Circulation Research Peer Review Report for DOI: 10.1161/CIRCRESAHA.123.323571

Title: Nitric Oxide Modulates Ca2+ Leak and Arrhythmias via S-Nitrosylation of CaMKII

June 22, 2022

Dr. Jeffrey Erickson
University of Otago
270 Great King Street
Dunedin 9016
New Zealand

RE: CIRCRES/2022/321457: Nitric Oxide modulates spontaneous Ca2+ release and ventricular arrhythmias during β adrenergic signalling through S-nitrosylation of Calcium/Calmodulin dependent kinase II

Dear Dr. Erickson:

Your manuscript has been carefully evaluated by 3 external reviewers and the editors as an Original Research article. We regret to inform you that the paper is not acceptable for publication in Circulation Research.

As you will gather from the reviews, the referees identified a number of substantive conceptual and methodological problems. Additional data are needed to confirm the conclusions. The editors concur. Major issues include the lack of detailed phenotyping and in vivo characterization of the mice. Additional studies are also needed to demonstrate that NO has similar effects to GSNO.

Given the nature of these concerns, which could not be adequately addressed without extensive new experimentation, the editors do not encourage revision. Nevertheless, if you feel that you can effectively address the reviewers' comments and are willing to perform the new experiments required, we would be willing to evaluate a resubmitted version on a de novo basis. The paper would be reviewed again, with no assurance of acceptance. Since the re-evaluation would be done de novo, the revised paper would be assigned a new number regardless of when it is resubmitted. One or more of the original reviewers would be re-consulted; the editors may also choose to obtain additional opinions from new reviewers. Please note that even after extensive modifications, we cannot guarantee that your manuscript will receive a priority sufficient for publication. Overall, fewer than 15% of all papers submitted to Circulation Research are eventually published.

As detailed in the reviewers' critiques, a responsive resubmission would require a substantial amount of new data. In particular, the editors feel that additional data would be necessary to address all the concerns of the reviewers. In particular, the editors feel that additional data would be necessary to address the concerns raised by the reviewers. In particular it would be important to provided additional phenotyping of the mice and additional in vivo characterization. It would also be necessary to increase the n values and to show that nitric oxide has a similar effect to GSNO. It would also be important to define the contribution of the NO-sGC-cGMP pathway.

To read the comments to authors from the reviewers, please see below.

If you choose to resubmit, please include a detailed response to each of the referees' and editors' comments, providing each comment verbatim in bold followed by your response and giving the exact page number(s),

paragraph(s), and line number(s) where each change was made. If you make substantive changes to the manuscript, please provide a clear description of what you did and where. If you insert important sentences, paragraphs, or sections in response to the comments, please also include them in your response. Please indicate clearly any deletions. Additionally, a marked up version of the resubmission with the changes highlighted or tracked should be uploaded as a supplemental file. If you do choose to resubmit, please do so online using the "Submit Resubmission" link available in your Author Tasks area or Post Decision Manuscripts folder.

Please ascertain that your resubmitted manuscript adheres to the Instructions to Authors as they appear online at https://www.ahajournals.org/res/author-instructions. The Editors strongly encourage you to adhere to the journal's Statistical Reporting Recommendations in your resubmission, which can be found here: https://www.ahajournals.org/statistical-recommendations.

We know that you will be disappointed by this decision. Circulation Research currently receives approximately 2,000 manuscripts a year, of which fewer than 15% can be published; as a consequence, relative priorities must be considered in making the final decision.

Despite our decision, we wish to thank you for having submitted this manuscript to Circulation Research.

Sincerely,

Jane E. Freedman, MD
Editor-in-Chief
Circulation Research
An American Heart Association Journal

****

Reviewer comments to the Authors:

Reviewer #1:
Authors sought to determine the role of S-nitrosylation of CAMKII using in isolated murine CM, ex vivo langendorff model, and in vivo assessment of cardiac function with echocardiography. Justification of the study is sound and experimental design is logical and well developed. I have a few concerns as described below.
1. Cardiomyocytes are paced at 0.5 Hz which is not a physiological heart rate for mouse heart. What is the justification of this heart rate? Would the results be same if the CM are paced at a higher rate such as 6-8 Hz?
2. While authors acknowledged that other important calcium handling proteins are also S-nitrosylated, effects of those proteins are not addressed in the study. Are levels of SERCA and RyR2 and their S-nitrosylation levels same between WT and KI mice? This is more important because of the depressed cardiac function of the KI mice at 12 weeks. Is there a possibility that altered SERCA or RyR2 levels influence the phenotypes of KI CM?

Reviewer #2:
I read this work with much interest and although I think it potentially important, there are a number of questions that need to be addressed so we can be more confident about the findings and their importance to ventricular arrhythmias and the conclusions the authors have made.

Much of the data comes from n = 3 and I find this somewhat uncomfortable as this really increases the chances of type I / II errors. In many of the cases, I'd personally like the replication to be double what it currently is. Indeed,

it would be reassuring that the same principal observations can be made when experiments are repeated at another time. This is an especially important issue given the data reproducibility crisis.

Erickson (Cell 2008) utilized a C290V mutant that was "indistinguishable from that with WT CaMKII" and there were studies with alkylating agents allowed the following concluding to be made: "oxidative activation of CaMKIIδ is independent of cysteines". I think careful discussion of how this fits with the new SNO data and concept is needed. It is difficult to think a cysteine that is a target for an oxidizing form of nitric oxide is unreactive with peroxide. Didn't previous studies from Erickson rule out a role for cysteine oxidation in CaMKII?

The study uses GSNO, which induces efficient trans-nitrosylation of proteins, but this is likely not the case with nitric oxide donors that are clinically relevant. Given the authors conclude this work has "important implications for the administration of NO donors in the clinical setting", it would be crucial to index protein S-nitrosylation when they are used and at a clinically relevant amount. I suspect that protein S-nitrosylation will not be detected under such conditions because GSNO or CysNO are commonly used in studies such as these because of their ability to induce the modification. In addition, the GSNO here is used as a concentration that is highly questionable in terms of biology or disease. The clinical nitric oxide donors will be an order of magnitude or more lower than the what is used here and it would be important to consider that soluble guanylyl cyclase and cGMP would be an important player that needs to be considered. I mean can the authors rule out contributions by the NO-sGC-cGMP pathway?

Related to the comment above, Figure 1A/B indexes NO release from GSNO, but I am not sure what it adds. One view is that it provides evidence that the 0.15 mM release NO at a steady state concentration that is more relevant to NO in cells. Firstly, the amount detected by the electrode analytical system is still supra-physiologic and it does not address the issue that GSNO is not a clinically used donor and given the conclusion made, such compounds (ideally several) should be tested for their ability to induce CaMKII-SNO. Unfortunately, I think that if a mouse is given a clinically used dose of such donors, that global protein S-nitrosylation or indeed CaMKII-SNO is unlikely to be observed. Quite simply the GSNO is used because it directly transfer (i.e. trans-nitrosylate) proteins it encounters. If the envisaged concept is that the low amount of authentic NO that was measured causes S-nitrosylation, one should consider that NO does NOT directly react with thiol. Many studies, including this have shown this: https://www.sciencedirect.com/science/article/pii/S0021925818879078?via%3Dihub have shown that authentic NO does not react with thiol. Thus, they state that "at pH 7.0, contrary to published reports, nitric oxide by itself does not react with thiols to yield nitrosothiol."

Activity assays with WT or mutant recombinant kinase in vitro would be an extremely valuable addition given the complexity of cell systems. In this connection do the authors truly detect kinase-SNO modification with clinical NO donors and what is the stoichiometry of the kinase S-nitrosylation. This is a crucial issue as there is evidence that protein-SNO is labile and not the regulatory end effector that it is often thought to be, as shown in this work: https://pubmed.ncbi.nlm.nih.gov/29358077/ In addition, there is substantive additional evidence for their instability as reviewed here: https://pubmed.ncbi.nlm.nih.gov/28189849/

Can the authors rule out methionine oxidation in the models studied here. Interventions with 0.15 mM GSNO is likely to cause systemic cellular protein oxidations, including disulfide-Prdx1/2 formation. This will decrease their peroxidase activity and thus this species will accumulate and signal.

Are there site-specific changes to S-nitrosylations of the kinase under conditions that alter susceptibility to arrhythmias? I am particulalrly thinking about conditions where pharmacological interventions are not used. Are changes in rate (without iso) associated with alterations to CaMKII-SNO?

I found it difficult to see anything in Figure 4D. I realize the detection is better than my eyes, but then I wonder about showing the images.

Reviewer #3:
This manuscript investigates the effects of NO on CaMKII and RyR function (sparks), and arrhythmias during B-AR activation. While there is a vast amount of data on the effects of NO, CaMKII, and RyR, and B-AR, the authors generated two novel mouse models with a single CaMKII S-nitrosylation point mutated. The authors generated excellent myocyte data. However, additional experiments must be performed for the paper to be considered for publication.

MAJOR COMMENTS:
1) there is a lack of detail on the generation of the novel CaMKII knockin models (i.e., background strain, etc.).

2)also there is a lack of characterization of these mice. There was echocardiography performed on one of the mice (C273S) with limited number of parameters listed in table 1. It appears that these hearts are dilated. Is there hypertrophy? fibrosis? What happens to these mice as they age? Can this explain some of the differences observed in this mouse vs WT. The other novel mouse model c290A must also be characterized.

3) It's surprising that the authors did not perform further experiments on their novel mouse models. These authors should perform telemetry and investigate arrhythmogenesis in vivo.

MINOR COMMENTS:

1) It is somewhat surprising that there was no difference in the ISO-induced Ca2+ transient amplitude or SR Ca content in the CaMKII KO myocytes considering there was a significant reduction in sparks.

2) Is the C273S mice similar to the CaMKII KO mice in regards to Ca handling during ISO stimulation?

3) Any thoughts on how one can S-nitrosylate CaMKII-C273 as a therapeutic in the clinics?

Responses to comments by the Editor and Reviewers

Editor comments:
As detailed in the reviewers' critiques, a responsive resubmission would require a substantial amount of new data. In particular, the editors feel that additional data would be necessary to address all the concerns of the reviewers. In particular, the editors feel that additional data would be necessary to address the concerns raised by the reviewers. **In particular it would be important to provided additional phenotyping of the mice and additional in vivo characterization. It would also be necessary to increase the n values and to show that nitric oxide has a similar effect to GSNO. It would also be important to define the contribution of the NO-sGC-cGMP pathway.**

We thank the editor and reviewers for their constructive feedback on our manuscript. In response to their suggestions, we have produced new data addressing key points raised in the review. Specifically, we have produced an entirely new figure characterizing our novel C273S mice, which includes a gene editing map, quantification of several calcium handling proteins, fibrosis stains, and cell size measurements to assess for hypertrophy (Figure 3). We also added new experiments with an additional clinically used NO donor (Na-nitroprusside; SNP) which is distinct from GSNO in its mechanism of nitrosylation. We have also added additional experimental data testing whether the observed GSNO effects might be mediated by soluble guanylate cyclase (sGC). We repeated some key experiments with a selective inhibitor of NO-dependent sGC activity, ODQ (1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one; Figure 5). Pretreatment of myocytes with 10 μM ODQ did not alter our prior observation that NO donors suppress ISO-induced increases in Ca sparks in WT, but not in CaMKIIδ-C273S, myocytes.

Reviewer #1 comments:
Authors sought to determine the role of S-nitrosylation of CAMKII using in isolated murine CM, ex vivo Langendorff model, and in vivo assessment of cardiac function with echocardiography. Justification of the study is sound and experimental design is logical and well developed. I have a few concerns as described below.

1. Cardiomyocytes are paced at 0.5 Hz which is not a physiological heart rate for mouse heart. What is the justification of this heart rate? Would the results be same if the CM are paced at a higher rate such as 6-8 Hz?

The Ca transients and sparks were recorded at room temperature, as is typical in this field, in part to ensure cell stability during experiments (doi:10.1161/CIRCRESAHA.117.312257/-/DC1, doi:10.1161/CIRCRESAHA.120.318402). At that temperature, pacing at physiological heart rates is not feasible. However, we believe that higher pacing rates would tend to increase the sensitivity of the myocytes to the NO effects, as CaMKII would be more active in these conditions (DOI: 10.1161/CIRCRESAHA.111.247148). We have added an acknowledgement of this point to the Discussion.

2. While authors acknowledged that other important calcium handling proteins are also S-nitrosylated, effects of those proteins are not addressed in the study. Are levels of SERCA and RyR2 and their S-nitrosylation levels same between WT and KI mice? This is more important because of the depressed cardiac function of the KI mice at 12 weeks. Is there a possibility that altered SERCA or RyR2 levels influence the phenotypes of KI CM?

We have added new data quantifying the levels of SERCA and RyR2 in the C273S animals (vs. WT, Figure 3). Interestingly, while RyR2 expression was unaffected by the C273S mutation of CaMKII and SERCA expression were increased in the mutant mice. This observation suggests that the C273S mutant may have undergone compensation to increase calcium uptake, possibly in response to elevated baseline CaMKII activation and enhanced calcium leak during diastole. Direct measurement of SERCA and RyR2 nitrosylation is very difficult due to the size and localization of these proteins. However, it is important to note that nitrosylation effects on SERCA and RyR2 would be present in all of our mouse models. Thus, we attribute the

differences we observe in cellular calcium handling and whole heart arrhythmia to nitrosylation of CaMKII.

Reviewer #2 comments:
I read this work with much interest and although I think it potentially important, there are a number of questions that need to be addressed so we can be more confident about the findings and their importance to ventricular arrhythmias and the conclusions the authors have made.

Much of the data comes from n = 3 and I find this somewhat uncomfortable as this really increases the chances of type I / II errors. In many of the cases, I'd personally like the replication to be double what it currently is. Indeed, it would be reassuring that the same principal observations can be made when experiments are repeated at another time. This is an especially important issue given the data reproducibility crisis.

We have added new data throughout that is derived from cohorts with larger n values to address this concern.

Erickson (Cell 2008) utilized a C290V mutant that was "indistinguishable from that with WT CaMKII" and there were studies with alkylating agents allowed the following concluding to be made: "oxidative activation of CaMKIIδ is independent of cysteines". I think careful discussion of how this fits with the new SNO data and concept is needed. It is difficult to think a cysteine that is a target for an oxidizing form of nitric oxide is unreactive with peroxide. Didn't previous studies from Erickson rule out a role for cysteine oxidation in CaMKII?

Indeed, the reviewer is correct that in the original Erickson et al. (Cell 2008) study, we did not observe an effect on CaMKII oxidation in a C290V mutant. However, much of this work was done with saturating levels of hydrogen peroxide while the Methionine residues at 281/282, key targets for CaMKII oxidation, were intact. Subsequently, both our group (Hegyi et al. Circ Res. 2021) and a team that included Mark Anderson (Rocco-Machado et al. J Biol Chem 2022), the senior author on the Cell 2008 paper, have published work more specifically examining oxidation of C290 and have concluded that there is a likely role for this site. We have amended the Discussion to acknowledge the potential role for C290 in CaMKII oxidation.

The study uses GSNO, which induces efficient trans-nitrosylation of proteins, but this is likely not the case with nitric oxide donors that are clinically relevant. Given the authors conclude this work has "important implications for the administration of NO donors in the clinical setting", it would be crucial to index protein S-nitrosylation when they are used and at a clinically relevant amount. I suspect that protein S-nitrosylation will not be detected under such conditions because GSNO or CysNO are commonly used in studies such as these because of their ability to induce the modification. In addition, the GSNO here is used as a concentration that is highly questionable in terms of biology or disease. The clinical nitric oxide donors will be an order of magnitude or more lower than the what is used here and it would be important to consider that soluble guanylyl cyclase and cGMP would be an important player that needs to be considered. I mean can the authors rule out contributions by the NO-sGC-cGMP pathway?

We have added data (Figure 5) showing the effects of a more clinically relevant NO donor, sodium nitroprusside (SNP), on calcium transients and sparks in WT and C273S mice. As the reviewer anticipated, the SNP was less potent in our assays than GSNO. However, SNP was able to inhibit Iso induced sparks in myocytes from WT, but not C273S, mice, as was observed with GSNO. We have also added data repeating our calcium transient and spark measurements using the soluble guanylyl cyclase inhibitor ODQ. Our data demonstrate that, even in the presence of ODQ, pre-treatment with nitric oxide donors ablate isoproterenol induced sparks. We have amended the Discussion to contextualize these results and acknowledge the possibility of cross-talk between NO, cGMP, and CaMKII.

Related to the comment above, Figure 1A/B indexes NO release from GSNO, but I am not sure what it adds. One view is that it provides evidence that the 0.15 mM release NO at a steady state concentration that is more relevant to NO in cells. Firstly, the amount detected by the electrode analytical system is still

supra-physiologic and it does not address the issue that GSNO is not a clinically used donor and given the conclusion made, such compounds (ideally several) should be tested for their ability to induce CaMKII-SNO. Unfortunately, I think that if a mouse is given a clinically used dose of such donors, that global protein S-nitrosylation or indeed CaMKII-SNO is unlikely to be observed. Quite simply the GSNO is used because it directly transfer (i.e. trans-nitrosylate) proteins it encounters. If the envisaged concept is that the low amount of authentic NO that was measured causes S-nitrosylation, one should consider that NO does NOT directly react with thiol. Many studies, including this have shown this: https://www.sciencedirect.com/science/article/pii/S0021925818879078?via%3Dihub have shown that authentic NO does not react with thiol. Thus, they state that "at pH 7.0, contrary to published reports, nitric oxide by itself does not react with thiols to yield nitrosothiol."

The reviewer is correct that different NO donors exert their effects through different mechanisms. Our new data in Figure 5 demonstrate that both GSNO and SNP are able to modulate Iso induced sparks, and that this spark modulation requires the C273 site on CaMKII. Defining the exact mechanism of CaMKII nitrosylation by various NO donors is an ongoing question. We have amended the Discussion to acknowledge these critical points.

Activity assays with WT or mutant recombinant kinase in vitro would be an extremely valuable addition given the complexity of cell systems. In this connection do the authors truly detect kinase-SNO modification with clinical NO donors and what is the stoichiometry of the kinase S-nitrosylation. This is a crucial issue as there is evidence that protein-SNO is labile and not the regulatory end effector that it is often thought to be, as shown in this work: https://pubmed.ncbi.nlm.nih.gov/29358077/ In addition, there is substantive additional evidence for their instability as reviewed here: https://pubmed.ncbi.nlm.nih.gov/28189849/

We have previously reported the results of activity assays using our CaMKII activity sensor Camui during conditions that would lead to nitrosylation (Erickson et al. J Biol Chem 2015). For this manuscript, we have added data demonstrating that the clinically relevant NO donor SNP appears to induce autonomous CaMKII activity and enhance Ca^{2+} sparks.

Can the authors rule out methionine oxidation in the models studied here. Interventions with 0.15 mM GSNO is likely to cause systemic cellular protein oxidations, including disulfide-Prdx1/2 formation. This will decrease their peroxidase activity and thus this species will accumulate and signal.

We cannot rule out methionine oxidation in our assays. However, it is important to note that the methionine sites on CaMKII that are subject to oxidation are present in all of our mouse models (aside from the CaMKII KO). That we see highly differential effects from the NO donors in the WT, C290A, and C273S myocytes and hearts despite the presence of the MM281/282 site in all of them suggests that the observed physiological effects do not derive from methionine oxidation.

Are there site-specific changes to S-nitrosylations of the kinase under conditions that alter susceptibility to arrhythmias? I am particulalrly thinking about conditions where pharmacological interventions are not used. Are changes in rate (without iso) associated with alterations to CaMKII-SNO?

These data were presented in a previous publication (Erickson et al. J Biol Chem 2015). In that manuscript, we showed data demonstrating the relationship between nitric oxide, CaMKII activity, and arrhythmia susceptibility. We have amended the Introduction of this submission to more carefully describe those findings. Changes in CaMKII-SNO with pacing rate would be interesting but technically challenging (see comments to Reviewer 1) and out of scope for this study.

I found it difficult to see anything in Figure 4D. I realize the detection is better than my eyes, but then I wonder about showing the images.

Now Figure 6D is shown to demonstrate an observation of interest: pre-treatment with GSNO in the C273S myocytes results in a surprisingly low frequency of calcium sparks. We attribute this observation to the high incidence of calcium waves, as observed in the far left of the scan. Other

groups have also described cellular conditions that result in high wave frequency and quenching of sparks, and thus we believe that some groups may be interested in seeing this example.

Reviewer #3 comments:
This manuscript investigating the effects of NO on CaMKII and RyR function (sparks), and arrhythmias during B-AR activation. While there is a vast amount of data on the effects of NO, CaMKII, and RyR, and B-AR, the authors generated two novel mouse models with a single CaMKII S-nitrosylation point mutated. The authors generated excellent myocyte data. However, additional experiments must be performed for the paper to be considered for publication.

MAJOR COMMENTS:
1) there is a lack of detail on the generation of the novel CaMKII knockin models (i.e., background strain, etc.).

We have included an entirely new figure (Figure 3) outlining the generation of the C273S mouse model. In addition, we have added more detail to the Methods section to address this point. The generation of the C290A mice is described in a parallel paper by Alim et al. *J Physiology* 2022. That paper is now cited in the present study and includes description of the CRISPR strategy used to generate the C290A model.

2)also there is a lack of characterization of these mice. There was echocardiography performed on one of the mice (C273S) with limited number of parameters listed in table 1. It appears that these hearts are dilated. Is there hypertrophy? fibrosis? What happens to these mice as they age? Can this explain some of the differences observed in this mouse vs WT. The other novel mouse model c290A must also be characterized.

Figure 3 also includes new data characterizing the C273S mouse model, including expression of Ca handling proteins, fibrosis, and cell size/hypertrophy. Characterization of the C290A mouse model has been published in Alim et al. *J Physiol* 2022, and this work has been cited here.

3) It's surprising that the authors did not perform further experiments on their novel mouse models. These authors should perform telemetry and investigate arrhythmogenesis in vivo.

We have added ECG experiments comparing the WT and C273S animals (Figure 7). Excitingly, our ECG data demonstrate that a subset of the C273S mice have apparent arrhythmias that are detectable in their ECG, indicative of enhanced baseline cardiac stress and calcium mishandling.

MINOR COMMENTS:
1) It is somewhat surprising that there was no difference in the ISO-induced Ca transient amplitude or SR Ca content in the CaMKII KO myocytes considering there was a significant reduction in sparks.

This observation was a bit surprising, but both this study and prior studies by our group and others show only modest effects, if any, of CaMKII on calcium transient amplitude despite substantial effects on calcium sparks/waves. This, we think, is because CaMKII sensitizes the RyR, such that with CaMKII inhibition (or KO) you may enhance SR Ca content, but obtain a lower fractional release of Ca, resulting in a similar Ca transient amplitude.

2) Is the C273S mice similar to the CaMKII KO mice in regards to Ca handling during ISO stimulation?

Our data indicate that the C273S mice have increased propensity for Ca leak in myocytes and arrhythmias in Langendorff perfused hearts during ISO stimulation, while CaMKII KO animals are protected in both of those assays.

3) Any thoughts on how one can S-nitrosylate CaMKII-C273 as a therapeutic in the clinics?

This is a good question. One key advantage to nitrosylation of the C273 site is that our data here and in our previous work (Erickson et al. *J Biol Chem*, 2015) indicates the C273 site is available for nitrosylation even when the kinase is inhibited. This suggests that a well-timed clinical dose of a NO donor prior to a cardiac stress may potently inhibit CaMKII and thus protect the heart from arrhythmias and other CaMKII related pathologies. We have amended the Discussion to better reflect on this point.

September 26, 2023

Dr. Jeffrey Erickson
University of Otago
270 Great King Street
Dunedin 9016
New Zealand

RE: CIRCRES/2023/323571D: Nitric Oxide modulates spontaneous Ca2+ release and ventricular arrhythmias during β adrenergic signalling through S-nitrosylation of Calcium/Calmodulin dependent kinase II

Dear Dr. Erickson:

Your manuscript has been carefully evaluated by 5 external reviewers and the editors as an Original Research manuscript. We regret to inform you that the paper is not acceptable for publication in its present form.

As you will gather from the reviews, the referees identified a number of conceptual and methodological problems. The editors concur. A suitably revised manuscript would need to address the concerns of the statistical and technical Reviewers. It would also be necessary to discuss in the manuscript the concerns raised by Reviewer 2.

Despite these concerns, the editors see this paper as potentially important and wish to encourage revision. If you would like to revise the manuscript in accordance with the suggestions of the reviewers and editors, we would be willing to evaluate a new version. The manuscript would be reviewed again, with no assurance of acceptance.

The Editors strongly encourage you to adhere to the journal's Statistical Reporting Recommendations in your revision, which can be found here: https://www.ahajournals.org/statistical-recommendations.

Upon revision, authors of manuscripts that contain cropped gels/blots will be required to submit a separate PDF file that contains the entire unedited gel for all representative cropped gels in the manuscript. Authors should label each gel as “Full unedited gel for Figure _” and highlight which lanes of the unedited gel correspond to those shown in the cropped images within the manuscript. For more information, please go to https://www.ahajournals.org/res/manuscript-preparation.

All research materials listed in the Methods should be included in the Major Resources Table file, which will be posted online as PDF with the article Supplemental Materials if the manuscript is accepted. A template Major Resources Table file (.docx) is available for download here: AHAJournals_MajorResourcesTable_2019.docx. Authors are required to upload the Table at the revision stage. Authors should reference the PDF in their Methods as follows: "Please see the Major Resources Table in the Supplemental Materials."

To read the comments to authors from the reviewers, please see below.

Please note that revised and resubmitted manuscripts are not assured of publication, and that fewer than 15% of all papers submitted to Circulation Research are eventually published.

Our current guidelines allow authors 90 days to complete the revision. If the manuscript is resubmitted within 90 days, one or more of the original reviewers will be re-consulted; the editors may also choose to obtain additional opinions from new reviewers. If you need more than 90 days to submit a revised paper, please notify the editorial

office. In general, extensions over the revision time limit will not be granted except under special circumstances at the editors' discretion.

You are encouraged to log in and start the revision submission process as soon as possible, because all authors must complete disclosure and publication agreement forms BEFORE the revision can be submitted. As corresponding author, you may start the revision process at any time and go to the Copyright & Licensing tab to contact coauthor(s) and send reminders. In addition, please review all revision submission tabs to ensure that you are prepared to provide the appropriate files, checklists, and other items when it is time to submit your revision.

If you choose to revise, please include a detailed response to each of the referees' and editors' comments, providing each comment verbatim in bold followed by your response and giving the exact page number(s), paragraph(s), and line number(s) where each revision was made. If you make substantive changes to the manuscript, please provide a clear description of what you did and where. If you insert important sentences, paragraphs, or sections in response to the comments, please also include them in your response. Please indicate clearly any deletions. Additionally, a marked up version of the revision with the changes highlighted or tracked should be uploaded as a supplemental file. Number each page in the top right corner, using your manuscript number followed by /R1 to denote a first revision.

We are piloting an integration with SciScore (https://www.sciscore.com) to provide authors automatically generated reports during revision submission containing a reproducibility score and tables on rigor adherence and key resources such as antibodies, experimental models, recombinant DNA, and software. You are also welcome to start the revision submission process at any time to receive your report. We strongly encourage you to use the provided report while revising your manuscript to improve the study's reproducibility and reporting quality.

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We wish to thank you for having submitted this manuscript to Circulation Research.

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Sincerely,

Jane E. Freedman, MD
Editor-in-Chief

Circulation Research
An American Heart Association Journal

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Reviewer comments to the Authors:

Reviewer #1:
No further comments. Authors responded to review comments satisfactorily.

Reviewer #2:
The authors have made reasonable responses to my initial comments. However, a still have three significant concerns or reflections on this study.

Firstly, given the very extensive work on methionine oxidation activating CamK to cause cardiac harm, as well as their previous Nature paper on glycation of the kinase activating it also being injurious – this work seems not very novel. The cysteine angle is, but this to me is an incremental advance.

Secondly, if I understand correctly the original work on hydrogen peroxide-induced methionine oxidation / activation of CamK ruled out cysteine oxidation and included experimental evidence that it did not occur. This was previously important because it meant that the oxidant had fidelity with the methionine and helpfully avoided complexity with additional redox mediators being involved. I understand that new evidence can supersede the older findings, but the issue that remains here is that it is extremely likely that the cysteines investigated here were a target of peroxide as well. Here we have nitrosative interventions that will cause substantive redox changes in the cell, likely compromising their reducing capacity and so methionine oxidation is also likely happening. Essentially, without studies with / perhaps crossing the Met mutant mice and the new transgenics I think there is a major question about the relative contributions of Met versus Cys oxidation. Given my point about novelty above, such studies may help address this concern.

Thirdly, I think it crucial to determine the stoichiometry of CamK SNO formation under conditions of physiological or disease-relevant nitrosative stress. In this connection, does the CamK-SNO transition to a more stable oxidation state such as disulfide that mediates the activation?

Reviewer #3:
All of my concerns have been addressed.

Statistical Reviewer:
1. If the statistical test assumes a normal distribution, please state the test of normality used. If the data are not normally distributed or if the sample size is too small to assess normality (<10), please use a non-parametric alternative. If relying on statistical properties for normality (e.g. the central limit theorem), explicitly state the property and what data it applies to.

Razali, N. M., & Wah, Y. B. (2011). Power Comparisons of Shapiro-Wilk, Kolmogorov-Smirnov, Lilliefors and Anderson-Darling Tests. Journal of Statistical Modeling and Analytics, 2, 21-33.

2. Please provide precise p-values (rather than P<0.0x). This can be obtained in GraphPad by increasing significant digits on the "Options" tab. Scientific notation with 2 significant figures (e.g. 1.2x10^-3) is strongly encouraged.

Further instruction can be found at https://www.graphpad.com/support/faq/how-can-i-get-exact-p-values-below-00001-for-t-tests-or-anova/

Technical Reviewer:
The current study was carefully evaluated for inclusion of guideline items present in the Circulation Research checklists for rigor, transparency, and reproducibility. The reviewer has identified a number of items that were either omitted or not adequately addressed in the text. Please see below for details:

• Please provide uncropped immunoblots for technical review.
• Molecular weight markers should be included on all blots; the ladder itself does not need to be shown on the gel but text can be used to indicate the proper sizes of bands. For example, these are missing in Figure 3D.
• How were representative images/figures chosen? Are they representative of the mean/average or the best/illustrative images? Please note this in the text somewhere.
• Per the journal's requirements, please complete and submit a "Major Resources Table". Please refer to the website for formatting instructions.

Responses to comments by the Editor and Reviewers

Editor comments:
As you will gather from the reviews, the referees identified a number of conceptual and methodological problems. The editors concur. A suitably revised manuscript would need to address the concerns of the statistical and technical Reviewers. It would also be necessary to discuss in the manuscript the concerns raised by Reviewer 2.

We thank the editor and reviewers for their constructive feedback on our manuscript. We have made a number of alterations and additions to the manuscript to address the concerns raised by the statistical and technical reviewers. We have also responded to concerns by Reviewer 2 below and in the manuscript.

Upon revision, authors of manuscripts that contain cropped gels/blots will be required to submit a separate PDF file that contains the entire unedited gel for all representative cropped gels in the manuscript. Authors should label each gel as "Full unedited gel for Figure _" and highlight which lanes of the unedited gel correspond to those shown in the cropped images within the manuscript.

The original gels have been submitted per the instructions.

All research materials listed in the Methods should be included in the Major Resources Table file, which will be posted online as PDF with the article Supplemental Materials if the manuscript is accepted. A template Major Resources Table file (.docx) is available for download here: AHAJournals_MajorResourcesTable_2019.docx. Authors are required to upload the Table at the revision stage. Authors should reference the PDF in their Methods as follows: "Please see the Major Resources Table in the Supplemental Materials."

Research materials listed in the Methods section have been added to the Major Resources Table, which has been submitted along with the manuscript.

Reviewer #1 comments:

No further comments. Authors responded to review comments satisfactorily.

Reviewer #2 comments:

The authors have made reasonable responses to my initial comments. However, a still have three significant concerns or reflections on this study.

Firstly, given the very extensive work on methionine oxidation activating CamK to cause cardiac harm, as well as their previous Nature paper on glycation of the kinase activating it also being injurious – this work seems not very novel. The cysteine angle is, but this to me is an incremental advance.

We respectfully disagree with the Reviewer on this point. The Reviewer is correct that oxidation and glycosylation dependent pathways for CaMKII activation have been reported previously, and that these mechanisms have been shown to contribute cardiac pathology. However, the effects of nitrosylation on CaMKII activation and subsequent contribution to the development of arrhythmia are novel. Moreover, unlike previously described mechanisms, nitrosylation has a dual regulatory role in the heart that has not been previously observed. This observation helps to explain key unresolved controversies in our field, as previous researchers have reported both beneficial AND detrimental effects of nitric oxide on cardiac arrhythmia risk. Given the critical role of CaMKII activation in the generation of arrhythmia, as well as our novel observation here that a pair of cysteines on CaMKII can be subject to nitrosylation with highly disparate effects on kinase activity, we believe that this work will be of critical interest to the field. Finally, nitric oxide donors are commonly used in the clinic for a number of cardiovascular applications. Our data may assist in refining the use of nitric oxide donors to maximize their effectiveness while minimizing downstream risk of arrhythmia.

Secondly, if I understand correctly the original work on hydrogen peroxide-induced methionine oxidation / activation of CamK ruled out cysteine oxidation and included experimental evidence that it did not

occur. This was previously important because it meant that the oxidant had fidelity with the methionine and helpfully avoided complexity with additional redox mediators being involved. I understand that new evidence can supersede the older findings, but the issue that remains here is that it is extremely likely that the cysteines investigated here were a target of peroxide as well. Here we have nitrosative interventions that will cause substantive redox changes in the cell, likely compromising their reducing capacity and so methionine oxidation is also likely happening. Essentially, without studies with / perhaps crossing the Met mutant mice and the new transgenics I think there is a major question about the relative contributions of Met versus Cys oxidation. Given my point about novelty above, such studies may help address this concern.

We agree with the Reviewer that there is likely interplay between oxidation and nitrosylation of CaMKII in the cellular environment. We have previously demonstrated that nitrosylation dependent effects on CaMKII activity can occur independent of the presence of the methionine oxidation sites (Erickson et al. Journal of Biological Chemistry. 2015). That said, there remains the possibility that an environment of cellular stress would induce both nitrosylation and oxidation of CaMKII. As the Reviewer suggests, one potential future study would be a collaborative effort with the Anderson group that generated the CaMKII oxidation resistant mice (methionine knockouts), which could then be assessed for nitrosylation effects both independently and crossed with our new transgenic animals. We have updated the Discussion (Page 12, Paragraph 3) of the manuscript to address this point.

Thirdly, I think it crucial to determine the stoichiometry of CamK SNO formation under conditions of physiological or disease-relevant nitrosative stress. In this connection, does the CamK-SNO transition to a more stable oxidation state such as disulfide that mediates the activation?

The Reviewer raises an interesting point here. We have previously published data that partially addresses the stoichiometry of CaMKII nitrosylation (Erickson et al. Journal of Biological Chemistry. 2015) using our FRET-based biosensor Camui. However, whether this nitrosylation can transition to another state (ex. Disulfide bond) is not know, nor is the time course for such a transition during acute or chronic cellular stress. A recent publication highlighted the possibility that CaMKII may be subject to disulfide bridge formation during oxidative stress (Rocco-Machado et al. Journal of Biological Chemistry. 2022). However, another group showed this year that CRISPR-Cas9 gene editing of the oxidation-sensitive methionine residues on CaMKII was sufficient to protect the heart from CaMKII oxidation effects (Lebek et al. Science. 2023), suggesting that the cysteine residues are more important for governing nitrosylation dependent effects as we describe in this manuscript. We have amended the Discussion (Page 12, Paragraph 3) to emphasize these points and suggest future studies.

Reviewer #3 comments:
All of my concerns have been addressed.

Statistical Reviewer:

If the statistical test assumes a normal distribution, please state the test of normality used. If the data are not normally distributed or if the sample size is too small to assess normality (<10), please use a non-parametric alternative. If relying on statistical properties for normality (e.g. the central limit theorem), explicitly state the property and what data it applies to.

We have reanalyzed all data sets with n<10 using non-parametric tests and revised all of the p values to reflect the new analysis. None of the central observations of the study were altered by this analysis. We have also adjusted the Methods section to describe the tests used as suggested by the Reviewer.

Please provide precise p-values (rather than P<0.0x). This can be obtained in GraphPad by increasing significant digits on the "Options" tab. Scientific notation with 2 significant figures (e.g. 1.2x10^-3) is strongly encouraged.

We have edited all of the p values to report with two significant figures in both the text and on the figures themselves.

Technical Reviewer:

Please provide uncropped immunoblots for technical review.

We have provided the uncropped blots as requested..

Molecular weight markers should be included on all blots; the ladder itself does not need to be shown on the gel but text can be used to indicate the proper sizes of bands. For example, these are missing in Figure 3D.

We have included molecular weight markers for all blots to indicate the proper size of the bands.

How were representative images/figures chosen? Are they representative of the mean/average or the best/illustrative images? Please note this in the text somewhere.

We have added a note in the Methods text to specify how representative images and figures were selected.

Per the journal's requirements, please complete and submit a "Major Resources Table". Please refer to the website for formatting instructions.

We have completed and submitted a Major Resources Table.

October 26, 2023

Dr. Jeffrey Erickson
University of Otago
270 Great King Street
Dunedin 9016
New Zealand

RE: CIRCRES/2023/323571DR1: Nitric Oxide modulates spontaneous Ca2+ release and ventricular arrhythmias during β adrenergic signalling through S-nitrosylation of Calcium/Calmodulin dependent kinase II

Dear Dr. Erickson:

Your revised manuscript has been carefully evaluated by the editors as an Original Research manuscript. While we are interested in your paper, further minor revision is required before we can accept the manuscript for publication in Circulation Research. Specifically, there are several formatting issues that need to be addressed. Please submit your revision by MONDAY, OCTOBER 30.

Please ensure that your manuscript adheres to the AHA Journals' implementation of the Transparency and Openness Promotion (TOP) Guidelines (available online at https://www.ahajournals.org/content/TOP-guidelines).

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- The limit on the total number of tables and/or figures is 8 for a Regular Article. Please move some of the figures/tables to an online supplement that can then be uploaded with the submission as a supplemental file.
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-Authors of manuscripts that contain cropped gels/blots will be required to submit a separate PDF file that contains the entire unedited gel for all representative cropped gels in the manuscript. Authors should label each gel as "Full unedited gel for Figure _" and highlight which lanes of the unedited gel correspond to those shown in the cropped images within the manuscript. If the manuscript is accepted, the PDF of the unedited gels will be included in the online supplemental materials. For more information, please go to https://www.ahajournals.org/res/manuscript-preparation.
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- A Graphical Abstract (to be uploaded as a separate supplemental file): The intent of the graphical abstract is to provide readers with a succinct summary of the study in a form that facilitates its dissemination in presentations. It should emphasize the new findings in the paper. Do not include data items; all content should be graphical. The graphical abstract should conform to the following format: A single figure panel, no more than a 15 cm square (15 cm x 15 cm); Font: prefer a san serif font that is no less than 12 point. Please upload as a graphic abstract file in JPG file format.
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We look forward to receiving the final revised version of your manuscript as soon as possible. Thank you for contributing to Circulation Research.

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