Author’s response to reviews

Title: Metformin restores electrophysiology of small conductance calcium-activated potassium channels in the atrium of GK diabetic rats

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Editor Comments:

1. Recently, authors (Calcium release channel RyR2 regulates insulin release and glucose homeostasis. Journal of Clinical Investigation 2015; 125(5), pp. 1968-1978), showed a functional role for RyR2 in metabolism-secretion coupling both in mice and in humans. In fact, T2DM represents a potent risk factor for the development of cardiovascular disease, and RyR2 channels are chronically leaky in heart failure. Therefore, these authors raised the possibility that the relationship between diabetes and cardiovascular disorders may be more complex than previously appreciated in that T2DM is both a risk factor for and a sequela of heart disease. Taken together, authors’ data provided compelling evidence that intracellular Ca2+ leak via RyR2 channels induces glucose intolerance associated with pancreatic β cell ER stress, mitochondrial dysfunction, and decreased insulin secretion. In my opinion, these results may be in line with your discussion, and this merits to be discussed in your article.
Response: Santuli et al. showed that the “leaky” RyR2 channel that was identified in the patients with heart failure could stimulate insulin release in mouse pancreatic β cells and impair glucose homeostasis in the transgenic mice. However, I would not agree that T2DM is a sequela of heart disease. “Leaky” RyR2 leads to dysfunction in both heart contraction and insulin release because RyR2 expressed in cardiomyocytes and pancreatic β cells is critical for the corresponding organ function. The data from Santuli et al. demonstrated the functions of RyR2 in the heart and pancreas, but did not support the concept that T2DM is a sequela of heart disease. On the other hand, it is well-documented that chronic hyperglycemia interferes with the function of cardiomyocytes, and thus T2DM is considered to be a risk factor for heart disease. Our data suggest that diabetes-induced alteration of SK channels may be one of the underlying mechanisms for T2DM-induced arrhythmia.

2. The K+ channels activity may be affected by alterations in inflammatory tone and oxidative stress, and this may be seen also in the setting of ventricular arrhythmias originating in patients without alterations of cardiac functionality, and structure (Metabolic syndrome is associated with a poor outcome in patients affected by outflow tract premature ventricular contractions treated by catheter ablation. BMC Cardiovasc Disord. 2014 Dec 6;14:176). These ionic channels alterations may affect the long term post ablative prognosis in humans. Please discuss this point.

Response: Thank you for the comment. This is a very good point for clinical consideration. The relation between ion channels and inflammatory tone, oxidative stress as well as post catheter ablation prognosis is discussed in the third paragraph of Discussion section.

3. Similarly, in humans T2DM may affect the functionality of ionic channels, conditioning systolic and diastolic electrical and anatomical properties of the heart (Impact of diabetes mellitus on the clinical response to cardiac resynchronization therapy in elderly people. J Cardiovasc Transl Res. 2014 Apr;7(3):362-8; Multipolar pacing by cardiac resynchronization therapy with a defibrillators treatment in type 2 diabetes mellitus failing heart patients: impact on responders rate, and clinical outcomes. Cardiovasc Diabetol. 2017 Jun 9;16(1):75). In humans, these alterations may be reported by devices interrogation at follow up, such as drop in impedance, sensing and pacing output of implanted channels (Cardiac electrophysiological alterations and clinical response in cardiac resynchronization therapy with a defibrillator treated
patients affected by metabolic syndrome. Medicine (Baltimore). 2017 Apr;96(14):e6558). Please discuss this point.

Response: As suggested, attention to ion channel alterations during the treatment of arrhythmia in T2DM patients is discussed in the third paragraph of Discussion.

4. T2DM plays these alterations by interactions also at epigenetic level (Cardiac Resynchronization Therapy Outcomes in Type 2 Diabetic Patients: Role of MicroRNA Changes. J Diabetes Res. 2016;2016:7292564). Why did you not study the epigenetic effects T2DM induced? This may be reported as study limitation. Please discuss.

Response: Thank you for the comment. In the current study, we focused on the SK channels in the cardiomyocytes of diabetic rats. The factors that regulate SK expression, such as transcription factors or microRNAs, during this pathologic process will be investigated in the future study. This future work proposal is mentioned in the second paragraph of Discussion section (lines 248-252).

5. At last, in your study there are not reported alterations in oxidative tone and inflammatory balance in atrial structure (Effects of Alpha Lipoic Acid on Multiple Cytokines and Biomarkers and Recurrence of Atrial Fibrillation Within 1 Year of Catheter Ablation. Am J Cardiol. 2017 May 1;119(9):1382-1386). Can you discuss this point?

Response: Thank you for the comment. Our study focuses on the SK channels in the diabetic cardiomyocytes. So far, there is not sufficient evidence substantiating the role of oxidative stress or inflammation in SK channel expression or activity, we decide not to go beyond the current data and literature at this moment. Nevertheless, the potential effect of oxidative tone and inflammatory balance on SK channel functionality is a very interesting question, and it is worth investigation in future studies.
Umberto Barbero (Reviewer 1):

1. The number of rats studied should be added in the abstract section.

Response: It is added in the Abstract section.

2. Given the small sample size, parametric distribution should be checked for.

Response: Thank you for the advice. Statistical analyses were re-performed. For multiple comparisons, one-way analysis of variance (ANOVA) followed by Fisher’s least significant difference test was used when the data conformed Gaussian distribution and homogeneity of variance, and nonparametric Kruskal-Wallis test with Dunn’s multiple comparisons was used otherwise. The p value marks in the tables and figures are corrected accordingly.

3. The authors should explain in the methods why they choose a high dose of metformin (instead of a lower dose of 100mg/kg/day)

Response: Metformin of 300 mg/kg/day is considered safe to rats. Previous studies have shown that long-term treatment with 300 mg/kg/day metformin could alleviate vascular dysfunction and rescue SK channel-mediated vasodilation in diabetic rats. Since we were interested in the effect of metformin on the SK channels, we chose this high dose that was shown to be effective on SK channel modulation. The reason for choosing this high dose and the relevant references are provided in the revised Methods section.

4. In the introduction, page 3 line 56 the authors say that diabetes "also confers an approximately twofold-increased risk of cardiovascular diseases" and cite an interesting but qualitative review. Since the relevance for this paper, may be more complete to cite also a quantitative analysis on
the topic (see for example "Assessing Risk in Patients with Stable Coronary Disease: When Should We Intensify Care and Follow-Up? Results from a Meta-Analysis of Observational Studies of the COURAGE and FAME Era. Scientifica (Cairo). 2016:2016:3769152” which reports indeed an OR of 1.93 for diabetes). That would add clinical relevance to the author's paper, especially because reporting the role of CRP as a cardiovascular risk factor, and metformin has demonstrated in experimental model to be able to diminish the level of CRP (Liu et al. Lipids Health Dis. 2014 Jul 15;13:115)

Response: Thank you for the recommended paper. It provides direct evidence for diabetes as a risk factor of cardiovascular disease, and it is cited.

5. I suggest to change the way the amount of metformin is reported (300 mg·kg-1·d-1) in a clearer way (for example 300 mg/kg/day).

Response: Thank you for the advice. The dose unit is edited as mg/kg/day.

6. The footnote of figure 2 should be extended to better explain differences between ECG.

Response: Thank you for the comment. This figure legend is expanded with more detailed description.

7. Page 5, line 94, the author use "Met": please add it in bracket before in the text to make clear is a short form of metformin. The same should be added in the abbreviation list.

Response: Met is only used for the group of metformin-treated rats because this short form is neat in the figures. The full name is added before “Met” to make it clearer, and it is added in the abbreviation list as suggested.
8. In the methods section the numbers of rat used as controls should be written.

Response: This information (n=6) is added in the Methods section.

9. In Figure 1, color coding of staining should be added in the footnotes.

Response: The collagen fibers were stained blue. This information is added in the figure legend as suggested.

10. In Figure 5 the section C is not clearly districts: it may be useful to move the capital letter "C" above

Response: The position of letter “C” is correct. Section A includes APA, RP, APD50 and APD90 before exposure to apamin, and section C includes changes in APD50 and APD90 after exposure to apamin. This is clarified in the revised figure legend.

Sebastian Garcia Zamora, M.D. (Reviewer 2):

1. In the method section you do not clarify how many GK rats received metformin and how many did not.

Response: This information is clarified as suggested.
2. In the statistics section you declare that your data was expressed as mean and SEM. However, your study has a relatively small "n" (n = 24) which means that you can not rely on the central limit theorem. I would like to know if all your data really has a "Gaussian" or "normal" distribution. If not, I think that you should use non-parametric tests when your data does not have this distribution.

Response: Thank you for the advice. Statistical analyses were re-performed. For multiple comparisons, one-way analysis of variance (ANOVA) followed by Fisher’s least significant difference test was used when the data conformed Gaussian distribution and homogeneity of variance, and nonparametric Kruskal-Wallis test with Dunn’s multiple comparisons was used otherwise. The p value marks in the tables and figures are corrected accordingly.