Oxidized Ca$^{2+}$/Calmodulin-Dependent Protein Kinase II Triggers Atrial Fibrillation

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Background—Atrial fibrillation (AF) is a growing public health problem without adequate therapies. Angiotensin II and reactive oxygen species are validated risk factors for AF in patients, but the molecular pathways connecting reactive oxygen species and AF are unknown. The Ca$^{2+}$/calmodulin-dependent protein kinase II (CaMKII) has recently emerged as a reactive oxygen species–activated proarrhythmic signal, so we hypothesized that oxidized CaMKII could contribute to AF.

Methods and Results—We found that oxidized CaMKII was increased in atria from AF patients compared with patients in sinus rhythm and from mice infused with angiotensin II compared with mice infused with saline. Angiotensin II–treated mice had increased susceptibility to AF compared with saline-treated wild-type mice, establishing angiotensin II as a risk factor for AF in mice. Knock-in mice lacking critical oxidation sites in CaMKIIb (MM-VV) and mice with myocardium–restricted transgenic overexpression of methionine sulfoxide reductase A, an enzyme that reduces oxidized CaMKII, were resistant to AF induction after angiotensin II infusion.

Conclusions—Our studies suggest that CaMKII is a molecular signal that couples increased reactive oxygen species with AF and that therapeutic strategies to decrease oxidized CaMKII may prevent or reduce AF. (Circulation. 2013;128:1748-1757.)

Key Words: angiotensin II | arrhythmias, cardiac | atrial fibrillation | calcium-calmodulin-dependent protein kinase type II | reactive oxygen species

Atrial fibrillation (AF) is the most common sustained arrhythmia. AF produces lifestyle-limiting symptoms and increases the risk of stroke and death, but current therapies have limited efficacy. The renin-angiotensin system is upregulated in cardiovascular disease, and elevated angiotensin II (Ang II) favors AF. Ang II activates NADPH oxidase, leading to increased reactive oxygen species (ROS), and fibrillating atria are marked by increased ROS. We recently identified the multifunctional Ca$^{2+}$- and calmodulin-dependent protein kinase II (CaMKII) as an ROS sensor and a proarrhythmic signal. Oxidation of critical methionines (methionines 281/282) in the CaMKII regulatory domain locks CaMKII into a constitutively active, Ca$^{2+}$-, and calmodulin-independent conformation that is associated with cardiovascular disease. On the basis of this information, we asked if oxidized CaMKII (ox-CaMKII) could be a biomarker and proarrhythmic signal for connecting increased atrial ROS to AF. We found that ox-CaMKII was increased in atrial tissue from patients with AF compared with patients in sinus rhythm and in atrial tissue from Ang II–infused compared with saline-infused mice. We used a validated mouse model of AF induction by rapid right atrial pacing and found that mice with prior Ang II infusion were at significantly higher risk of AF compared with vehicle-infused mice. We tested AF induction in Ang II– and vehicle-infused mice with genetically engineered resistance to CaMKII oxidation by knock-in replacement of methionines 281/282 with valines in CaMKIIb (MM-VV), the isoform associated

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with cardiovascular disease,11–14 or by myocardium-targeted antioxidant therapy by transgenic overexpression of methionine sulfoxide reductase A (MsrA), an enzyme that reduces ox-CaMKII.15,16 Collectively, our results support the view that Ang II promotes AF induction by increasing ROS, ox-CaMKII, CaMKII activity, sarcoplasmic reticulum Ca2+ leak, and delayed afterdepolarizations (DADs). Our findings provide novel insights into an ROS- and Ang II–dependent mechanism of AF by linking oxidative stress to dysfunctional intracellular Ca2+ signaling via ox-CaMKII and identify a potential new approach for treating AF by targeted antioxidant therapy.

Methods

Human Samples and Immunodetection of ox-CaMKII

The human samples were provided by the Georg-August-University Goettingen and the University of Heidelberg after approval by the local ethics committee of the Georg-August-University Göttingen and the Medical Faculty Mannheim, University of Heidelberg (No. 2011-216 N-MA). Each patient gave written informed consent. The investigation conforms to the principles outlined in the Declaration of Helsinki. Right atrial appendage tissue samples were obtained from patients with sinus rhythm or with AF undergoing thoracotomy (Table) as published previously.17 For immunostaining experiments, a total of 9 samples were studied, including 5 patients with sinus rhythm and 4 patients with AF (Table, middle). For immunoblotting, a total of 51 samples were studied from 25 patients with sinus rhythm and 26 patients with AF (Table, right). We reviewed the patient charts to obtain relevant clinical information. See the online-only Data Supplement for detailed methods.

Mouse Models and Experimental Methods

All mice used in the study were available to us in C57Bl/6 background. All experiments were performed in male mice 8 to 12 weeks of age. In total, we studied 262 mice. Numbers for each experimental group are provided in the figures or figure legends. See the online-only Data Supplement for detailed methods.

Statistics

Data are presented as mean±SEM. P values were assessed with a Student t test (2 tailed), ANOVA, or 2-way ANOVA, as appropriate, for continuous data. The effect of Ang II compared with saline on ox-CaMKII, CaMKII, and the ratio of ox-CaMKII to CaMKII was tested within each mouse genotype (strain) and compared among the 4 genotypes using 2-way ANOVA. The factors that were tested in the ANOVA model were genotype (wild-type [WT], MM-VV, p47−/−, and p47−/− transgenic mice with myocardium-delimited MsrA overexpression [MsrA TG]), treatment (Ang II versus saline), and genotype-treatment interaction effect. A significant genotype-treatment interaction indicated that the effect of Ang II (versus saline) differed significantly among the strains. Post hoc comparisons after ANOVA were performed with the Bonferroni test. Discrete variables were analyzed by the Fisher exact test. Statistical analyses were made with GraphPad Prism or SAS version 3.9, and the null hypothesis was rejected for values of P<0.05. See the online-only Data Supplement for detailed methods.

Results

ox-CaMKII Is Increased in AF

Patients with AF have increased atrial CaMKII activity18,19 and high circulating levels of serum markers for oxidative stress.4,5. We first obtained right atrial tissue from patients undergoing cardiac surgery (Table) and measured ox-CaMKII using a validated antiserum against oxidized methionines 281/282 in the CaMKII regulatory domains.6 These pilot immunofluorescence studies on atrial tissue samples made available on consent by patients with AF or normal sinus rhythm (Table, middle) showed significantly (P<0.05) higher (≈2.5 fold) ox-CaMKII levels in patients with AF (Figure 1A and 1B). On the basis of these initial findings, we measured ox-CaMKII in atrial tissue from a larger cohort of patients (Table, right; see also Figure I

Table. Summary of Patient Characteristics

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sinus Rhythm (n=5)</th>
<th>Atrial Fibrillation (n=4)</th>
<th>Sinus Rhythm (n=25)</th>
<th>Atrial Fibrillation (n=26)</th>
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<tr>
<td>Age, y</td>
<td>67±6</td>
<td>72±3</td>
<td>70±2</td>
<td>74±1</td>
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<tr>
<td>Male sex, n (%)</td>
<td>4 (80.0)</td>
<td>3 (75.0)</td>
<td>11 (44.0)</td>
<td>15 (57.7)</td>
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<td>Rhythm, n (%)</td>
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<td>Persistent/permanent AF</td>
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<td></td>
<td>Unclassified AF</td>
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<td>6 (23.1)</td>
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<td>Surgery, n (%)</td>
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<td>19 (76.0)</td>
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<td></td>
<td>Valve surgery</td>
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<td>9 (34.6)</td>
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<tr>
<td></td>
<td>CABG+valve surgery</td>
<td>4 (16.0)</td>
<td>8 (30.8)</td>
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<td>4 (100.0)</td>
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<td>Drug treatment, n (%)</td>
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Echocardiography

LA diameter, mm | 42±3 | 52±1* |
EF >45%, n (%) | 13 (52.0) | 10 (38.5) |
EF 35%–45%, n (%) | 7 (28.0) | 5 (19.2) |
EF <35%, n (%) | 0 (0.0) | 2 (7.7) |
EF (average), % | 58±6 | 48±8 |

Values are mean±SEM when appropriate. ACEI indicates angiotensin-converting enzyme inhibitor; AF, atrial fibrillation; ARB, angiotensin receptor blocker; CABG, coronary artery bypass graft; EF, ejection fraction; and LA, left atrial.

*P<0.05, Student t test, sinus rhythm vs AF from the same column.

Purohit et al ox-CaMKII and AF 1749
in the online-only Data Supplement) in sinus rhythm (n=25) or AF (n=26) using Western blots and confirmed that AF patients have significantly elevated expression of ox-CaMKII, whereas there was no difference in total CaMKII (Figure 1C–1F). The patient characteristics in the 2 groups (Table) were similar in terms of age, presence of hypertension and diabetes mellitus, and left ventricular ejection fraction, recognized risk factors for AF.20 The subgroup of AF patients who were not treated with angiotensin-converting enzyme inhibitor or angiotensin receptor blockers showed the highest levels of ox-CaMKII and total CaMKII (Figure IA and IB in the online-only Data Supplement). Taken together, these findings showed a positive association between AF and increased expression of atrial ox-CaMKII and a loss of this association in AF patients treated with angiotensin-converting enzyme inhibitors or angiotensin receptor blockers.

Ang II Treatment Enhances AF Susceptibility
To test the hypothesis that ox-CaMKII contributes to AF, we developed a mouse model of AF by infusing wild-type (WT) mice with Ang II (2000 ng·kg⁻¹·min⁻¹) or an equal volume of normal saline via osmotic minipumps for 3 weeks. We previously established that this dose of Ang II caused a significant increase in atrial ox-CaMKII and resulted in serum Ang II levels similar to those measured in heart failure patients.21 The subgroup of WT mice that were not treated with angiotensin-converting enzyme inhibitor showed the highest levels of ox-CaMKII and total CaMKII (Figure IA and IB in the online-only Data Supplement). Taken together, these findings showed a positive association between AF and increased expression of atrial ox-CaMKII and a loss of this association in AF patients treated with angiotensin-converting enzyme inhibitors or angiotensin receptor blockers.

ox-CaMKII Is Critical for AF
To test whether ox-CaMKII was required for AF induction in our model, we used oxidation-resistant knock-in MM-VV mice (Figure II in the online-only Data Supplement).22 CaMKII with the MM-VV mutation is resistant to oxidative activation but retains normal Ca²⁺- and calmodulin-dependent activation and is capable of transitioning into a Ca²⁺- and calmodulin-independent enzyme after threonine 287 autophosphorylation.23 The MM-VV mice were significantly resistant to AF induction after Ang II infusion compared with WT method (Figure 2A–2C).10 Mice treated with Ang II showed significantly higher AF induction rates compared with saline-treated mice (64% [9 of 14] versus 18% [2 of 14]; P=0.018, Fisher’s exact test; Figure 2D). Ang II is known to contribute to hypertension, left ventricular hypertrophy, and heart failure, all established clinical risk factors for AF.20 Therefore, we measured blood pressure (BP) by the tail-cuff method and assessed left ventricular size and systolic function by echocardiography. As expected, Ang II treatment significantly increased systolic BP (P<0.01; Figure 2E) and left ventricular mass (P<0.001; Figure 2F). Ang II–treated mice maintained a normal left ventricular ejection fraction, similar to saline-infused control mice (Figure 2G). These data showed that Ang II infusion increased the susceptibility of mice to AF induction by rapid right atrial pacing and established a framework for us to test the hypothesized role of ox-CaMKII in promoting AF.
controls (Figure 3A), suggesting that ox-CaMKII is required for increased AF susceptibility in Ang II–infused mice. WT mice treated with Ang II showed significantly higher (≈2.7 fold; 95% confidential interval, 1.4–5.1) levels of atrial ox-CaMKII compared with saline-treated mice. As expected, Ang II infusion increased ox-CaMKII less in MM-VV (≈2.1 fold; 95% confidential interval, 1.1–4.0) than in control WT mice (Figure 4C). The residual increase in ox-CaMKII in the MM-VV mice likely results from expression of CaMKII γ myocardial CaMKII isoform not affected by the MM-VV mutation.23 However, despite the greater increase in ox-CaMKII in WT compared with MM-VV mice, Ang II–related ROS production was increased in both WT and MM-VV mice to a similar degree (Figure IV in the online-only Data Supplement). Interestingly, Ang II–treated WT mice showed a significant decrease in total CaMKII levels (Figure IIIA and IIIB in the online-only Data Supplement), suggesting feedback inhibition of total CaMKII expression.

Atrial lysates from MM-VV mice showed significantly less Ca2+- and calmodulin-independent activity after Ang II treatment but retained WT-level CaMKII activity increases in response to isoproterenol (Figure IIA in the online-only Data Supplement). At 8 weeks, MM-VV mice had body weight (Figure IIB) and BP (Figure 3B) that were similar to those of WT mice, suggesting CaMKII methionine 281/282 oxidation did not affect basal BP or developmentally appropriate growth. CaMKII is known to regulate the chronotropic response to stress, and mice with CaMKII inhibition have a smaller increase in heart rate with isoproterenol treatment compared with controls.24 Isolated Langendorff-perfused hearts from WT and MM-VV mice had similar resting heart rates (Figure IIC in the online-only Data Supplement) and comparable heart rate increases after isoproterenol treatment (Figure IID in the online-only Data Supplement), suggesting that CaMKII-dependent physiological heart rate increases do not require CaMKII δ methionine oxidation. L-type Ca2+ currents were similar in
II–treated p47−/− mice did not show an increase in ox-CaMKII expression similar to that of saline-treated controls (Figure 4). The Ang II–induced increases in ROS production seen in WT atria were absent in atria from MsrA TG mice (Figure IV in the online-only Data Supplement), suggesting that MsrA-sensitive targets represent an important component of Ang II–mediated atrial oxidation. The protection from AF in MsrA TG mice appeared to be independent of prosor effects of Ang II because MsrA TG and WT mice showed similar increases in BP (Figure 3B). Taken together, these findings suggest that NADPH oxidase–dependent ROS and elevated ox-CaMKII are critical for the proarrhythmic actions of Ang II in pacing-induced AF and that targeted antioxidant therapy, by MsrA overexpression, can reduce or prevent AF in Ang II–infused mice.

**Ang II Increases Ca2+ Sparks and Triggered Action Potentials**

CaMKII contributes to increased sarcoplasmic reticulum Ca2+ leak in mice with a ryanodine receptor 2 (RyR2) mutation modeled after a human arrhythmia syndrome, catecholaminergic polymorphic ventricular tachycardia, in a goat model of AF and in atrial myocytes isolated from patients with AF. Atrial myocytes from patients with AF show increased CaMKII activity and increased CaMKII-dependent RyR phosphorylation at serine 2814. Furthermore, CaMKII inhibition with KN-93 reduced the open probability of single RyR2 channels and prevented the increased frequency of sarcoplasmic reticulum Ca2+ sparks in atrial myocardium biopsied from AF patients. On the basis of this knowledge, we asked whether increased RyR2 Ca2+ leak also contributed to the mechanism of AF in WT Ang II–infused mice and measured diastolic Ca2+ sparks, a marker of RyR2 Ca2+ leak. Atrial myocytes from Ang II–treated WT mice showed a significant (P<0.05) increase in spontaneous Ca2+ sparks compared with atrial myocytes from saline-treated control mice (Figure 5A and 5B). Other Ca2+ spark parameters and sarcoplasmic reticulum Ca2+ content were not different between the saline- and Ang II–treated WT mice (Figure VII in the online-only Data Supplement). In contrast to findings in WT mice, the atrial myocytes isolated from Ang II–treated MM-VV mice did not show an increase in Ca2+ sparks compared with saline-treated MM-VV mice (Figure 5A and 5B). A significantly greater proportion of atrial myocytes isolated from Ang II–treated WT mice showed DADs compared with atrial myocytes from saline-treated mice (P=0.03, Fisher exact test; Figure SC and 5D). In contrast, atrial myocytes from Ang II–infused MM-VV mice did not show a significant increase in DADs compared with the atrial myocytes from

**NADPH Oxidase and MsrA Regulate ox-CaMKII and AF Susceptibility**

Ang II increases intracellular ROS in myocardium by activating NADPH oxidase, and p47−/− mice, lacking functional NADPH oxidase, are resistant to Ang II–dependent increases in ROS and ox-CaMKII. Atrial lysates from Ang II–treated p47−/− mice did not show an increase in ox-CaMKII (Figure 4), and the p47−/− mice were also resistant to Ang II–mediated increases in AF (Figure 3A) but showed similar increases in BP (Figure 3B), overall heart weight (Figure 3C), and estimated left ventricular mass (Figure VI in the online-only Data Supplement) after Ang II treatment compared with WT controls. ox-CaMKII is reduced by MsrA, and MsrA TG mice have increased atrial MsrA protein (Figure IIIC in the online-only Data Supplement) and are resistant to ROS-induced myocardial injury. We found that Ang II–treated MsrA TG mice showed decreased AF induction compared with Ang II–treated WT mice (Figure 3A) and had atrial ox-CaMKII expression similar to that of saline-treated controls (Figure 4). The Ang II–induced increases in ROS production seen in WT atria were absent in atria from MsrA TG mice (Figure IV in the online-only Data Supplement), suggesting that MsrA-sensitive targets represent an important component of Ang II–mediated atrial oxidation. The protection from AF in MsrA TG mice appeared to be independent of prosor effects of Ang II because MsrA TG and WT mice showed similar increases in BP (Figure 3B). Taken together, these findings suggest that NADPH oxidase–dependent ROS and elevated ox-CaMKII are critical for the proarrhythmic actions of Ang II in pacing-induced AF and that targeted antioxidant therapy, by MsrA overexpression, can reduce or prevent AF in Ang II–infused mice.

**Figure 4. Oxidized Ca2+/calmodulin-dependent protein kinase II (ox-CaMKII) in atria after angiotensin II (Ang II) or saline treatment.** A, Atrial lysate immunoblots from wild-type (WT), MM-VV, p47−/−, and myocardium-restricted transgenic overexpression of methionine sulfoxide reductase A (MsrA TG) mice treated with Ang II or saline for 3 weeks and probed with an antiserum for ox-CaMKII. For quantification, ox-CaMKII bands were normalized to the total protein loading as assessed with Coomassie staining of the membrane. B, Increase in ox-CaMKII with Ang II treatment expressed relative to the saline-treated group. From each genotype, 4 saline-treated mice were used as controls. *P<0.05, WT Ang II vs WT saline ( ); in all other genotypes, P>0.05. Ang II vs saline; in addition, P=0.02 for WT Ang II vs MsrA TG Ang II and P=0.05 for MM-VV Ang II vs MsrA TG Ang II. C, Fold change in ox-CaMKII (over total CaMKII) in WT, MM-VV, and MM-VV mice (Figure V in the online-only Data Supplement). Data were analyzed with 2-way ANOVA (for treatment and genotype) and Bonferroni post hoc comparisons. The numbers in the bars indicate the sample size in each group. From each genotype, 4 saline-treated mice were used as controls. ***P<0.001 vs WT saline; **P<0.01 vs MsrA TG saline. Data were analyzed with 2-way ANOVA (for treatment and genotype) and Bonferroni post hoc comparisons. The numbers in the bars indicate the sample size in each group.
saline-treated MM-VV mice. We interpret these data to suggest that the proarrhythmic effects of Ang II infusion depend on an increase in ox-CaMKII, sarcoplasmic reticulum Ca\textsuperscript{2+} leak, and DADs.

**Mice With CaMKII-Resistant RyR2 Are Protected From AF After Ang II Infusion**

Enhanced CaMKII-mediated phosphorylation of serine 2814 on RyR2 is associated with an increased susceptibility to acquired arrhythmias, including AF.\(^{31}\) On the basis of our findings that atrial myocytes from Ang II–infused WT mice developed more Ca\textsuperscript{2+} sparks than atrial myocytes from saline-infused mice, we hypothesized that the proarrhythmic actions of ox-CaMKII require access to RyR2 serine 2814. We tested this hypothesis by treating mutant S2814A knock-in mice (lacking serine 2814)\(^{9}\) with Ang II or saline and performing right atrial burst pacing. The S2814A mice were highly resistant to Ang II–mediated AF (Figure 6A). Similarly, AC3-I mice with transgenic myocardial expression of a CaMKII inhibitory peptide\(^{32}\) were also resistant to the proarrhythmic effects of Ang II infusion on pacing-induced AF (Figure 6A). S2814A, AC3-I, and WT mice all developed similar BP increases (Figure 6B) and cardiac hypertrophy (Figure 6C) in response to Ang II, indicating that these mice were not resistant to the hemodynamic effects of Ang II but were nevertheless protected from AF.

**Discussion**

AF usually develops in patients with underlying structural heart disease such as left ventricular hypertrophy, coronary artery disease, valve disease, and congestive heart failure.\(^{20}\) Elevated ROS is a common feature of these conditions.\(^{33}\) The dose of Ang II used in our model produces a 4-fold increase in plasma Ang II compared with saline controls,\(^{7}\) similar to increases in Ang II observed in heart failure patients compared with nonhypertensive control subjects.\(^{21}\) Despite the extensive evidence of elevated ROS in structural heart disease, clinical trials with antioxidants have generally been unsatisfactory.\(^{34–36}\) One potential obstacle to developing effective antioxidant therapies is the lack of detailed understanding of molecular pathways that are affected by ROS. The renin-angiotensin system is one of the best-understood pathways that contribute to ROS production in AF patients.\(^{37}\) In the present study, we created a model of AF by infusing mice with Ang II for 3 weeks and assembled a cohort of genetically altered mice to rigorously test a novel molecular pathway that links oxidative stress to AF (Figure 7). Our present study provides strong evidence that CaMKII is a critical ROS sensor for transducing increased ROS into enhanced AF susceptibility in mice and suggests that atrial ox-CaMKII could contribute to AF in patients.

CaMKII and increased ROS are now widely recognized to contribute to cardiac arrhythmias.\(^{38,39}\) Recent studies suggest that patients with persistent AF have elevated markers of...
oxidative stress in serum and depleted levels of atrial glutathione. Under increased oxidative stress, CaMKII is activated by oxidation of methionines (methionines 281/282), which lock it into a constitutively active conformation, suggesting a possible role for ox-CaMKII as an ROS-activated proarrhythmic signal in AF. Our laboratory recently demonstrated that ox-CaMKII plays a major role in sinus node dysfunction, adverse post–myocardial infarct remodeling, and cardiac rupture. In the present study, we investigated the role of ox-CaMKII in AF. Human atria and Ang II–treated WT mouse atria showed significantly elevated ox-CaMKII (Figure 4). Atrial myocytes from Ang II–treated WT mice had a higher frequency of spontaneous Ca^2+ sparks and DADs compared with controls (Figure 5). From these findings, we hypothesized that the oxidation of methionines 281/282 on CaMKIIδ causes diastolic sarcoplasmic reticulum Ca^2+ leak and DADs, cellular AF triggers. To definitively test this hypothesis, we used a recently developed knock-in mouse (MM-VV) in which CaMKIIδ, the myocardial CaMKII isoform implicated in myocardial disease, is resistant to oxidative activation. Ang II treatment did not increase Ca^2+ sparks and calmodulin-independent CaMKII activity (Figure IIA in the online-only Data Supplement), Ca^2+ sparks (Figure 5A and 5B), or DADs (Figure 5C and 5D) or enhance AF susceptibility in MM-VV mice (Figure 3A). It is important to note that the MM-VV mutant form of CaMKIIδ selectively ablates the response to oxidation while retaining other aspects of CaMKII molecular physiology such as activation by Ca^2+ and calmodulin and constitutive activation by threonine 287 autophosphorylation. Thus, the residual AF observed in Ang II–infused MM-VV mice could be a result of non–oxidation-dependent mechanisms for CaMKIIδ activation in our model. We found that atrial tissue from AF patients treated with angiotensin-converting enzyme inhibitors or angiotensin receptor blockers did not show elevated ox-CaMKII, suggesting that Ang II stimulation oxidizes CaMKII in human atria and that ox-CaMKII–independent pathways are operative in AF patients. AF in patients is more complex than AF in our Ang II–infused mice. In particular, patients present with variable chronicity, tissue, and structural changes. In contrast, the triggers for our mice are uniform (ie, Ang II infusion and rapid right atrial pacing) and result in a similar, modest degree of hypertrophy. We interpret the data showing that an increase in ox-CaMKII in AF patients is reduced or eliminated by clinical antagonist drugs that reduce Ang II signaling as validating our findings in mice that Ang II increases ox-CaMKII. However, we suppose that the presence of AF in patients on angiotensin-converting enzyme inhibitors or angiotensin receptor blockers means that other pathways also result in AF. Our sample is not

**Figure 6.** Ca^2+/calmodulin-dependent protein kinase II (CaMKII) activation and ryanodine receptor 2 serine 2814 are required for atrial fibrillation (AF) in angiotensin II (Ang II)–infused mice. A, AC3-I and S2814A mice were treated with Ang II for 3 weeks and then burst paced to induce AF. AC3-I and S2814A mice were resistant to Ang II–mediated AF promotion compared with WT Ang II–treated mice (*P<0.05 vs all, Fisher exact test). B, AC3-I and S2814A mice show similar systolic blood pressure (sBP) elevation after treatment with Ang II. Final sBP measurements were performed on 3 consecutive days before AF induction, as shown in A. C, Ang II treatment causes similar cardiac hypertrophy in AC3-I and S2814A mice compared with saline controls (***P<0.001 vs AC3-I saline and **P=0.01 vs S2814A saline). The numbers in the graph indicate the number of mice in each group.

**Figure 7.** Schematic illustrating the proposed mechanism of atrial fibrillation (AF) in angiotensin II (Ang II)–infused mice. Ang II binding activates NADPH oxidase (NOX) to increase reactive oxygen species (ROS), leading to oxidation of methionines 281/282 in Ca^2+/calmodulin-dependent protein kinase II (ox-CaMKII). Elevated ox-CaMKII phosphorylates serine 2814 on ryanodine receptor 2 (RyR2), causing enhanced diastolic Ca^2+ leak that promotes AF, triggering delayed afterdepolarizations. Genetically modified mice were used to test key steps of the proposed pathway. ATR indicates Angiotensin II receptor; MsrA TG, myocardium-restricted transgenic overexpression of methionine sulfoxide reductase A; and SR, sarcoplasmic reticulum.
powered to ask whether AF resistance to Ang II antagonist drugs represents later-stage disease, but this is our hypothesis. Furthermore, CaMKII can be activated independently of oxidation, although oxidation appears to be the primary pathway for activating CaMKII during Ang II infusion. Thus, it is unknown whether CaMKII is also important for AF progression in the group of patients treated by Ang II antagonist drugs who exhibit normal levels of ox-CaMKII.

Although we did not see higher total CaMKII in AF patients (compared with patients in sinus rhythm), the subgroup of AF patients who were not treated with angiotensin-converting enzyme inhibitors or angiotensin receptor blockers showed significantly elevated CaMKII levels, supporting prior studies that reported elevated CaMKII activity in AF. In contrast to the situation in patients, total CaMKII expression was reduced in mice after subcutane Ang II infusion. Although the mechanism for the variable response of CaMKII expression in mice and patients is unclear, the change in CaMKII expression in mice and in humans in response to manipulation of the Ang II pathway supports the idea that CaMKII is a fundamental component of Ang II signaling. The relatively small number of patient samples is not powered for analysis of AF subtypes, but human AF may transition from paroxysmal to persistent and permanent (chronic) forms. In contrast, our mouse model is simpler because it is triggered by a single upstream event (ie, Ang II infusion) and is elicited in a highly controlled environment by rapid atrial pacing. The resistance of MM-VV mice to AF provides new evidence that oxidative activation of CaMKII is important for initiation of AF; whereas the finding that ox-CaMKII is elevated in atrial tissue from AF patients and particularly in AF patients naive to Ang II antagonist therapies suggests that this pathway may also participate in human AF. Thus, our findings in MM-VV mice provide strong, mechanistic evidence that oxida-

Clinical, there appears to be an overlap in sinus node dysfunction and AF. Almost half of patients enrolled in the Mode Selection Trial (MOST) with sinus node dysfunction had a history of AF, but a clear mechanistic link between increased risk of AF and sinus node dysfunction is unknown. In recent studies, we showed that Ang II– and diabetes mellitus–induced CaMKII oxidation caused sinus node dysfunction by increased pacemaker cell death and fibrosis, whereas MM-VV mice are resistant to sinus node dysfunction evoked by hyperglycemia. Here, we provide evidence that ox-CaMKII increases the susceptibility for AF via increased diastolic sarcoplasmic reticulum Ca2+ release, showing that the proarrhythmic actions of ox-CaMKII may occur in cardiomyocytes by increasing sarcoplasmic reticulum Ca2+ leak or by enhanced cell death. Our findings suggest that the clinical association between sinus node dysfunction and AF might have a mechanistic basis because sinus node dysfunction and AF are downstream consequences of elevated ox-CaMKII.

Acknowledgments

We are grateful to Dr Miriam B. Zimmerman for her help with the statistical analysis of the data. We are also grateful to Kathy Zimmerman and Melissa Davis for performing the mouse echocardiograms. We acknowledge Jinying Yang for her assistance in maintaining mouse colonies. We thank Chantal Allamargot and the University of Iowa Microscopy Research Facility for their assistance in microscopic imaging and Shawn Roach for graphic art. We also thank the cardiac surgeons of Heart Centers Göttingen and Heidelberg for the kind provision of human atrial tissue samples.

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Disclosures

Dr Anderson is a cofounder of Allosters Therapeutics, a biotech company aiming to develop enzyme inhibitors to treat arrhythmias. The other authors report no conflicts.

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1755

Purohit et al ox-CaMKII and AF

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Atrial fibrillation is associated with hyperactivity of renin–angiotensin II signaling, enhanced oxidant stress, and increased activity of the multifunctional Ca\textsuperscript{2+} and calmodulin-dependent protein kinase II (CaMKII). Excessive CaMKII activity promotes arrhythmia initiation by enhancing Ca\textsuperscript{2+} leak from intracellular stores. We recently identified a mechanism whereby CaMKII is activated by oxidation of regulatory domain methionines in response to angiotensin II stimulation and, motivated by these findings, developed new mouse models to test the potential role of oxidation-activated CaMKII in atrial fibrillation. We identified increased oxidized CaMKII in atria from patients with atrial fibrillation compared with nonfibrillating control subjects and determined that atrial fibrillation patients treated with angiotensin-converting enzyme inhibitors or angiotensin receptor blockers did not have increased atrial oxidized CaMKII. These findings suggest that CaMKII is oxidized by the renin-angiotensin II pathway and is associated with atrial fibrillation in the subgroup of patients not treated with angiotensin-converting enzyme inhibitors or angiotensin receptor blockers. Angiotensin II–infused mice also showed increased atrial oxidized CaMKII and high rates of atrial fibrillation after rapid right atrial pacing. In contrast, genetically engineered mice with oxidation-resistant CaMKII and mice with atrial overexpression of a methionine-reducing enzyme, methionine sulf oxide reductase A, were resistant to atrial fibrillation induction, intracellular Ca\textsuperscript{2+} leak, and angiotensin II infusion–induced increases in oxidized CaMKII. These findings suggest that CaMKII is a critical component of a proarrhythmic oxidant pathway and that CaMKII inhibition could be an effective antioxidant and antiarrhythmic therapy.
Oxidized Ca\textsuperscript{2+}/Calmodulin-Dependent Protein Kinase II Triggers Atrial Fibrillation

Anil Purohit, Adam G. Rokita, Xiaohun Guan, Biyi Chen, Olha M. Koval, Niels Voigt, Stefan Neef, Thomas Sowa, Zhan Gao, Elizabeth D. Luczak, Hrafnhildur Stefansdottir, Andrew C. Behunin, Na Li, Ramzi N. El-Accaoui, Baoli Yang, Paari Dominic Swaminathan, Robert M. Weiss, Xander H.T. Wehrens, Long-Sheng Song, Dobromir Dobrev, Lars S. Maier and Mark E. Anderson

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SUPPLEMENTAL MATERIAL
Methods

**Immunofluorescence staining.** Human right atrial appendage tissue was placed in Tyrode’s solution. Tissue was then cryopreserved and sectioned at 10μm thickness. For all immunofluorescence staining, the slides were air dried for 5 minutes, fixed in formalin for 30 minutes, and washed in PBS, 3 times, 5 minutes each. The sections were incubated with a blocking buffer (3% gelatin from cold water fish skin, 0.1% Triton X, and 2 mg/ml BSA in PBS) with normal serum. The tissue was incubated overnight in the primary antibody (rabbit anti–mouse ox-CaMKII). The next day slides were washed in PBS and the tissue incubated with appropriate secondary antibodies for 1 hour. The slides were then washed and cover-slipped with VECTASHIELD (Vector Laboratories) with DAPI mounting medium.

**Immunoblotting for human samples.** Patient atrial myocardium was homogenized in Tris buffer as previously reported containing (in mM) Tris-HCl 20, NaCl 200, NaF 20, Na3VO4 1, DTT 1, 1% Triton X-100 (pH 7.4) and complete protease-inhibitor cocktail (Roche). Protein concentration was determined by BCA assay (Pierce Biotechnology). Protein concentration was adjusted to 1 μg/μl with phosphate buffered saline (PBS) and 5 X Laemmli buffer without β-mercaptoethanol was added (1:20) and samples were heated to 95°C for 30 min. Denaturated tissue homogenates were subjected to Western blotting (8% SDS-polyacrylamide gels) using anti-CaMKII (1:15000, gift from D. M. Bers, University of California, Davis, CA) and anti-ox-CaMKII (1:15000) antibodies.
Chemi-luminescent detection was done with SuperSignal West Pico Substrate (Pierce).

**Mouse Models and Experimental Methods.** p47⁻/⁻ mice were purchased from Jackson Labs. Mice with transgenic myocardial CaMKII inhibition (AC3-I)¹, MM-VV knock-in mice² and MsrA transgenic mice³ were generated by our laboratory as described. S2814A knock-in mice were generated by the Wehrens laboratory.⁴ All animal studies were reviewed and approved by the IACUC of the University of Iowa.

**HR and BP measurements.** One week prior to the start of each experiment, mice were trained on the tail cuff BP equipment (Visitech BP-2000 blood pressure analysis system). HR and BP recorded for four consecutive days. The readings from days 1-2 were discarded and days 3-4 were averaged for the baseline value. Saline or Ang II pumps were implanted after collecting the baseline data. HR and BP were measured for at least 4 days a week (to maintain habituation to the apparatus and handling) for 3 weeks. The last 3 days of data were averaged to assess the effect of 3 weeks of treatment.

**Mini-osmotic pump implantation.** Mini-osmotic pumps (Alzet model 1004, 0.11 µl/hr, 28 days) containing saline or Ang II (2000 ng/kg/min) were inserted subcutaneously under anesthesia (ketamine/xylazine- 87.5/12.5 mg/kg), as previously reported.⁵

**Transthoracic Echocardiography:** We recorded transthoracic echocardiograms in conscious mice three weeks after Ang II pump implantation, as previously
described. Images were acquired and analyzed by an operator blinded to mouse genotype and treatment.

**In vivo Electrophysiology (EP studies).** Mice were anesthetized with isoflurane (2% for induction and 1.5% for maintenance of anesthesia; Isotec100 Series Isoflurane Vaporizer; Harvard Apparatus). During EP studies the mouse body temperature was monitored by an intra-rectal probe and controlled using mousepad circuit board equipped with a heating element (Mousepad, THM 100, Indus Instruments, USA). All studies were performed at 37.0 ± 0.5°C. We used a Millar 1.1F octapolar EP catheter (EPR-800; Millar Instruments) inserted via the right jugular vein, as previously described. A computer-based data acquisition system (Powerlab 16/30; ADI instruments) was used to record a 1-lead body surface ECG and up to 4 intracardiac bipolar electrograms (Labchart Pro software, version 7; ADI instruments). In brief, right atrial pacing was performed using 2 ms current pulses delivered by an external stimulator (STG-3008; Multi Channel Systems). Using an automated stimulator, inducibility of atrial fibrillation was tested by decremental burst pacing. Burst pacing started at a 40 ms cycle length, decreasing by 2 ms every 2 seconds to a cycle length of 20 ms. Burst pacing was repeated one minute after the previous burst concluded or the termination of AF. Burst pacing was performed for a total of five times in each mouse. AF was defined as the occurrence of rapid and fragmented atrial electrograms with irregular AV-nodal conduction and ventricular rhythm for at least 1 second. If at least 2 bursts (out of 5) produced AF, the mouse was considered inducible. Mice with < 2 bursts of AF were considered non-inducible.
**Immunoblotting for mouse samples.** Mice were sacrificed at the end of Ang II or saline infusion 3 weeks after implantations of the mini-osmotic pumps. Hearts were excised, and the atria were separated from the ventricles and flash frozen in liquid nitrogen. Atria were then homogenized in modified RIPA buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% v/v NP-40, and 0.5% w/v deoxycholate), containing a mixture of protease and phosphatase inhibitors. Equal amounts of protein were fractionated on NuPAGE Bis-Tris gels (Invitrogen) and transferred onto PVDF membranes (Bio-Rad). After blocking nonspecific binding with 5% w/v non-fat milk powder in TBS-T (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% v/v Tween-20), blots were incubated in primary antibodies (ox-CaMKII [1:500] and CaMKII [1:250]) overnight at 4°C. The same procedure was applied for atrial immunoblots from untreated MsrA transgenic and WT littermate mice incubating with a primary anti-MsrA antibody (1:1000, abcam). Blots were washed in TBS-T and incubated with appropriate HRP-conjugated secondary antibodies. Protein bands were detected using ECL reagent (Lumi-Light, Roche), and loading was monitored by Coomassie staining of the blots after antibody probing. Atrial lysates from Ang II treated mice were run side by side on the same gel with lysates from saline treated mice from the same genotype. Quantification was performed using Image J analysis software (version 1.46, NIH).

**ROS detection.** Murine left atria, acquired as described above, were mounted and flash frozen in Tissue Freezing Medium (Triangle Biomedical Sciences, Durham). The tissue was then cryo sectioned (30 μm), rinsed with PBS and
incubated in DHE (2 × 10^{-2} M, Invitrogen) for 15 minutes at room temperature in darkness as previously described with minor alterations. Fluorescence was measured using a laser scanning confocal microscope (Zeiss 510 and 710; excitation at 488 nm and detection at 585 nm by using a long-pass filter). For quantification and analysis, tissue fluorescence was corrected for the nuclear fluorescence signal and normalized to the area of the imaged tissue section using Image J analysis software (version 1.46, NIH).

**Atrial myocyte isolation.** Atrial myocytes were isolated from adult mouse hearts using an established protocol with minor modifications.

**Voltage clamp.** Voltage and current signals were measured with an Axon 200B patch-clamp amplifier controlled by a personal computer using a Digidata 1320A acquisition board driven by pClamp 8.0 (Axon Instruments). We used perforated (amphotericin B) patch for \( I_{\text{Ca}} \) and action potential studies. All experiments were conducted at T=35°C. Recording pipettes, fabricated from borosilicate glass, had tip diameters of 2–3 μm and resistance of 2–4 MΩ, when filled with recording solution. All solutions were adjusted to 275–295 mOsm.

**Ca^{2+} Imaging.** Calcium imaging was performed as described previously. Briefly, atrial myocytes were loaded with Fluo-3 AM (5 μM, 20 min) at room temperature. After de-esterification, the cells will be perfused with normal Tyrode solution (1.8 mM Ca^{2+}). Confocal Ca^{2+} imaging will be performed with a laser scanning confocal microscope (LSM 510 Meta, Carl Zeiss) equipped with a NA=1.35, 63x lens. Line-scan measurement of Ca^{2+} transients, SR content and sparks were acquired at a sampling rate of 1.93 ms/line along the longitudinal
axis of the myocytes. Ca\(^{2+}\) sparks were measured under resting conditions.
Steady state Ca\(^{2+}\) transients were achieved by a 30 sec pacing at 1 Hz.
Sarcoplasmic reticulum Ca\(^{2+}\) content was measured as a global Ca\(^{2+}\) release
induced by 20 mM caffeine. All digital images were processed with IDL 6.0
(Research System Inc).

**Statistics.** Data are presented as mean ± SEM. P values were assessed with a
Student’s t-test (2-tailed), ANOVA or two-way ANOVA, as appropriate, for
continuous data. Post hoc comparisons after ANOVA were performed using the
Bonferroni test. Discrete variables were analyzed by Fisher’s exact test. The null
hypothesis was rejected for p < 0.05. From the fitted ANOVA model, tests of
mean contrast were performed to test for differences between Ang II versus
saline within each genotype (mouse strain) with p-values adjusted using
Bonferroni’s method to account for the number of tests performed (i.e. adjusted
for 4 tests corresponding to each genotype). Similarly, tests of mean contrast
were also used for pair-wise comparison of the Ang II versus saline differences
between the genotypes with the p-value adjusted using the Bonferroni step-down
method (adjusted for pairwise comparisons among all genotypes, or a total of 6
comparisons). The two-way ANOVA was performed on the natural log (Ln)
transformation of the (normalized to Coomassie) ox-CaMKII, CaMKII, and ox-
CaMKII/CaMKII ratio data. This was done because the data were not normally
distributed and there was inequality of variance among the genotype-treatment
groups. By applying the Ln transformation, the data distribution was normalized
and homogeneity of variance was achieved. Since the mean estimates from the
ANOVA are in the Ln transformed scale, these were back-transformed to obtain
means in the original scale, which corresponds to the geometric mean. The ratio
of Ang II relative to saline group means in the original scale were computed by
back-transformation of the difference between Ang II and saline treatment group
means in the Ln scale.
References


Figure legend for supplementary figures:

Supplementary Figure 1. Subgroup analysis of CaMKII expression in patients. Immunoblotting was performed on atrial tissue obtained from patients in SR and AF using antisera against ox-CaMKII (A) and total CaMKII (B). Patients with AF were divided into two subgroups based on their use of angiotensin converting enzyme inhibitors (ACE-i) or angiotensin receptor blockers (ARB), and the respective ox-CaMKII (A) and total CaMKII (B) levels were compared with levels in the SR group (*p<0.05 and p values as shown above, one-way ANOVA with post-hoc Tukey test). The % values indicate the mean ox-CaMKII/GAPDH ratio (panel A) and CaMKII/GAPDH ratio (panel B) in the subgroups as normalized to the corresponding mean values in the SR groups. C. Complete films for total CaMKII and GAPDH immunoblots with red boxes highlighting the bands shown in Figure 1E. All lanes were included in the analysis. D. Complete films for ox-CaMKII and GAPDH immunoblots with red boxes highlighting the bands shown in Figure 1C.

Supplementary Figure 2. Characterization of MM-VV mice.

A. Similar increase in Ca^{2+} and calmodulin independent CaMKII activity with Isoproterenol treatment (daily injections) in WT and MM-VV mice, but no increase with Ang II in MM-VV mice as compared to WT littermates (*p<0.05 ANOVA, post-hoc Tukey test). B. MM-VV and WT mice had similar body weights at 8 weeks of age. C. Similar baseline heart rates (HR) in Langendorff-perfused hearts from WT and MM-VV mice at 8 weeks of age. D. Similar increases in HR
(\% over baseline shown in E) in response to 10 mM isoproterenol (Student’s t-test) between WT and MM-VV Langendorff-perfused hearts.

**Supplementary Figure 3. Atrial CaMKII and MsrA expression**

**A.** Immunoblots for total CaMKII from atrial lysates isolated from mice treated with Ang II or saline for 3 weeks. For quantification, CaMKII bands were normalized to the total protein loading as assessed with Coomassie staining of the membrane.  

**B.** Summary data for total CaMKII expression in Ang II treated mice relative to saline treated mice of the same genotype. From each genotype 4 saline treated mice were used as controls. **p<0.01 versus WT saline, in addition p = 0.002 for WT Ang II versus MsrA TG Ang II and p = 0.013 for WT Ang II versus p47\(^{-/-}\) Ang II.** Data were analyzed using two-way ANOVA (for treatment and genotype) with Bonferroni post-hoc comparison.

**C.** Immunoblots for MsrA from atrial lysates isolated from MsrA TG and WT littermates. Coomassie staining of the membrane was used as a loading control.

**Supplementary Figure 4. Atrial ROS in Ang II and saline treated mice.**

**A.** Representative DHE fluorescence images taken in cryo-preserved left atrial myocardium from WT, MM-VV, p47\(^{-/-}\) and MsrA TG mice after 3 weeks of Ang II or saline infusion. **Horizontal calibration bars indicate 100 \(\mu\)m.**

**B.** Summary of left atrial DHE fluorescence data. For each genotype, the bars represent the mean intensity of DHE fluorescence in the Ang II group as relative to DHE fluorescence in the saline group. From each genotype 4 saline treated mice were
used as controls. *p<0.05 versus saline from the WT and the MM-VV group separately.

**Supplementary Figure 5. L-type Ca\(^{2+}\) current facilitation.**  
**A.** Peak L-Type Ca\(^{2+}\) currents recorded from atrial myocytes of WT and MM-VV mice (N=2 and 3 mice per group, respectively).  
**B.** Summary data for maximal facilitation of L-Type Ca\(^{2+}\) currents. Atrial myocytes from WT and MM-VV mice show similar peak L-type Ca\(^{2+}\) current density and facilitation (same amount of mice per group as in A). Facilitation is abolished by the CaMKII inhibitor KN-93 (500nM). *p<0.001 for WT versus WT + KN-93 and for MM-VV versus MM-VV + KN-93, and #p<0.05 WT versus MM-VV (Student’s t-test). Each point represents an N=5-7 cells.  
**C.** Exemplary L-Type Ca\(^{2+}\) current tracings from the 1\(^{st}\) and 4\(^{th}\) voltage command step (-40 to 0 mV) in panel B to demonstrate facilitation.

**Supplementary Figure 6. Hypertrophic response to Ang II.**

LV mass as estimated by echocardiography performed after 3 weeks of Ang II or saline treatment (all comparisons versus saline controls, *p<0.05 and ***p<0.001, Student’s t-test).

**Supplementary Figure 7. Ca\(^{2+}\) spark parameters and sarcoplasmic reticulum Ca\(^{2+}\) content in WT mice.**

**A.** Average spatial width (FWHM, full-width at half-maximal amplitude in \(\mu m\)).  
**B.** Average duration (FDHM, full duration at half-maximal amplitude in ms).  
**C.** Average amplitude of spontaneous Ca\(^{2+}\) sparks in WT atrial myocytes treated with 3 weeks of Ang II or saline. Ang II N=46 cells from 6 mice and saline N=23
cells from 5 mice; no significant differences between Ang II and saline treatments. **D.** Sarcoplasmic reticulum (SR) Ca$^{2+}$ transient and Ca$^{2+}$ content (rapid SR depletion with 20 mM caffeine spritz) were similar in atrial myocytes from Ang II and WT saline treated mice (N=4 mice each group).
Supplementary Figure 2

A

Ca\textsuperscript{2+}/CaM independent CaMKII activity

\[ P < 0.05 \]

\[ P < 0.05 \]

\begin{tabular}{c|c|c|c}
 & Saline & Ang II & Isoproterenol \\ 
WT & 5 & 6 & 4 \\ 
MM-VV & 5 & 6 & 4 \\ 
\end{tabular}

B

Body weight (mg)

\begin{tabular}{c|c}
 & WT & MM-VV \\ 
7 & 8 \\ 
\end{tabular}

C

Heart Rate (bpm)

\begin{tabular}{c|c}
 & WT & MM-VV \\ 
5 & 7 \\ 
\end{tabular}

D

% change in HR with Iso

\begin{tabular}{c|c}
 & WT & MM-VV \\ 
5 & 7 \\ 
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Supplementary Figure 3

A

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B

CaMKII

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Supplementary Figure 4

A

Saline

WT  MM-VV  p47⁻/-  MsrA TG

Ang II

B

relative DHE fluorescence intensity in %

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* p < 0.05 compared to WT
Supplementary Figure 6

![Supplementary Figure 6](image)

Supplementary Figure 7

![Supplementary Figure 7](image)