR2 dysfunction and Ca^{2+} leak in patients is unknown and requires extensive work in subsequent studies. Moreover, multiple possible complex processes (CaMKII activation, ROS signaling, etc.) may contribute to the genesis of myocardial defects in prediabetic individuals. Further experimental and translational studies are needed to evaluate the possibility that CaMKII-dependent SR Ca^{2+} leak may also constitute a core mechanism in the development and progression of ventricular arrhythmias in prediabetic patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AF	Atrial Fibrillation
AV	Atrioventricular
Ca^{2+}_i	Intracellular Calcium
Ca_iT	Intracellular Calcium Transient
CaMKII	Ca^{2+} Calmodulin dependent protein kinase
CaSp	Calcium Sparks
CaW	Calcium Waves
DAD	Delay after depolarizations
EAD	Early after depolarizations
ECG	Electrocardiogram
LV	Left ventricle
RyR2	Ryanodine Receptor 2
PLN	Phospholamban
NCX	Sodium-Ca2+ exchanger
ROS	Reactive Oxygen Species
SR	Sarcoplasmic Reticulum

T2DM	Type 2 Diabetes Mellitus
WT	Wild Type

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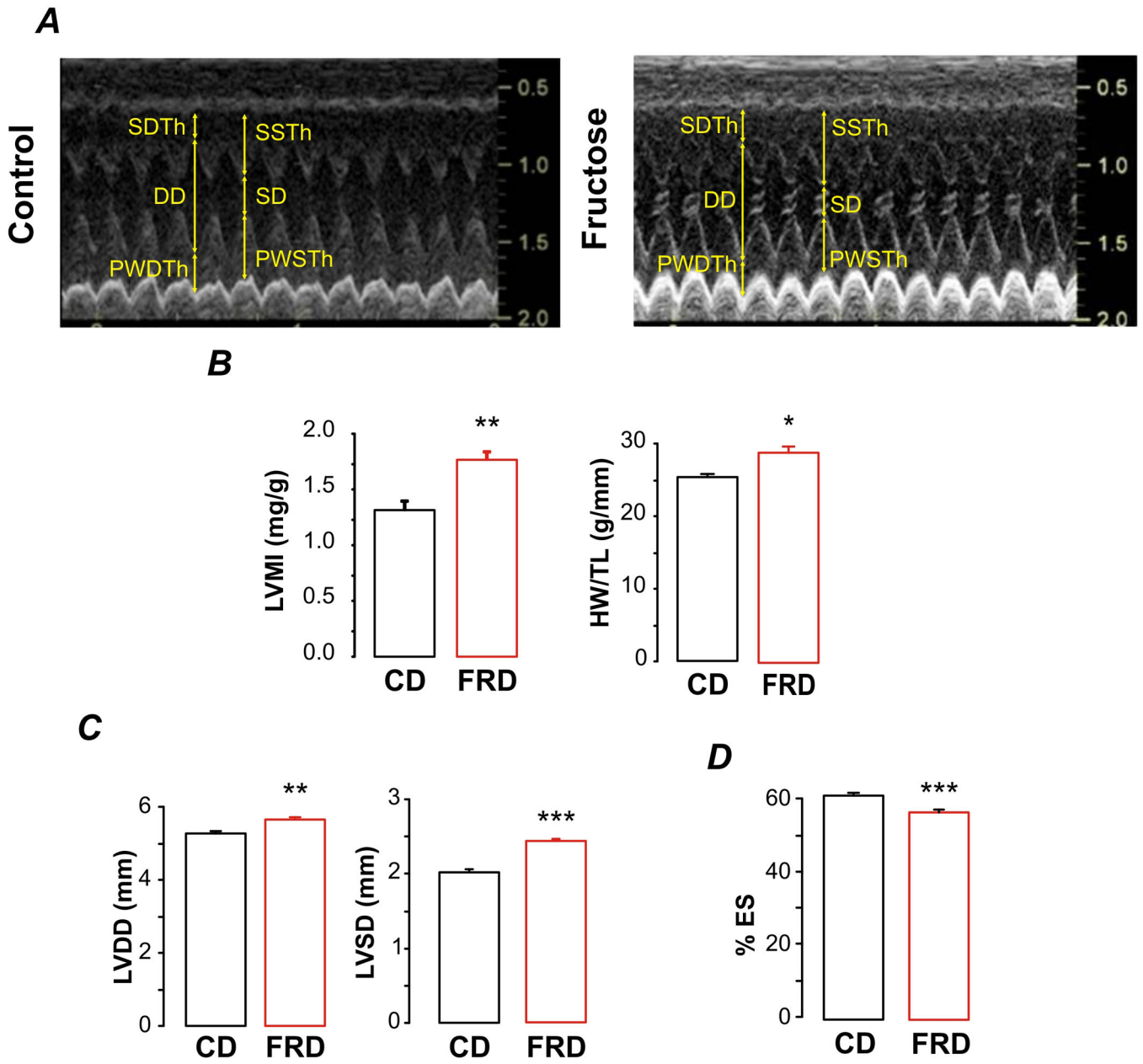

Figure 1. Structural, functional and electrical alterations in the Fructose-rich diet rat model

A, representative echocardiographic left ventricle images from control diet (CD) and Fructose-rich diet (FRD) treated rats; ***B***, Hypertrophy assessed by left ventricular mass index (LVMI) and heart weigh/tibial length ratio (HW/TL); ***C***, Dilation evaluated by left ventricle diastolic diameter (LVDD) and left ventricle systolic diameter (LVSD); ***D***, Percent of endocardial shortening. The diastolic and systolic left ventricular (LV) diameters were increased in fructose-rich diet compared to control diet rat hearts, in association with significant increases in hypertrophy indices. Moreover, systolic function was reduced in fructose-rich diet with respect to control diet rats. Bar data are mean ± SEM. n=6 to 8 rats per group, *p<0.05, ***p<0.001 with respect to CD.

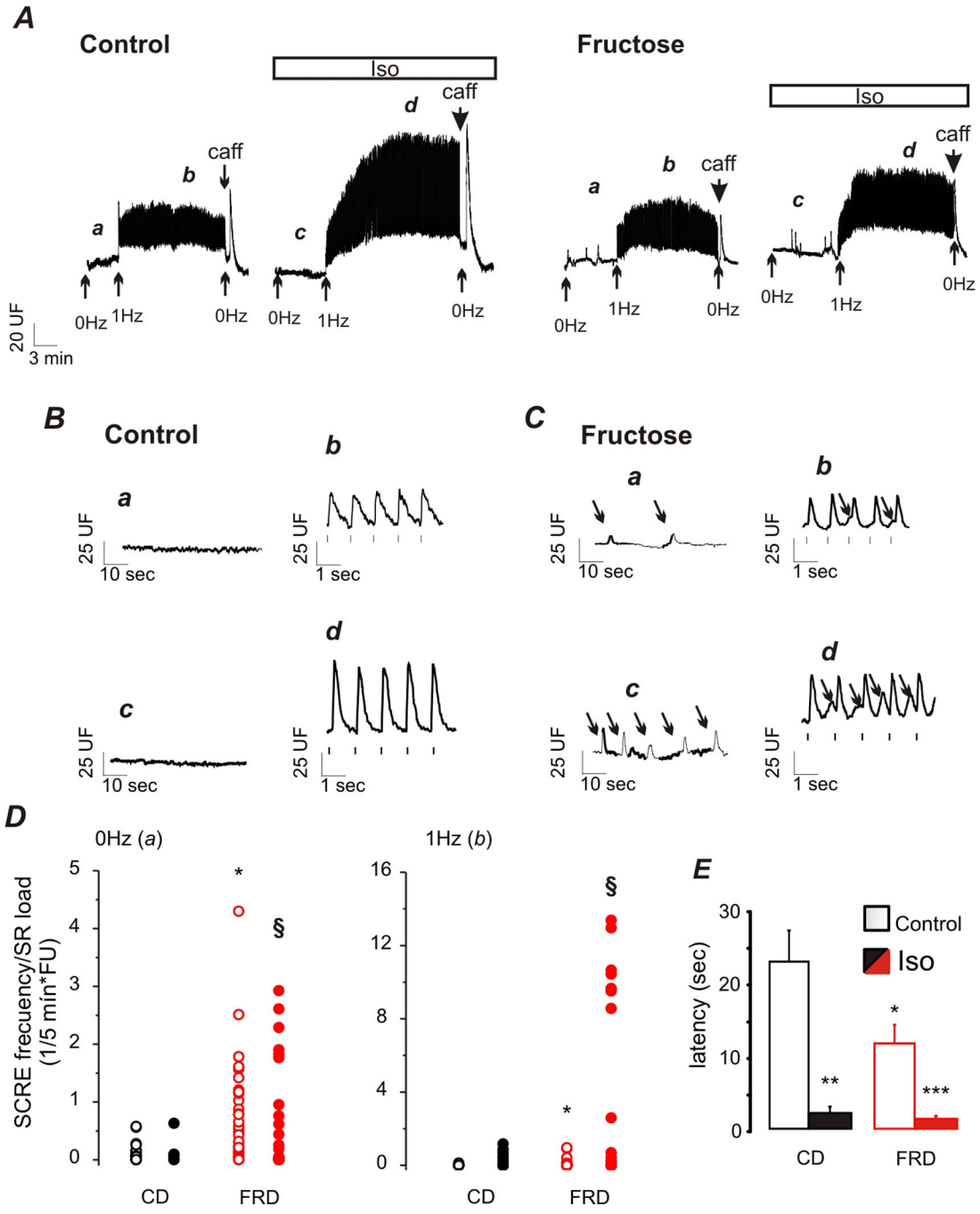

Figure 2. SCRE are increased in myocytes from FRD vs CD rats

A, Protocol followed to study Ca^{2+}_i in myocytes from CD and FRD rats. Ca^{2+}_i transient were recorded under quiescent condition (***a*** and ***c***) and at 1Hz field electrical stimulation (***b*** and ***d***) in the absence and presence of Isoproterenol (Iso). A Caffeine pulse (caff) was performed to evaluate sarcoplasmic reticulum Ca^{2+} content. ***B***, CD and ***C*** FRD myocytes. Ca_iT at the times indicated by letters ***a-d*** in panel ***A***, in an expanded scale. Arrows indicate SCRE. ***D***, Value plots of the frequency of SCRE at 0 Hz and 1 Hz normalized by the SR Ca^{2+} content, in the presence and absence of Iso. ***E***, Latency of SCRE. Myocytes from FRD

rats showed a significant increase in SCRE vs. CD in all conditions tested. n= 25 to 40 myocytes from 4–6 rats per group, *p<0.05, **p<0.01, ***p<0.001 vs. CD without Iso; § p<0.05 vs. CD with Iso.

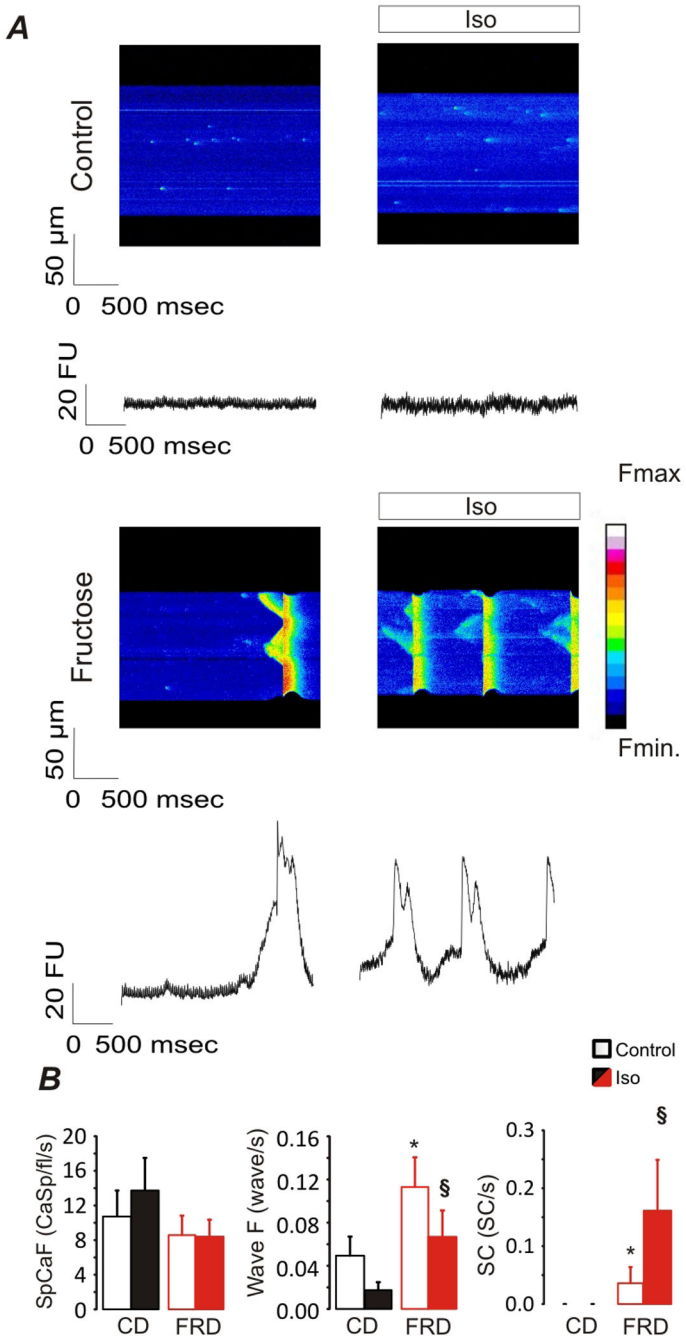

Figure 3. Local SR Ca^{2+} release events are increased in myocytes from FRD vs. CD rats

A, Line scan images from myocytes of CD and FRD rats in the presence and absence of Isoproterenol (Iso), with their respective plot profiles. ***B***, average values of CaSpF, Wave F and SC frequency. FRD cardiomyocytes showed a significantly increased Ca^{2+} Wave F and SC compared to CD myocytes. Bar data are mean ± SEM. n= 20 to 26 myocytes from 4 rats per group, *p<0.05 compared with CD, § p<0.05 compared with CD+Iso.

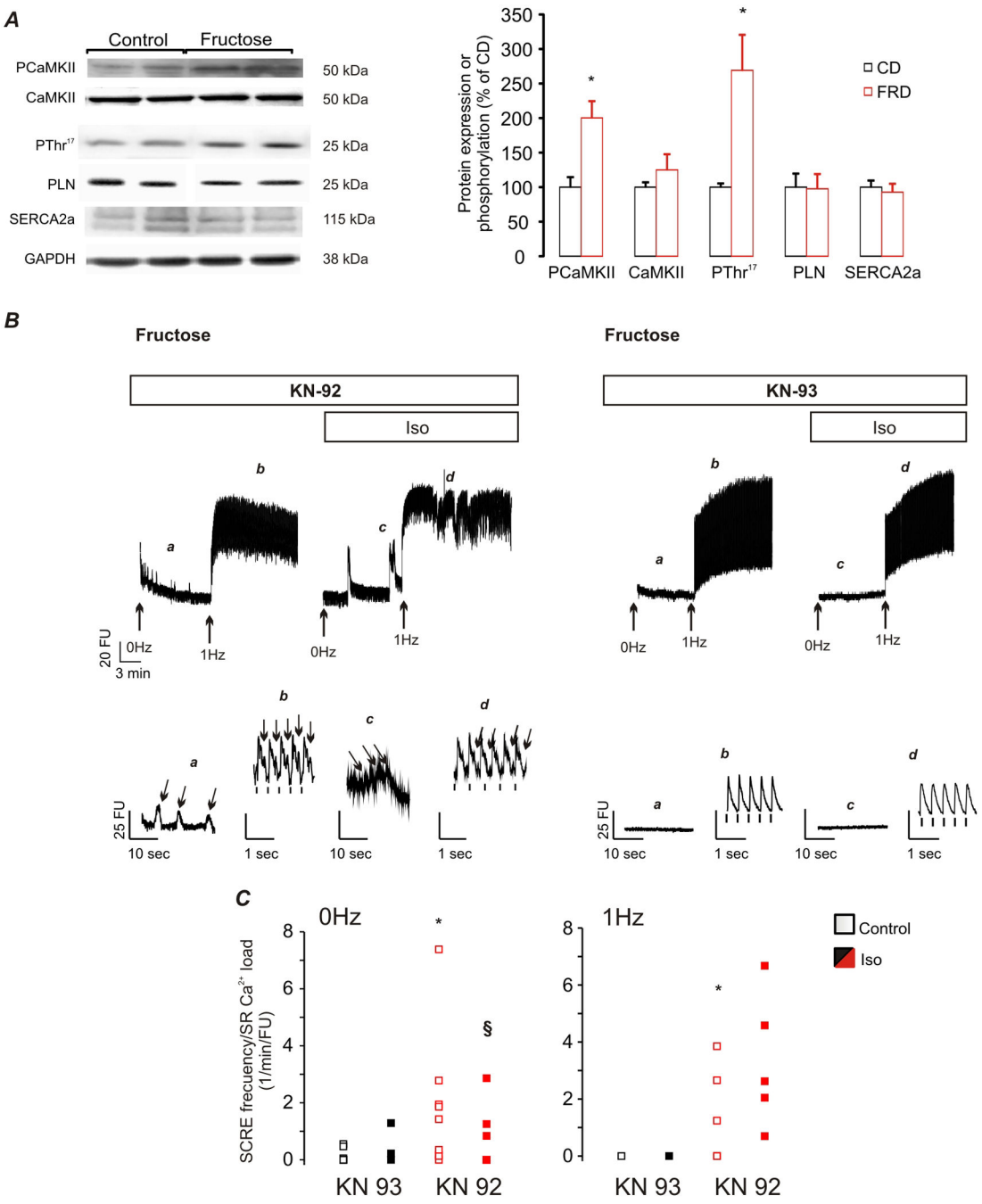

Figure 4. Inhibition of CaMKII activity prevents Spontaneous Ca^{2+} release events (SCRE) in fructose-rich diet (FRD) myocytes

A, Representative inmunoblots of PCaMKII, total CaMKII, $PThr^{17}$, total PLN, SERCA2a and GAPDH as loading control on the left. Average of CaMKII expression and activity, SERCA2a expression and PLN expression and CaMKII-dependent phosphorylation of control diet (CD) and FRD rat hearts, on the right. ***B***, Typical continuous recordings of Ca^{2+}_i transients from rat myocytes perfused in the presence of KN-92 and KN-93. Below, Ca_iT in an expanded scale at the times indicated by letters ***a-d***. Arrows indicate SCRE.***C***, Value plots

of the frequency of SCRE at 0 Hz and 1 Hz normalized by the SR Ca^{2+} content, in the presence and absence of isoproterenol (Iso), of myocytes incubated with KN-92 and KN-93. CaMKII activity increased in FRD vs. CD rat hearts and CaMKII inhibition prevented the increase of SCRE in FRD under basal and Iso conditions. Data of inmunoblots are mean ± SEM. n= 10 to 17 myocytes from 3 rats per group. *$p<0.05$ compared with CD, § $p<0.05$ compared with CD+Iso.

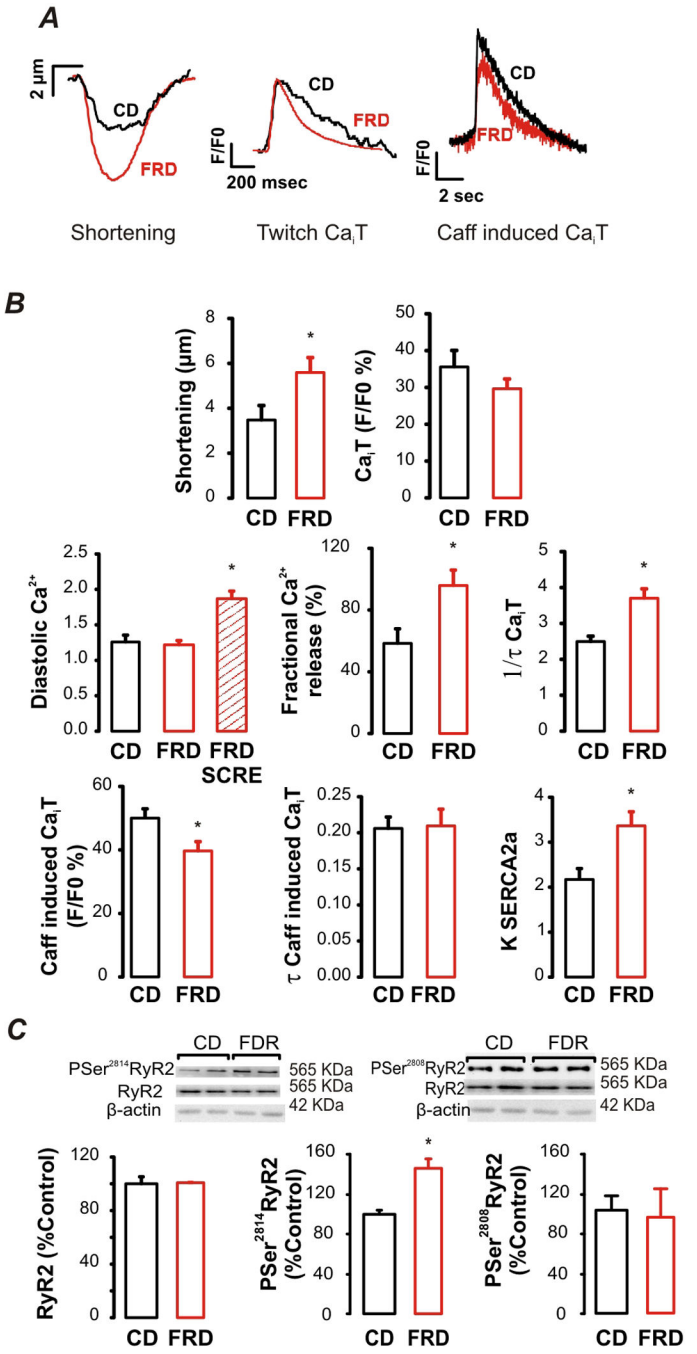

Figure 5. Fructose treatment increases CaMKII-dependent phosphorylation of RyR2 and decreases SR Ca^{2+} content

A, Representative individual twitch contractions, Ca^{2+} transients (Ca_iT) and caffeine induced Ca_iT of control and fructose-rich diet (CD and FRD) rat myocytes. ***B***, average values of cell shortening, Ca_iT amplitude, diastolic Ca^{2+}, fractional Ca^{2+} release, rate constant of Ca_iT decay ($1/\tau$ of Ca_iT), caffeine induced Ca_iT amplitude, τ of caffeine induced Ca_iT and SERCA2a activity (K SERCA2a), of myocytes from CD and FRD rats. Data are mean ± SEM. n= 25 to 40 myocytes from 4–6 rats per group. The stripped bar in the middle panel represents the increase in diastolic Ca^{2+} that occurs during the episodes of spontaneous Ca^{2+}

release in FRD myocytes. Data are mean ± SEM of the average diastolic Ca^{2+} from the multiple spontaneous release events of each cardiac myocyte. n=18 myocytes from 6 rats. *p<0.05 compared with CD. ***C***, Representative inmunoblots and average results of total RyR2 (on the left), $PSer^{2814}$ residue of RyR2 (on the middle) and $PSer^{2808}$ (on the right). β-actin was used as loading control. Data are mean ± SEM. n= 4 rats per group, *p<0.05 compared with CD.

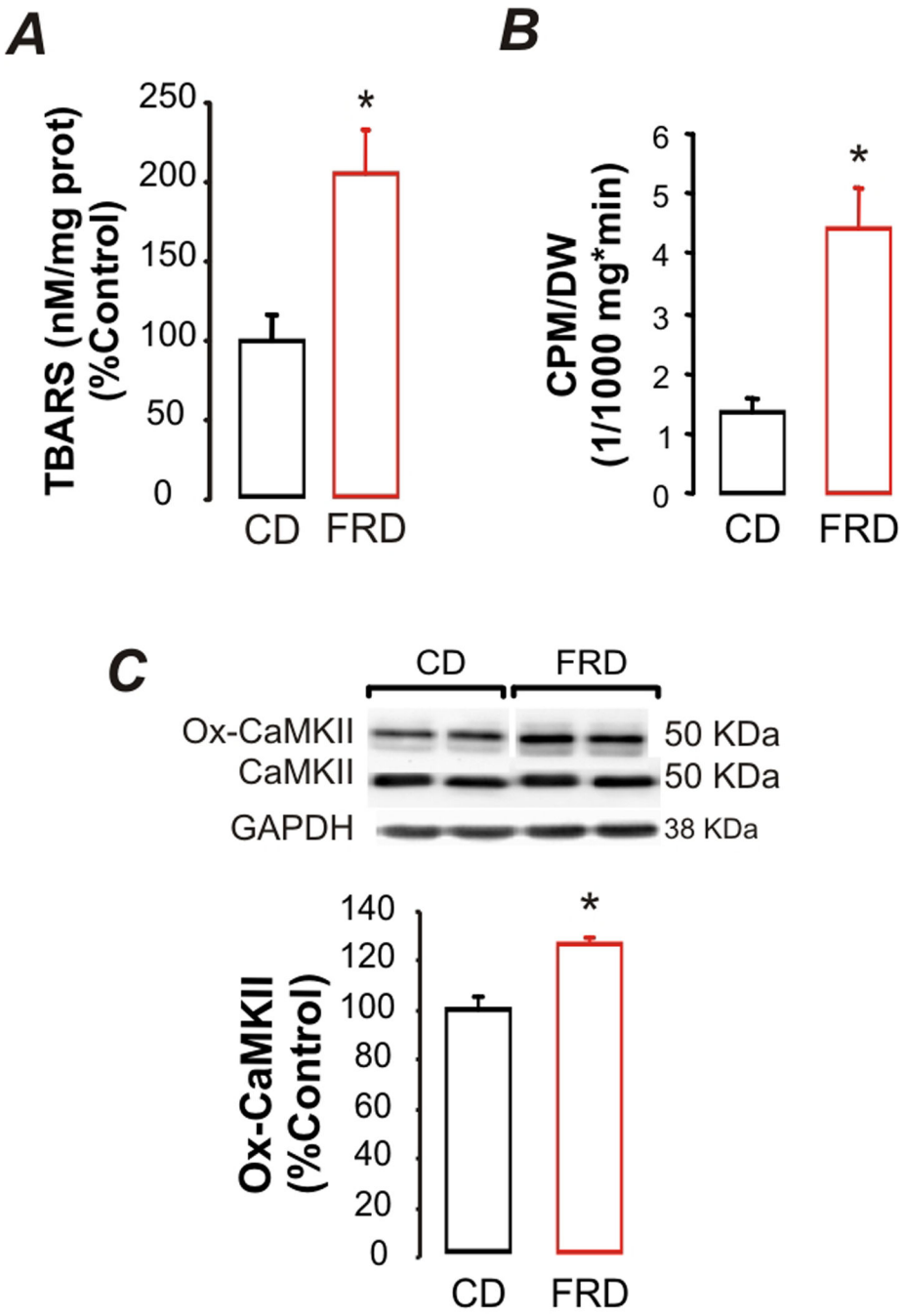

Figure 6. Lipid peroxidation, ROS production and ox-CaMKII are enhanced in FRD vs. CD animals

A, average data for lipid peroxidation measured by TBARS; ***B***, ROS assessed by chemiluminescence; and ***C***, representative inmunoblots and average data for oxidized CaMKII (ox-CaMKII) and total CaMKII from CD and FRD treated rats. Data are mean ± SEM. n= 4 rats per group, *p<0.05.

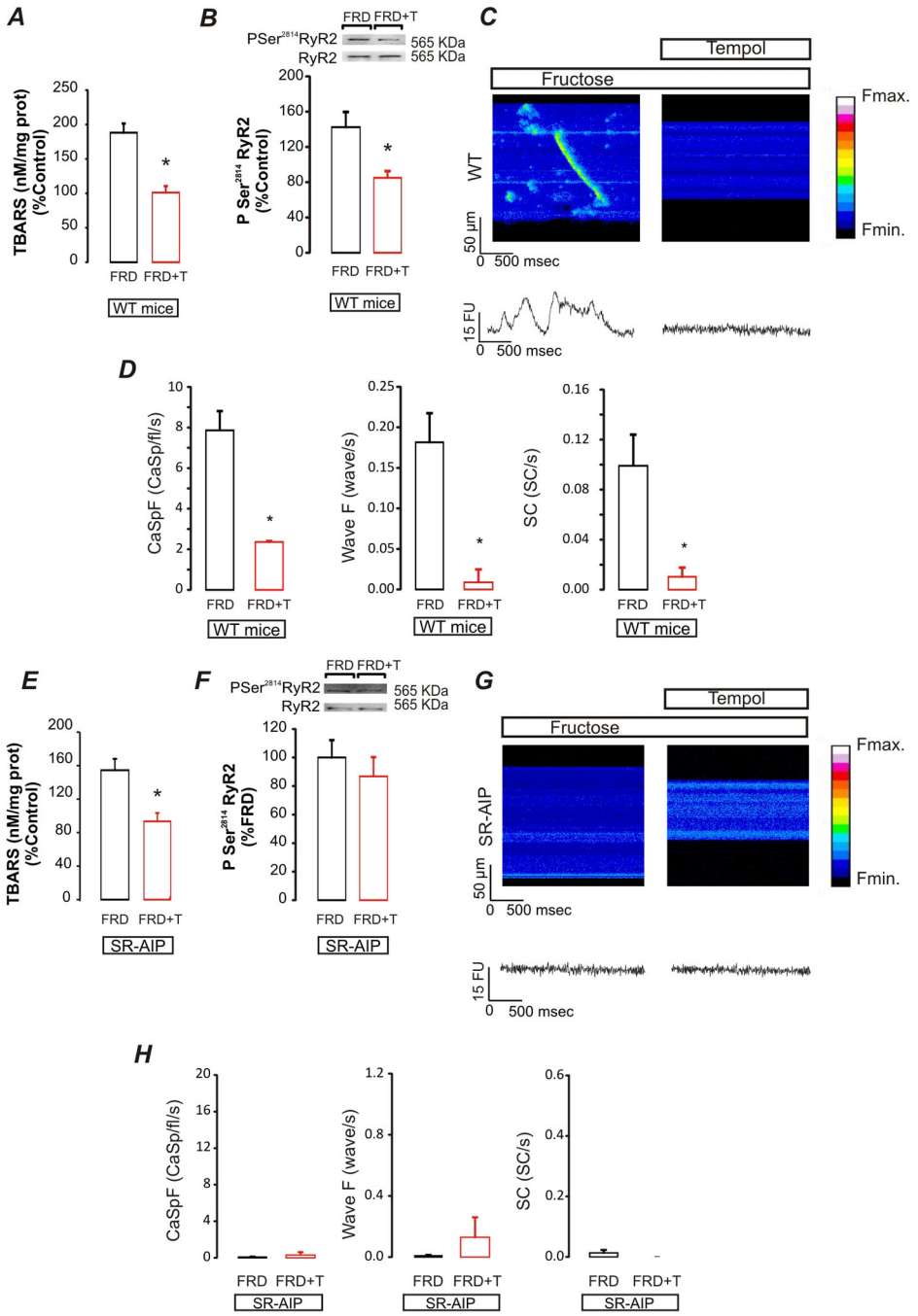

Figure 7. Role of ROS in spontaneous Ca^{2+} release events and in CaMKII activation

A, Lipid peroxidation increased in fructose-rich diet (FRD) WT with respect to control diet (CD) mice. ***B***, CaMKII-dependent phosphorylation of RyR2 at Ser^{2814} site in the presence and absence of Tempol. ***C***, Line scan images of myocytes from FRD and FRD + Tempol (FRD+T) treated WT mice. Below the images, are depicted their respective plot profiles. Tempol prevented the increased spontaneous Ca^{2+} release events produced by fructose treatment.***D***, Average values for CaSpF, Wave F, spontaneous contractions (SC) frequency. ***E***, lipid peroxidation in CD and FRD SR-AIP mice. ***F***, CaMKII-dependent phosphorylation

of RyR2 at Ser^{2814} site in the presence and absence of Tempol in SR-AIP mice. ***G***, Line scan images and their respective plot profiles from myocytes of FRD and FRD + Tempol (FRD +T) treated SR-AIP mice. ***H***, average values for CaSpF, Wave F and SC frequency. SR-AIP mice do not show any intracellular Ca^{2+} release event, in spite of the increase in ROS. Of note, values of CaSpF, Wave F and SC in either FRD or CD mice, are close to 0 (compare with **panel *D***). Data are mean ± SEM. n= 10 to 15 myocytes from 4 mice per group.

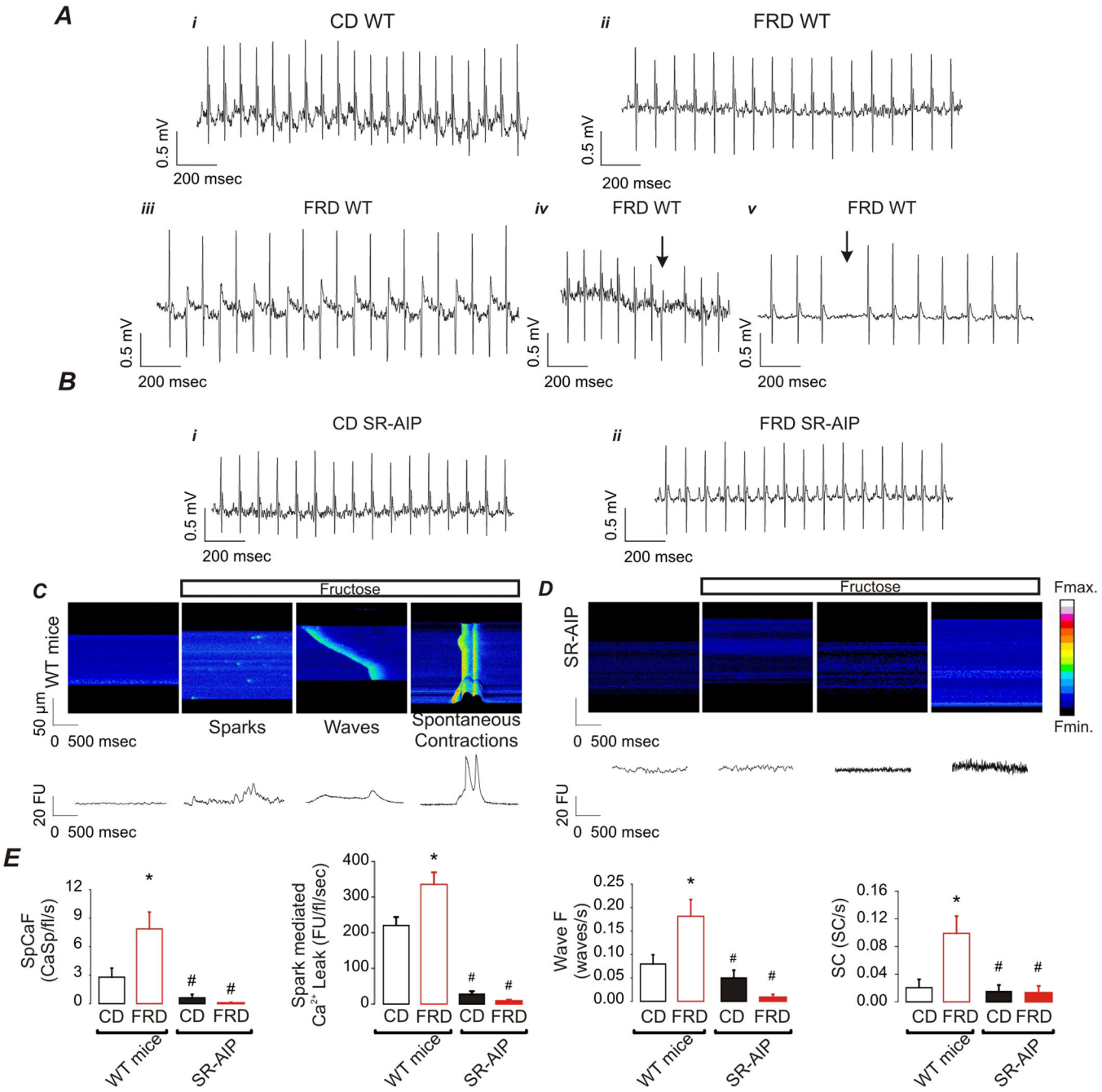

Figure 8. FRD induces cardiac arrhythmias and Local SR Ca^{2+} release events in WT but not in SR-AIP mice

A, and ***B***, representative ECG records from CD and FRD treated WT and SR-AIP mice. ***A***, normal sinus rhythm in CD WT mice (***i***), FRD WT mice with bradycardia (***ii***), bidirectional tachycardia (***iii***), ventricular ectopic beats (***iv***, arrow) and AV block (***v***, arrow). ***B***, regular sinus rhythm in CD (***i***) and FRD (***ii***) SR-AIP mice. n=3 to 5 mice per group. ***C*** and ***D***, Line scan images from myocytes of CD and FRD WT and SR-AIP mice, respectively. Below the images are depicted their respective plot profiles. ***E***, average values for CaSpF, Ca^{2+} leak,

Waves F and SC frequency of myocytes from CD and FRD WT and SR-AIP mice. SR-AIP mice were protected from arrhythmias and local SCRE. Data are mean ± SEM. n= 14 to 21 myocytes from 4 mice per group, *$p<0.05$ compared with CD WT mice; #$p<0.05$ compared with FRD WT mice.