Author's response to reviews

Title: Amiloride derivatives enhance insulin release in pancreatic islets from diabetic mice

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Author's response to reviews: see over
Dear Editors,

We would first like to thank the reviewers for their careful reading of the manuscript and many thoughtful comments. We agree with all the comments, and have done our best to modify the manuscript to address these concerns. We have carefully evaluated each of the reviewers’ comments. The resulting changes in the title, text, and figures are described below. We hope that the revised manuscript is more clear and informative, and will be found acceptable for publication. Thank you.

**General points made by the reviewers:**

Upon reading the reviewers’ comments we agreed that the title of the manuscript may have been misleading. Both title and text have now been modified to be less optimistic, and to clearly indicate that this work was performed in isolated pancreatic islets. While we believe that amiloride derivatives do have a strong potential to correct the secretory defect in certain cases of type 2 diabetes, we understand that the *in vitro* work done in this study is not adequate to make a definitive claim to that effect. Currently we are performing similar experiments on other mouse models of type 2 diabetes with more pronounced secretory defects. A more suitable model for the *in vivo* studies will need to demonstrate consistent hyperglycemia and hypoinsulinemia, and we are currently working with some promising mouse lines. Once a suitable mouse model is established, we will obtain all relevant blood parameters before and after amiloride treatment. However, that work is still open-ended at this point. We believe that the new *in-vitro* data reported in the current manuscript offers valuable insight into a potential method to correct the secretory defect in type 2 diabetes. While we are continuing this work in human islets and *in vivo* studies in rodent models, the current manuscript may also prove useful to other researchers working on diabetic models of different species.

As the reviewers suggested we have included more detail about the mouse models, experimental design and results. The main purpose of this study was to determine whether previously-reported *in vitro* effects of amiloride are applicable to the diabetic situation, and what we had previously measured in detail was the response of isolated islets (7,8). Therefore, we focused on the response of isolated islets from diabetic mice, and the results from these commercially available models show promise in the ability of amiloride to enhance insulin release in NIDDM. We want to perform the *in-vivo* studies in a better model of NIDDM with a pronounced secretory defect, as well as continue the *in-vitro* studies on human islets.

The modified manuscript is attached, where the additions/modifications to the text are denoted in blue font.

The specific concerns from each reviewer are addressed below.
Reviewer 1:

The reviewer realized a discrepancy between the title of the manuscript and the studies done. The title promises in vivo studies leading to conclusions for treatment of type 2 diabetes. In reality all studies done were based on islets isolated from two diabetic mouse strains.

The title and text have been modified to clearly indicate that the results were obtained in *in-vitro* studies on isolated islets.

The information relating to these animals is very poor. The reviewer misses information regarding the age of the animals, body weight, glucose levels, and insulin levels. The "problem" described by the authors on page 10 is based on the facts mentioned above. The animals develop diabetes over time and these changes have a strong influence on insulin secretion. The different phases of insulin secretion are strongly influenced by the age/diabetic status. In an early stage the first phase of insulin secretion is intact. Within a short time frame the first phase is impaired. The relevance of the data shown is dependent on these facts. It is well known that a type 2 diabetic patient has an impaired first phase of insulin secretion.

Detailed information on the mouse models has been added to the text in the methods and discussion sections. Additional information and literature is available on the Jackson laboratory website (23, 24). The mice of both NIDDM models we used were old enough to develop diabetes (9+ weeks for KK mice, and 5-9 months for NON mice). The presence of diabetes was confirmed by clinical signs such as weight gain, hyperphagia, polyurea and polydypsia. Blood glucose and insulin levels in these strains, as reported in other studies, have been added to the text in the discussion section.

What procedure was done to control the real dose given in the drinking water? It is not possible to keep only one animal in a cage. Was the compound plasma level determined? If not, a precise dosing is not possible. An oral gavage is the only alternative.

Animals were housed two to a cage and their behavior was monitored. All animals were observed to drink frequently, and dose was calculated using estimated water intake. Although the plasma levels of the compound was not measured, islets from every animal in the treated group showed significantly higher insulin response to glucose compared with the untreated control group, indicating that each animal took in an effective dose.

It is difficult for the reviewer to assess the data under these circumstances. Based on these data the conclusion seems to be very optimistic that amiloride derivatives have potential therapeutic value.

The conclusion, title and text have been modified to be less optimistic. However, the fact that amiloride consistently improves insulin secretion in all situations tested (in this study and previous studies) suggests potential therapeutic value, and merits further investigation through *in-vivo* studies.
The islet studies are well done. Why don’t the authors show ph-measurements in NON islets?

pH measurements in NON islets are included in Figure 1.

It has been shown that normalization of blood glucose in db/db mice resulted in a normalization of insulin secretion from islets subsequently isolated. What are the blood glucose levels of your mice after the one-week treatment? Can the improved insulin secretion be due to lowered blood glucose levels?

We did not measure blood parameters simply because this study was designed to determine whether previously-reported in vitro effects of amiloride are applicable to the diabetic situation, and what we had previously measured in detail was the response of isolated islets. Amiloride derivatives consistently enhance NSIS in normal rodent islets, and enable/uncover certain secretory functions that are normally absent (7,8). The main purpose of this study was to determine whether amiloride elicits the same response in islets from diabetic animals. The results from these commercially available models show that amiloride consistently elicits the same favorable effects in isolated islets, and thus show promise in correcting the secretory defect in diabetes in vivo.

The next step is to determine whether amiloride can normalize blood glucose and insulin levels in type 2 diabetes. However, the models used in the current study have elevated basal insulin release compared to wild type islets, and thus not ideal for the proposed work. Therefore the in vivo studies will be conducted in a more suitable model of type 2 diabetes with a more pronounced secretory defect that will ideally be phenotypically closer to human diabetes. The proposed work involves breeding and genotyping a large number of mice, and would take several months to complete. In the meantime we believe the in vitro data reported in the current study would be useful to other researchers working on NIDDM in different species.

Blood parameters in untreated diabetic mice, as indicated in literature, are now included in the discussion section.

Do the depicted differences in ph-changes between WT islets and islets from diabetic animals (Fig 1) go along with a change in the kinetics of insulin secretion? (Perifusion of isolated islets)

While it would be beneficial to determine this, our lab is currently not equipped for perifusion studies. This work may be performed as part of the proposed study in collaboration with a different lab.

Fig 2: depiction of fractional insulin release is an accepted method. A comparison of diabetic and non-diabetic animals, however, requires the display of absolute amounts of released insulin, especially if a compound is thought to normalize secretion.

The corresponding absolute values in ng/ml are now included in the figure legends.
Reviewer 2:

a) Whether BSA has been added in KRBH buffer during insulin secretion assay?

Yes. The text has been modified in the methods section.

b) How many islets were taken during the insulin secretion assay?

Our isolation technique yields 60-120 islets per mouse, depending on the strain. (80-120 for WT and KK mice, 60-80 for NON mice). The secretion studies were done using 4 islets per tube, and 4-5 tubes per each experimental condition, each typical experiment consisting of 4-8 conditions. n denotes the number of times each experiment was repeated using a different mouse (or group of mice). The text in the methods section has been modified.

Ans. Yes but I wish to know that whether the islet monolayer which is used 14 days after culture has insulin secretion capacity or not? As pH assessment is done on the monolayer while secretion assay is done on fresh islets, the functionality of the two should be compared before correlating the results.

Yes, the cultured islet monolayer has secretory capacity comparable to that of fresh islets, as indicated in previous studies (19-21) and our preliminary work. We compared insulin secretion in fresh islets and islets cultured for up-to three weeks under the conditions used for the current work. Insulin response to basal and high glucose with and without DMA in the cultured islets showed the same pattern as in fresh islets. While the overall secretory capacity decreases over time, the cultured islets still show a healthy insulin response for at least three weeks in culture.

Ans. Yes. But no biochemical parameter has been measured in vivo like effect of the treatment on blood glucose, serum insulin or glucose tolerance hence we are not fully satisfied how it will be used for type 2 diabetes therapy. I feel it will be useful in a combinatorial therapy and that part should be discussed.

It has been claimed that in vivo treatment enhances the insulin secretion capacity of NON islets. Along with ex-vivo data they should provide some in vivo data such as glucose tolerance test, proving the claim.

The title, text and conclusion have been modified to sound less optimistic. Please see response to Reviewer 1 (comment 6).

Reviewer 3:

Minor Essential Revisions
Corrected.

Fig 3:
In the legend the complete words for the abbreviations G, a KIC and BCH should be given in order to alleviate reading.

Corrected.

Page 10:

However the ? fold increase in islets from NON mice ……
Please check the sentence

Sentence has been modified.

Discretionary Revisions

More information should be given on the traits and genetics of the type 2 diabetes mouse models used in the study, if available. Particularly, is there anything known about genetic effects which may have impact on Na+/H+ - exchanger, intracellular pH calcium influx and potassium retention?

More information on the mouse models has been added to the text in the methods and discussion sections. Additional information and literature is available on the Jackson laboratory website (23, 24). There is no reported information on genetic effects that may impact on Na+/H+ exchanger, intracellular pH, calcium influx and potassium retention. Our data show significant abnormalities in the pH$_i$-regulation in isolated islets from both models, suggesting the possibility of such genetic effects.

Values of glucose and insulin plasma concentrations in mice treated with and without DMA and amiloride should be given, if available. Glucose values might be higher based on data in the literature. The increased insulin release, found by the authors, probably exclude an interfering “glucose-toxic” effect.

Please see response to Reviewer 1 (comment 6).