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Dietary Saturated Fat Promotes Arrhythmia by Activating NADPH Oxidase 2 (NOX2)

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Abstract

Background -—Obesity and diets high in saturated fat increase the risk of arrhythmias and sudden cardiac death. However, the molecular mechanisms are not well understood. We hypothesized that an increase in dietary saturated fat could lead to abnormalities of calcium homeostasis and heart rhythm by a NADPH oxidase 2 (NOX2)-dependent mechanism.

Methods -—We investigated this hypothesis by feeding mice high fat diets. In vivo heart rhythm telemetry, optical mapping, and isolated cardiac myocyte imaging was used to quantify arrhythmias, repolarization, calcium transients, and intracellular calcium sparks.

Results -—We found that saturated fat activates NOX, whereas polyunsaturated fat does not. The high saturated fat diet increased repolarization heterogeneity and ventricular tachycardia (VT) inducibility in perfused hearts. Pharmacologic inhibition or genetic deletion of NOX2 prevented arrhythmogenic abnormalities *in vivo* during high statured fat diet and resulted in less inducible VT. High saturated fat diet activates Ca2+/calmodulin-dependent protein kinase (CaMK) in the heart, which contributes to abnormal calcium handling, promoting arrhythmia.

Conclusions -—We conclude that NOX2 deletion or pharmacologic inhibition prevents the arrhythmogenic effects of a high saturated fat diet, in part mediated by activation of CaMK. This work reveals a molecular mechanism linking cardiac metabolism to arrhythmia, and suggests that

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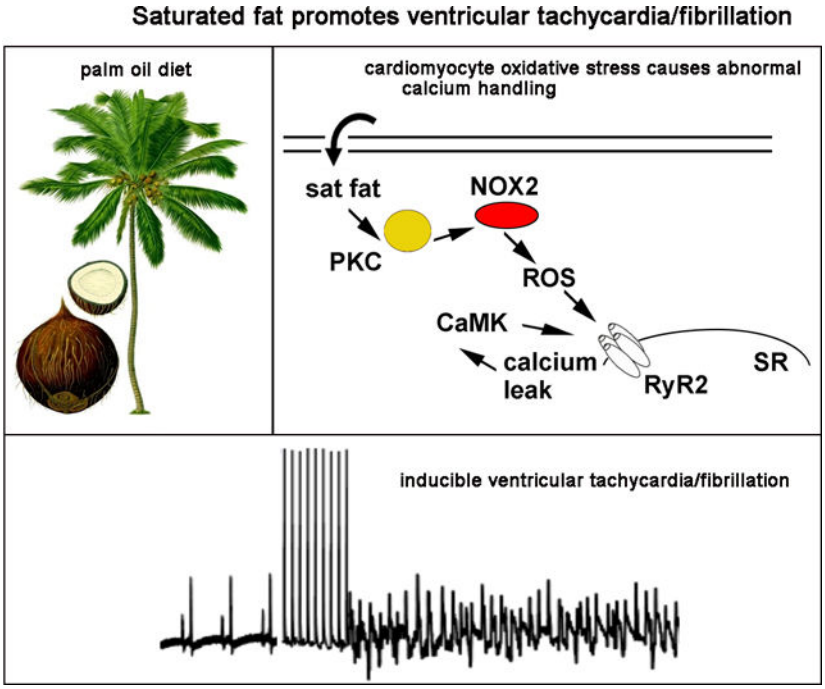
NOX2 inhibitors could be a novel therapy for heart rhythm abnormalities caused by cardiac lipid overload.

Graphical Abstract



Keywords

NAD(P)H oxidase; repolarization; fatty acid; reactive oxygen species; arrhythmia

Journal Subject Terms:

Arrhythmias; Lipids and Cholesterol; Oxidant Stress

Introduction

Many epidemiologic studies have shown that obese patients have approximately twice the risk of sudden cardiac death (SCD) than age matched controls[1–4]; SCD is often caused by ventricular arrhythmias. The increased risk of SCD is greater than the increased risk of myocardial infarction, suggesting that arrhythmic events are increased more than coronary events in obese humans. Excessive lipid accumulation is found in cardiomyocytes from obese and diabetic patients, and is believed to contribute to heart failure and arrhythmia[5–8]. Epidemiology studies also show that higher saturated fat intake leads to an increased risk of sudden cardiac death[9–12], suggesting that the effects of dietary saturated fat may be sufficient to cause abnormal heart rhythm, without obesity.

The most common electrophysiologic abnormalities found in obese patients are increased frequency of ventricular ectopy and prolongation of the QT interval[13–15]. We have

previously shown that wild type (WT) mice with high-fat diet induced obesity (DIO) have long QT and increased ventricular ectopy[16], mimicking the abnormalities found in obese humans. Oxidative stress is a plausible mechanistic link between lipid metabolism and cardiovascular pathology[17–19]. Mild, transient increases in cardiac reactive oxygen species (ROS) may be involved in adaptive processes, but it is postulated that long-term increases in cardiac ROS are detrimental[20]. There are several sources of ROS in cardiomyocytes, including NADPH oxidase (NOX), nitric oxide synthase (NOS), and byproducts of mitochondrial metabolism. We hypothesized that increasing saturated fatty acid in the diet could increase oxidative stress in cardiomyocytes by activating NOX2, resulting in abnormalities of calcium homeostasis and heart rhythm, before the onset of obesity.

Materials and Methods

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Materials

The OxyBlot Protein Oxidation Detection Kit and apocynin were purchased from Sigma. The anti-PKC antibodies were purchased from Cell Signaling (PKC alpha) and Santa Cruz (PKC beta and delta). Ion channel antibodies were purchased from Alomone. The anti-CaMK antibodies were purchased from Fisher Scientific. The anti-PKA antibodies were purchased from Cell Signaling. RyR2 blots were performed as previously described[21].

Animal care and cardiomyocyte isolation

Animal protocols were approved by the Columbia University Institutional Animal Care and Use Committee and were carried out in accordance with the NIH guidelines for the care and use of laboratory animals. Wild type (WT) and B6.129S-*Cybb*^{tm1Din}/J (NOX2KO) mice were purchased from Jackson labs and were used for experiments at 12–16 weeks of age. High fat diets were special orders from Research Diets Inc: D04051707, 60 kcal% from palm oil, 20% kcal protein, 20% kcal carbohydrate; and D01112603, 60 kcal% from olive oil, 20% kcal protein, 20% kcal carbohydrate. Control chow was Picolab Rodent diet 20 from LabDiet, which has 13% kcal from fat, 25% protein, and 62% carbohydrate. Animals were fed ad libitum. Isolation of cardiomyocytes was performed as previously described[6]. Animals were randomly assigned to treatment groups. No animals were excluded from the analysis. Echocardiograms, optical mapping, and histology quantification were performed by blinded operators.

Echocardiograms and blood glucose measurements

Echocardiograms were performed on mice using a high-resolution imaging system with a 30-MHz imaging transducer (Vevo 770; VisualSonics). Isoflurane anesthesia was used. The operator was blinded to group assignment. Whole blood glucose was measure at the time of sacrifice with one-touch ultra-glucometer (Johnson&Johnson).

Histology

Heart tissue was fixed with 4% paraformaldehyde, embedded in paraffin wax, and then sectioned. Sections were incubated with trichrome stain using standard methods by the Columbia University Pathology core facility. Fibrosis was quantified by measuring mean blue pixels in multiple slices using Adobe Photoshop 11.0.2.

NOX activity measurements

NADPH-dependent superoxide production was measured in cellular homogenates using lucigenin-enhanced chemiluminescence as previously described[22]. Cells were homogenized in Krebs buffer, pH 7.4 (130 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1.5 mM $CaCl_2$, 5 mM glucose, 35 mM phosphoric acid, and 20 mM HEPES). Homogenates were centrifuged at 1000 × g at 4°C for 10 min to remove the unbroken cells and debris. The pellet was resuspended in Krebs buffer containing 0.5 mM lucigenin followed by the addition of 0.1 mM NADPH as the substrate. Protein content was measured using the Bradford protein assay reagent. The samples were transferred to a 96-well plate at 50 μg of protein per well. Photon emission in terms of relative light units was measured in a Tecan 200 microplate reader after 5 min incubation in the dark. There was no measurable activity in the absence of NADPH. Superoxide anion production was expressed as relative lucigenin luminescence.

Heart rhythm telemetry

Telemetry devices (Data Sciences International, model EA-F10) were implanted in mice under sterile conditions with inhaled isoflurane for anesthesia. The two subcutaneous leads were positioned to approximate ECG limb lead II. The mice recovered for one week after surgery before initiating recordings. ECG intervals were measured manually using Ponemah 3 software from recordings with minimal artifact at approximately the same time of day. Intervals were averaged from 4 consecutive beats and QTc was calculated by the formula $QTc=QT/(RR/100)$[23]. PVC and arrhythmia counts were tallied manually by a blinded reader.

Optical mapping protocol

The operator was blinded to group assignment. Mice were injected with heparin prior to administration of isoflurane. Hearts were isolated and perfused via a Langendorff apparatus with warm oxygenated Krebs-Henseleit buffer (pH 7.4; 95% O_2, 5% CO_2, 36–38°C), and were placed in a glass chamber in a Tyrode bath for superfusion. One AgCl wire was attached to the metal aortic cannula, and another AgCl wire was positioned near the surface of the heart to record an ECG. Blebbistatin (5–10 μM) was perfused to reduce motion, and Di-4-ANEPPS (100 μM) was perfused to record optical membrane potentials. Hearts were uniformly shone with green excitation lasers (532 nm) to activate Di-4-ANEPPS. Emitted fluorescence was captured through a 715-nm pass filter using a complementary metal-oxide-semiconductor (CMOS) camera (MICAM Ultima, SciMedia). Movies were acquired at 1000 frames per second for a duration of 4–5 sec, with 100 × 100-pixel resolution (0.095 mm per pixel). Optical movies were acquired at different pacing cycle lengths. Susceptibility to pacing-induced VF/VT was assessed by 3 attempts of burst pacing (20 Hz, 10 s) at twice the excitation threshold of the left ventricle (Pulsar 6i, FHM, Brunswick, ME).

Optical mapping data processing and analysis

Recorded optical movies were processed using custom software based on PV-WAVE (Precision Visuals - Workstation Analysis and Visualization Environment, Visual Numerics, Inc)[24]. The background fluorescence was subtracted from each frame, and spatial (5 × 5 pixels) and temporal (9 frames) conical convolution filters were used to increase signal-to-noise ratio. Optical mapping movies were spatio-temporarily filtered to reduce noise. Phase movies were obtained after Hilbert transformation of the fluorescent signal and action potential duration (APD) maps were generated as previously reported[25]. APD dispersion (APD_{max} –APD_{min}) was calculated after drawing a custom 10×10 pixel area.

Cardiomyocyte contractility and calcium spark recording and analysis

Cardiomyocyte isolations were performed as previously described[26]. Isolated cardiomyocyte contractility was measured using an Ionoptix system as previously described[26]. Spontaneous contractions were measured for 40 seconds after a brief pacing interval. Calcium transients were measured using Fura2 (Invitrogen). SR calcium load was evoked with rapid application of caffeine (10 mM). The operator was blinded to group assignment. Sparks were recorded with a Leica SP2 confocal microscope (Wetzlar, Germany) equipped with a 63×x1.4 NA objective. Isolated cardiac myocytes experiments were performed at room temperature. Cardiomyocytes were loaded with fluo-4 (5 μM for 10 minutes) in modified Tyrode solution containing 1 mM calcium. Line scan images were recorded for 10 seconds with Leica TCS software and quantified with the Sparkmaster plugin of ImageJ.

Statistical analysis

Results are given as mean ± SEM. The unpaired *t* test was used for comparisons of 2 means. a 2-tailed value of $P < .05$ was considered statistically significant. For groups of 2 or more, analysis of variance (ANOVA) was used with *post hoc* testing (Prism v5, GraphPad Software Inc., La Jolla, CA).

Results

High saturated fat diet rapidly causes ventricular ectopy and long QT without obesity

To determine the effects of high-fat diet on cardiac function, we fed adult WT C57Bl6 mice regular chow or one of two high fat diets: 60 kcal% from palm oil (42% saturated fat, high saturated fat diet = HSFD); and 60 kcal% from olive oil (14% saturated fat, high unsaturated fat diet = HUFD). Palm oil is commonly used as a cooking oil in many parts of the world and is composed of approximately 44% palmitate (C16, a saturated fat) and 37% oleate (C18:1, a monounsaturated fat). The olive oil diet was used as a control because olive oil has generally been found to have neutral or benign effects on cardiovascular health in humans. The 4-week duration of high-fat diet did not result in obesity (mean increase in weight 3.2–3.8 g for all groups, supplemental table 1), nor did we observe significant differences in serum glucose (supplemental Fig1A).

Serum free-fatty acids (FFA) were significantly increased after 4 weeks of the HSFD (Fig1A). Serum lipidomic analysis showed that mice on the HSFD had 2.6 times as much palmitate in the bloodstream as regular chow controls, which was statistically significant

(Fig 1A). The HSFD also caused several significant changes in the ventricular tissue lipid content compared to regular chow. Lipidomic analysis of ventricular tissue samples showed that both palmitate and oleate levels were significantly increased, consistent with the composition of palm oil (supplemental Fig1B).

We have previously demonstrated increased ventricular ectopy and long QT in obese mice[16]. To determine if the HSFD was sufficient to cause similar heart rhythm abnormalities without obesity, we recorded heart rhythm in WT mice before and after the HSFD or HUFD. Mice were implanted with heart rhythm telemetry monitors and allowed to recover for one week. Following baseline heart rhythm recordings on regular chow, mice were fed the HSFD or HUFD diets. Telemetry recordings showed that mice developed a statistically significant increase in QT on the HSFD diet (Table 1). The mice also had a significant increase in ventricular ectopy in the form of premature ventricular complexes (PVCs) (Fig1D). No atrial arrhythmias were detected during this time. In contrast, mice fed the HUFD diet did not show increased PVCs or QT duration (Fig1D). These findings indicate that a HSFD can cause the same arrhythmogenic abnormalities as diet-induced obesity without substantial weight gain, and that a high fat diet does not cause cardiac abnormalities if it is composed of olive oil.

Pharmacologic NOX2 inhibition during high saturated fat diet prevents heart rhythm abnormalities and NOX2KO mice are protected from arrhythmogenic changes of saturated fat

To determine if the high-fat diets increased oxidative stress, we measured NOX activity in ventricular tissue and found that it was increased by the HSFD, but not by the HUFD (Fig1B). NOX2 protein levels in the heart were not changed significantly by diet (supplemental Fig1C). Total cellular oxidative stress, as measured by protein carbonyl groups in ventricular lysates, showed no significant difference between HSFD and regular chow (supplemental Fig1D). This suggests that the oxidative stress caused by NOX activation does not cause an increase in total protein oxidation. Since the HUFD did not cause abnormalities, we focused the remainder of our study on the effects of the HSFD.

To test the *in vivo* relevance of NOX activation, we quantified NOX activity in ventricular lysates from mice given regular chow or HSFD, with or without the NOX inhibitor apocynin in the drinking water. As hypothesized, NOX activity is increased in ventricular lysates from mice on high fat saturated fat diet and apocynin prevents the increase in NOX activity (supplemental Fig2). After telemetry surgery, we treated another group of WT mice with apocynin (5mM in drinking water) to block NOX2 activity. Drug treatment was started one day before switching mice to the HSFD and we recorded heart rhythm for four weeks. Apocynin prevented the increase in ventricular ectopy caused by HSFD; PVC frequency was unchanged from baseline recordings on regular chow for these mice (less than 0.3 PVC/hour for all mice at all time points) (Fig1D). The QT interval is also preserved by apocynin (Table 1). This supports NOX2 activation as a critical factor in arrhythmia caused by dietary saturated fat.

We also tested the role of NOX2 with NOX2 KO mice. ECG parameters at baseline, on regular chow, were the same at WT mice (Table 1). With HSFD, however, the NOX2KO

mice did not exhibit PVCs or long QT on the high saturated fat diet (Table 1 and Fig1D), demonstrating that deletion of NOX2 is protective in vivo.

High saturated fat diet promotes ventricular arrhythmia and repolarization dispersion in a NOX2-dependent manner

To determine if dietary saturated fat increases the substrate for arrhythmia we used a pacing protocol with perfused hearts to quantify ventricular arrhythmia susceptibility. These experiments showed an increase in ventricular tachycardia and ventricular fibrillation (VT/VF) inducibility in WT hearts after 4 weeks of HSFD (Fig2A,B,C and supplemental movie). None of the control chow hearts had inducible VT/VF, indicating that this was not an aggressive pacing protocol, whereas 5/6 HSFD hearts had inducible VT/VF. Both monomorphic VT and VF with rotor activity were induced in HSFD hearts. To further evaluate the substrate, we quantified repolarization heterogeneity with action potential dispersion in the LV epicardium. HSFD significantly increased repolarization heterogeneity. Hearts from NOX2 KO mice fed the same HSFD exhibited a small number of VT/VF episodes, and did not exhibit increased repolarization heterogeneity compared to control hearts (Fig2D,E), indicating that NOX2 is an important component of the arrhythmia mechanism.

Four weeks of high saturated fat diet does not change cardiac histology or cause cardiac hypertrophy

The histologic appearance of left ventricular (LV) cardiac tissue was normal after 4 weeks of HSFD. Trichrome staining showed only minimal perivascular fibrosis in both control and HSFD hearts and quantification of trichrome staining indicated no significant difference in cardiac fibrosis. (Fig3A,B). Total ventricular weight and heart weight to body weight ratios were not significantly different, demonstrating that this duration of HSFD does not cause cardiac hypertrophy (Fig3C). Echocardiography showed that there was no statistically significant change in ejection fraction or end-diastolic volume, indicating that this duration of HSFD does not cause systolic heart failure (Fig3D,E and supplemental table 2). In summary, the heart is structurally and histologically normal after HSFD.

High saturated fat diet causes spontaneous contractions from abnormal sarcoplasmic reticulum calcium leak in cardiomyocytes.

To determine if high saturated fat diet could increase ectopic beats in a cell-autonomous manner, we isolated cardiac myocytes from adult mice fed the high saturated fat diet (or regular chow for controls). We recorded contractions in isolated adult mouse ventricular myocytes, selecting cardiomyocytes with normal morphology that were not spontaneously contracting at baseline. After a short period of pacing at 1Hz (20–30 seconds to achieve steady-state contraction), pacing was stopped and spontaneous contractions were counted. Cardiomyocytes from high saturated fat diet mice had significantly more contractions than cardiomyocytes from control mice (Fig4A). Spontaneous contractions can be caused by abnormal intracellular calcium handling. To examine calcium handling, we measured calcium sparks, which were significantly increased in cardiomyocytes from WT mouse hearts after HSFD. Sparks in NOX2KO cardiomyocytes were not increased after high saturated fat diet (Fig4).

Since calcium leak generally involves post-translation modification of RYR2, we examined RYR2 proteins. RyR2 was oxidized in the WT hearts after HSFD (Fig4E). In contrast, NOX2KO hearts had minimal oxidation of RyR2 after HSFD, corresponding to the lack of significant increase in sparks.

To characterize cellular calcium handling, we also evaluated single-cell contractility, calcium transients, and sarcoplasmic reticulum (SR) calcium load. There were no significant differences in these measurements with the exception of a mild decrease in the return velocity of the calcium transients in the cardiomyocytes from HSFD hearts compared to chow control, which may suggest early stage diastolic dysfunction (table 2). The NOX2 cardiomyocytes did not show any significant differences compared to WT. Calcium transient amplitude and SR calcium content were similar comparing WT HSFD to WT chow and comparing WT to NOX2 KO (table 2, sup fig. 3).

High saturated fat diet activates cardiac PKC isoforms and CaMKIIdelta

Our group and others have shown that saturated fat activates NOX2 via PKC isoforms in cardiomyocytes[27, 28]. We performed western blots from membrane preparations of ventricular tissue, since activated PKC translocates to the membrane, which showed that PKC beta and delta isoforms are activated in the heart after 4 weeks of HSFD (supplemental Fig3). PKC alpha was not activated. Because previous reports have shown that the kinase Src is activated in the heart by disease states associated with oxidative stress, causing arrhythmogenic remodeling[17, 29], we also examined Src, but did not find significant activation (supplemental Fig4). In summary, dietary saturated fat activates PKC isoforms in the heart, which are known to activate NOX2.

CaMKIIdelta activation, caused by calcium or oxidative stress, is a well-established pathway in several forms of heart disease[30, 31]. Phosphorylation of CaMKII at threonine 286, which indicates activation, was significantly increased in mouse hearts after 4 weeks of high saturated fat diet (Fig5). The olive oil diet did not activate CaMKII. Oxidation of CaMKII as detected by immunoblot was not significantly increased by either high fat diet. To investigate the protective effect of NOX2 deletion in the heart, we examined CaMKII activation, which demonstrates that NOX2 deletion reduces CaMKII activation by HSFD (Fig5). Since oxidation of CaMKII was not increased, the activation of CaMK by NOX is more likely an indirect effect mediated by increased calcium leak from SR caused by oxidation of RyR2.

To determine if CaMK activation plays a role in the abnormal calcium handling, we isolated cardiomyocytes from mice fed HSFD and treated them with the CaMK inhibitor KN93 for one hour. CaMK inhibition reduced calcium sparks in cardiomyocytes, indicating that CaMK activation does have a significant role in the calcium handling abnormalities caused by high fat diet (fig 5E).

Potential roles for PKA and NOX4

Since the PKA pathway has been shown to have interactions with CaMK and NOX2[32], we examined PKA activation by quantifying phosphorylation. We found a nonsignificant trend

toward activation (sup fig 5A,B). Although we cannot exclude a role for PKA in this model system, it seems less likely that PKA is a major component.

NOX2 and NOX4 are both present in the rodent heart. We found that there is a small, nonsignificant increase in NOX4 in the hearts of WT mice after HSFD (sup fig 5C,D). We also found that NOX2 KO mice have a significant increase in NOX4 at baseline, which does not increase after HSFD (sup fig 5E,F). Since the NOX2 KO mice have more NOX4 protein, and these mice are protected, it seems unlikely that the increase in NOX4 is pathologic in this context but there could be some contribution to oxidative stress.

Discussion

Overall, these results support an arrhythmogenic effect of a high saturated fat diet, prior to the onset of obesity, due to NOX2 activation. Our findings demonstrate that pharmacologic NOX2 inhibition is anti-arrhythmic. Mechanistically, we show that dietary saturated fat changes the lipidomic profile of cardiac muscle, activating PKC isoforms in the heart. It is likely that PKC activation is upstream of NOX activation, based on prior work[27].

Our study shows the saturated fat is sufficient to promote arrhythmia, without fibrosis, hypertrophy or a decrease in contractility. The absence of contractile dysfunction and hypertrophy after a short duration of high-fat diet is consistent with prior literature[33, 34]. In contrast to saturated fat, unsaturated fat (olive oil diet) does not promote arrhythmia at the four-week time-point. Thus the type of fat is critically important, at least at this shorter time-point.

NOX2 activation in turn causes both abnormal calcium handling and abnormal repolarization (figure 5F). These findings may explain the mechanism of increased risk of sudden cardiac death in humans with obesity and/or high saturated fat intake, which is a consistent finding in several epidemiologic studies[1–4]. One issue regarding the nutritional aspect of the project is that the diets are not matched for carbohydrates. It is impossible to adjust percent fat without also changing percent carbohydrate and/or percent protein. Early the project we used two high-fat diets and the diet made from olive oil did not cause heart rhythm abnormalities in vivo, as shown in figure 1. This makes it very unlikely that reducing carbohydrates has an effect on heart rhythm, at least at this time-point.

Abnormal calcium homeostasis and heart rhythm

We show that a high saturated fat diet is sufficient to induce QT prolongation and ventricular ectopy. Prolongation of the QT interval and ventricular ectopy are both associated with increased mortality in the general population[35, 36]. PVCs themselves are probably not a cause of mortality, but appear to be a predictor of sudden cardiac death from sustained ventricular arrhythmias, which we induced in hearts from WT mice after feeding them the saturated fat diet. The increase in PVC frequency and QT interval caused by high saturated fat diet in our animal model mimics human heart disease caused by obesity. Frequent PVCs may be an early-warning sign of abnormal cardiac calcium homeostasis. We show that a high saturated fat diet increases sarcoplasmic reticulum calcium leak by causing post-translational modification of RyR2, which is known to promote arrhythmia.

Saturated fat, NOX2, and CaMK

Our data indicate that CaMK is activated in the heart by dietary saturated fat and CaMK activation is increased by NOX2 activation in vivo. Although our findings are novel, these data are consistent with prior work which has shown that NOX2 can be upstream of CaMK activation caused by angiotensin in isolated cardiomyocytes[32]. The strong protective effect in the NOX2KO mouse indicates that this is the major isoform that is activated in the rodent heart during high fat diet.

CaMK activation is known to increase SR calcium leak which could promote ventricular ectopy and our sparks experiments are consistent with this mechanism. The fact that CaMK is phosphorylated, but not oxidized, in our animal model indicates that NOX2 activation does not activate CaMK directly through increased ROS. Rather, CaMK activation is probably mediated by increased SR calcium leak from oxidized RyR2, but then CaMK further increases SR calcium leak in a positive feedback loop.

Abnormal repolarization

Abnormal calcium handling is probably the trigger for VT/VF in this model, but abnormal repolarization may be required as a substrate for sustained VT/VF. Our work shows that a high saturated fat diet is sufficient to cause abnormal repolarization in WT mice, without structural abnormalities. The pathways connecting saturated fat to abnormal repolarization may involve CaMK activation, which is known to modulate the function of the cardiac sodium channel (including late sodium current) and several potassium channels[37]. More research will be needed to examine the complex interactions of CaMK and repolarization in the context of high fat diet.

Translational potential

Although clinical trials of antioxidants for cardiovascular disease have been disappointing, for the most part, there is some clinical evidence supporting beneficial effects on heart rhythm[38, 39]. It may be that antioxidants are only beneficial in certain forms of cardiovascular disease, where increased ROS has a central role in the pathophysiology. Pharmacotherapy that specifically targets the source of cardiac ROS could be more effective. Our results demonstrate that pharmacologic inhibition of NOX2 is anti-arrhythmic *in vivo*.

Conclusion

We conclude that dietary saturated fat activates NOX2 which results in arrhythmia by causing increased sarcoplasmic reticulum calcium leak and abnormal ventricular repolarization. Genetic deletion of NOX2 or pharmacologic NOX2 inhibition prevents the pro-arrhythmic effect of high saturated fat diet in vivo. The mechanisms revealed by this work may have therapeutic implication for heart disease caused by diabetes and obesity and support NOX2 inhibition as a potential anti-arrhythmic therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations and Acronyms:

APD	action potential duration
CaMK	calcium/calmodulin-dependent protein kinase
DIO	diet induced obesity
HSFD	high saturated fat diet
HUFD	high unsaturated fat diet
NOX2	NADPH oxidase 2
PVC	premature ventricular complexes
ROS	reactive oxygen species
SCD	sudden cardiac death

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What is known:

- Clinical research demonstrates that obese people have an increased risk of sudden cardiac death
- Other studies indicate that high-fat diets increase the risk of sudden cardiac death. The mechanisms are not understood.

What the study adds:

- In mice a, high saturated fat diet causes increased ventricular ectopy (PVCs), prolongation of the QT interval, and increased susceptibility to induced ventricular tachycardia.
- These abnormalities are dependent on oxidative stress from activation of NOX2, by promoting abnormal calcium handling and repolarization heterogeneity.
- CaMK activation appears to be a downstream component of the pathophysiology and could contribute to both abnormal calcium handling and abnormal repolarization.

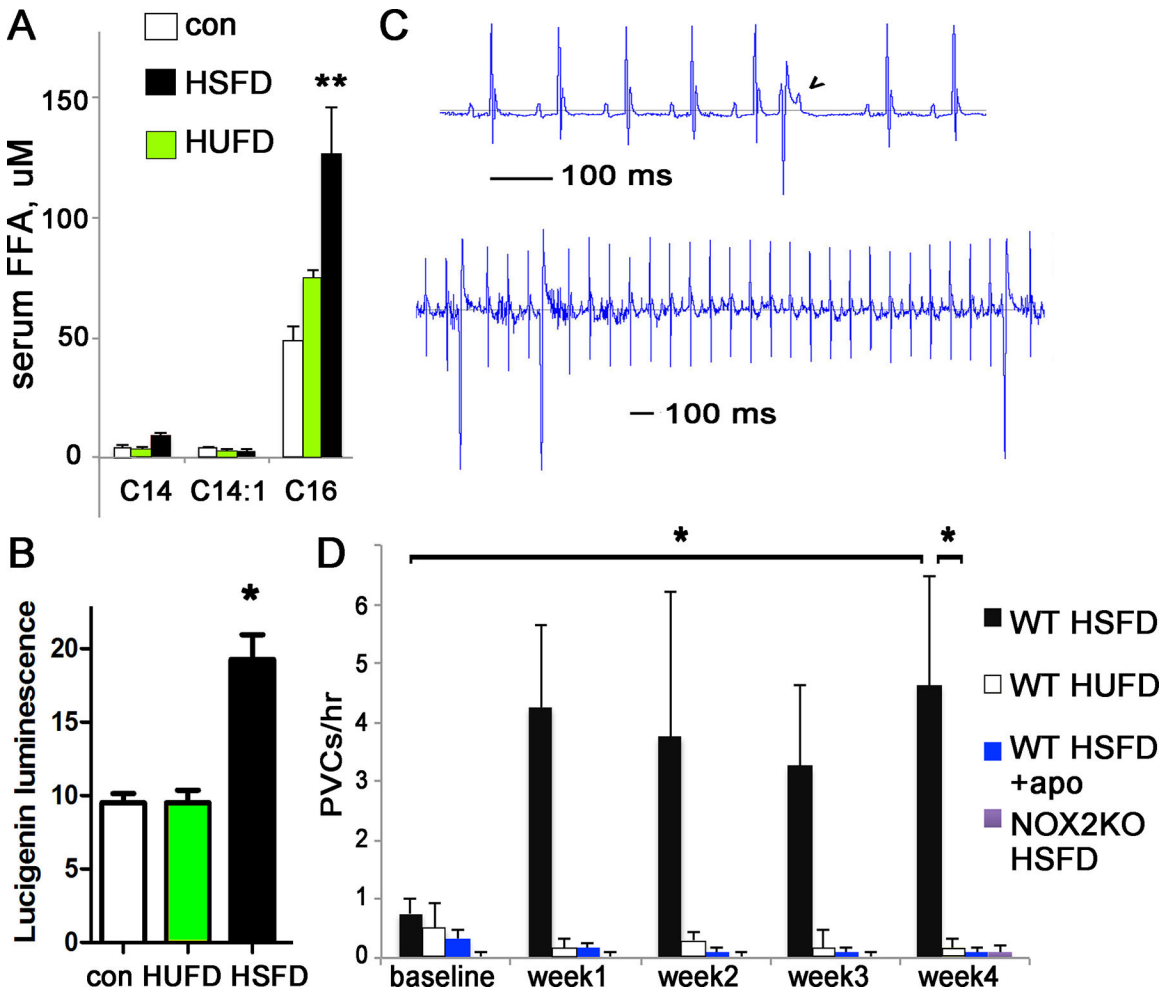

Figure 1. High saturated fat diet causes activation of NOX and ventricular ectopy

A. Graph of serum palmitate after 4 weeks of diet. **B**. NOX2 activity is increased in ventricular lysates from mice on high fat diets. **C**. Example of PVCs in a WT mouse fed PA diet; note the p-waves continue at the same rate despite the PVC (wide early QRS). Lower section shows frequent monomorphic PVCs in a mouse fed PA diet; note the time scale is compressed compared to upper section. **D**. Graph of PVCs/hour in unrestrained mice, before and during palm oil or olive oil diet.

For all panels n=4 animals for each group, error bars are SEM, * indicates $p<0.05$, ** indicates $p<0.01$

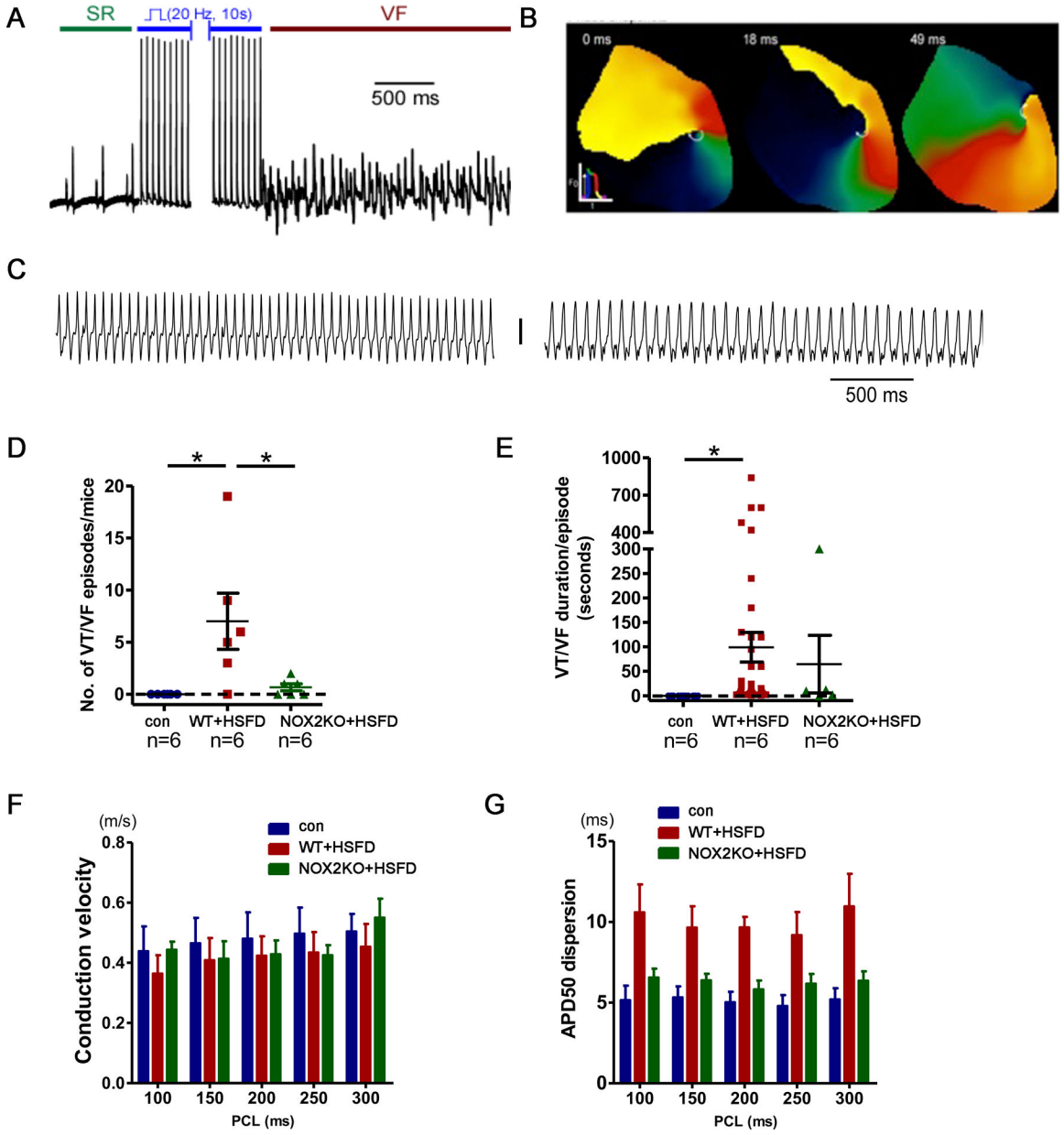

Figure 2. VT/VF in high saturated fat diet (HSFD diet) hearts

A. Example of VF in an HSFD heart after pacing. **B**. Optical mapping phase snapshots showing a rotor during VF. **C**. Examples of monomorphic VT after HSFD. **D**. Quantification of induced VT/VF, the means are different by ANOVA, * indicates significant difference by post-hoc testing. **E**. Quantification of duration of induced VT/VF, the means are different by ANOVA, * indicates significant difference by post-hoc testing. **F**. Conduction velocity is not significantly different between groups. **G**. APD dispersion is increased in WT hearts after HSFD but not NOX2KO hearts. Control and HSFD groups are significantly different by 2-way ANOVA with post-hoc testing. PCL = pacing cycle length

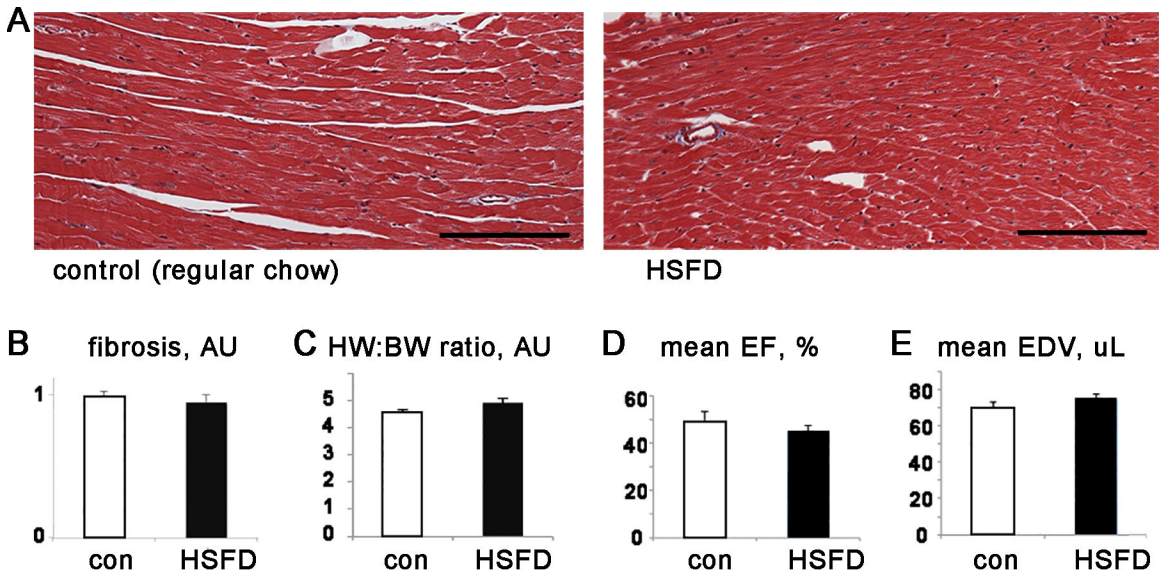

Figure 3. Four weeks of high saturated fat diet does not cause fibrosis, hypertrophy, or systolic dysfunction.

A. Representative histology, trichrome stain of LV tissue, scale bar = 100 μm. **B**. LV fibrosis quantification. **C**. Graph of heart weight to body weight ratio. **D**. Graph of mean ejection fraction (EF). **E**. Graph of LV end-diastolic volume (EDV). For B, n=4 each group, C,D,E n=7–8 each group, error bars are SEM, none of the comparisons are statistically significant

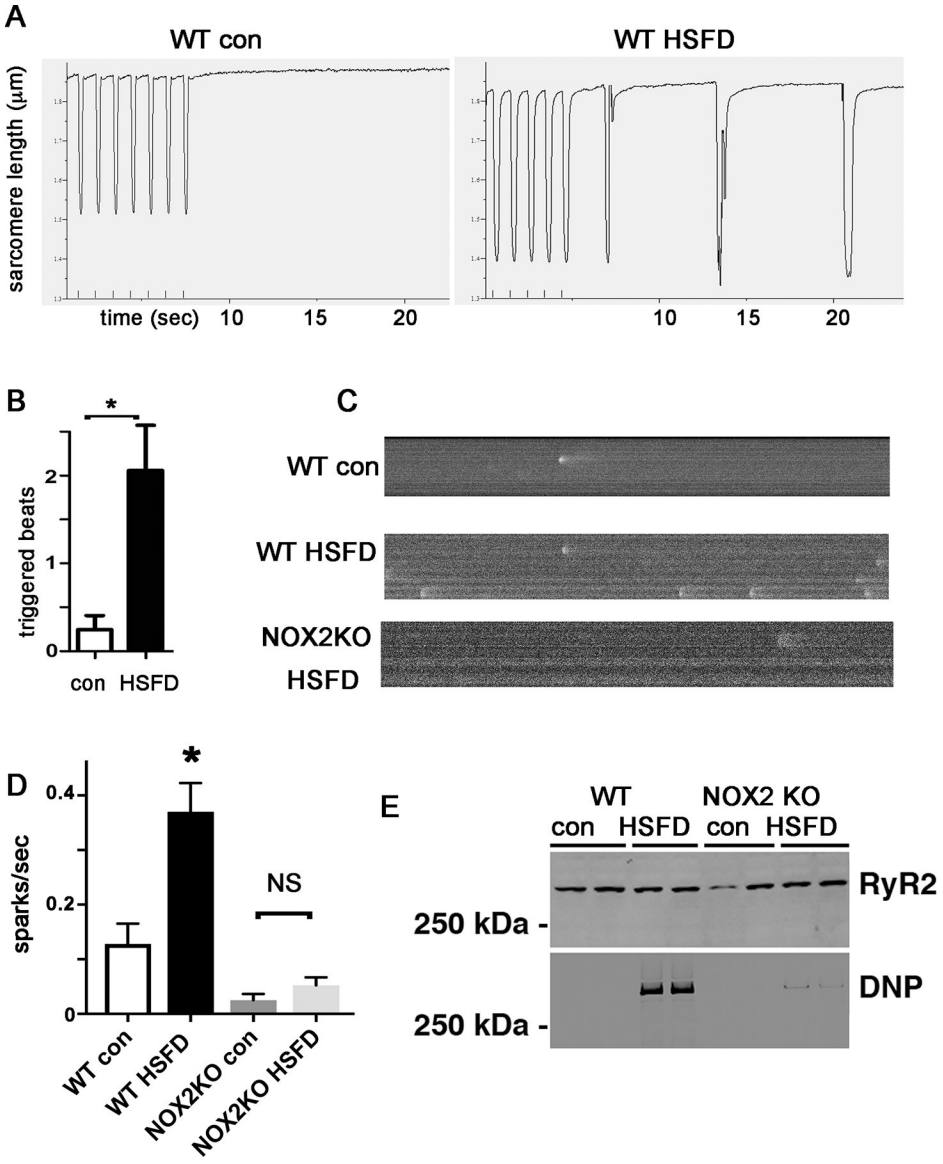

Figure 4. Spontaneous contractions and increased calcium sparks in cardiomyocytes from high saturated fat diet hearts

A. Raw data from isolated cardiac myocytes, pacing indicated by small vertical marks at the bottom of the rectangle, followed by cessation of pacing. Note that the high-fat diet cardiomyocyte demonstrates spontaneous contractions but the control does not. **B**. Graph of triggered beats from HSFD cardiomyocyte. N= 29–36 cells, from 3 hearts each group, error bars are SEM. **C**. Representative images of sparks from control and HSFD cardiomyocytes. **D**. Graph of calcium sparks/second from control and HSFD cardiomyocytes. N= 15–18 cells, from 3 hearts each group. * indicates $p<0.05$, error bars are SEM. **E**. Blots of RyR2 and oxidized RyR2 from mouse hearts. DNP = dinitrophenylhydrazine, indicating carbonyl from protein oxidation.

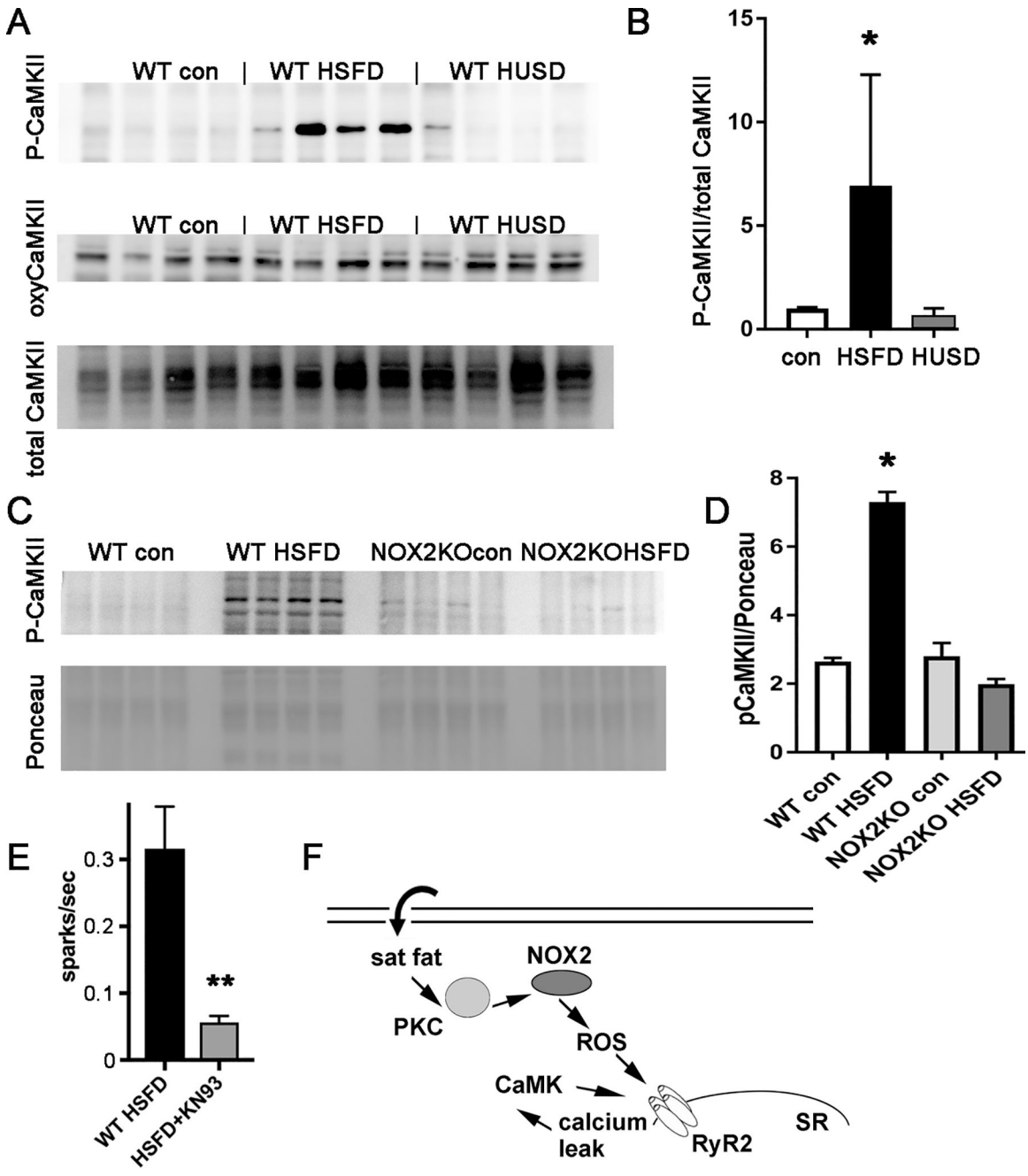

Figure 5. CaMKII is hyperphosphorylated in the WT heart after high saturated fat diet, but not after high unsaturated fat diet.

A. Representative images of immunoblots. **B**. Graph of immunoblots of P-CaMKII, normalized to total CaMK, from heart lysates; n=4 each group, error bars are SEM, the means are significantly different by ANOVA, * indicates significantly different from chow control by post-hoc test. **C**. Representative immunoblots showing that NOX2 KO hearts do not have a significant increase in P-CaMK after saturated fat diet. **D**. Graph of immunoblots of P-CaMKII, normalized to Ponceau, * indicates significantly different from WT control. **E**. Sparks quantification from WT cardiomyocytes after 4 weeks HSFD under control

conditions or with the CaMK inhibitor KN93. ** indicates P <0.01 by t-test, N=31–36 cells from 3 different cardiac isolations. **F**. Schematic of proposed pathways: dietary saturated fat activates NOX2 via PKC. NOX2 activation increases reactive oxygen species (ROS) and causes SR calcium leak, activating CaMK, which worsens SR calcium leak. CaMK = calcium/calmodulin kinase, PKC = protein kinase C, RyR2 = ryanodine receptor 2, SR = sarcoplasmic reticulum.

Table 1.

QT prolongs with HSFD diet in WT mice;

intervals are in milliseconds,

		RR	PR	QRS	QT
WT HUFD	basal	104.3 ± 0.9	35.1 ± 0.6	11.3 ± 0.3	42.8 ± 0.4
	4 wks	105.7 ± 0.3	35.7 ± 1.5	12.2 ± 0.2	43.0 ± 0.6
WT HSFD	basal	104.5 ± 1.0	33.8 ± 0.3	11.8 ± 0.3	43.8 ± 1.4
	4 wks	105.3 ± 1.3	36.3 ± 0.5 *	12.5 ± 0.3	49.5 ± 1.0 *
WT HSFD +apo	basal	104.0 ± 1.5	33.0 ± 0.4	11.5 ± 0.3	45.0 ± 0.4
	4 wks	103.5 ± 0.6	34.0 ± 0.4	11.6 ± 0.4	43.9 ± 0.4
NOX2KO HSFD	basal	107.0 ± 0.9	32.8 ± 0.2	12.0 ± 0.2	42.9 ± 0.7
	4 wks	106.4 ± 1.2	33.2 ± 0.8	11.2 ± 0.1	40.9 ± 1.1

* = $p<0.05$ compared to baseline.

Table 2.

Contractility and calcium transient measurements from of WT chow, WT HSFD, and NOX2 KO mice on chow.

	CONTRACTILITY		CALCIUM TRANSIENT			SR CALCIUM	
	AMP	RETv	AMP	DEPv	RETv	AMP	Ratio (transient vs total)
WT CHOW	14.5 ± 0.5	3.6 ± 0.2	45.2 ± 3.8	19.8 ± 2.3	−1.8 ± 0.2	64.4 ± 4.2	0.7 ± 0.04
WT HSFD	14.2 ± 0.6	4.5 ± 0.3	39.2 ± 2.5	19.0 ± 2.0	−1.4 ± 0.1*	60.5 ± 4.8	0.7 ± 0.04
NOX2KO CHOW	14.6 ± 0.4	3.7 ± 0.2	39.3 ± 2.5	24.7 ± 1.9	−1.7 ± 0.1	63.1 ± 4.4	0.7 ± 0.03

AMP= amplitude, DEPv = depolarization velocity, RETv = return velocity

N=3 hearts, n=29 cells for WT chow; N=4, n=44 for WT HSFD; N=3, n=30 for NOX2 KO CHOW (*p<0.05 vs control).