Author's response to reviews

Title: Partial deficiency of HIF-1alpha stimulates pathological cardiac changes in the streptozotocin-induced diabetic mice

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Version: 2 Date: 27 December 2013

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

The manuscript MS#: 1181649703110301 “The role of hypoxia inducible factor 1 alpha in diabetic cardiomyopathy” has originally been submitted to Cardiovascular Diabetology. Based on the editor’s suggestion, I am using the opportunity to transfer the revised manuscript to an alternative journal within the BioMed Central portfolio, BMC Endocrine Disorders. The manuscript has been renamed to “Partial deficiency of HIF-1α stimulates pathological cardiac changes in the streptozotocin-induced diabetic mice” based on reviewer’s recommendation. It has been thoroughly revised based on the reviewers’ comments. Revisions are explained below, including my detailed replies to reviewers’ comments.

The manuscript is original research; it has not been submitted elsewhere in print or electronic form to another journal.

This is the first study providing evidence that genetic variation in the activity of hypoxia-inducible factor 1 (HIF-1) alters cardiac responses to diabetes. The combinatory effect of Hif1a haploinsufficiency and diabetes affected the both diastolic and systolic parameters of the left ventricle, myocardial remodeling, and the gene expression profile of the heart. As such, this paper should be of interest to both basic and clinician scientists engaged in cardiovascular and diabetes research.

Thank you again for considering this paper for publication in BMC Endocrine Disorders. Please address all correspondence concerning the manuscript to me via e-mail at gpavlinkova@img.cas.cz.

Sincerely,

RNDr. Gabriela Pavlinkova, Ph.D.

**Detailed response to the reviewers’ recommendations:**

Thank you very much for your detailed comments on the manuscript. The manuscript has been thoroughly revised based on the reviewers’ recommendations. I have attempted to address all comments and recommendations. The revisions are summarized below. Reviewers’ suggestions...
are listed in direct quotes. My answers and revisions made are explained in bullet points below each comment or recommendation.

**Reviewer: 1**

“The title of the manuscript is quite vague; “the role of HIF-1α” is not specific. More specific title such as “Inhibition of HIF-1α stimulates pathological cardiac remodeling in STZ treated diabetic mice” would be better.”

- The title is changed to be more informative and specific.

“The results of manuscript are related with HIF-1α, diabetic cardiomyopathy with respect to the Vegfa, apoptosis, collagen 1 and connexin 43. Background should be concise showing what is done in these aspects specifically HIF-1α mediated cardiac protection in diabetes and what is not clear, which this manuscript is attempting to study. Please remove inflammation part from background.”

- The introduction of the paper is rewritten and the particular aspects of apoptosis, collagen I deposition, fibrosis, Vegfa, and HIF-1α-mediated cardiac protection in diabetes are extended in the Introduction.
- The inflammation part was removed from the Background.

“Regarding increased apoptosis in diabetic *Hif-1a*+/− hearts as compared to diabetic WT hearts, how it could be protective? In the discussion, authors mentioned that WT mice are less affected by diabetes than *Hif-1a*+/. If %FS of WT and *Hif-1a*+/- mice are considered in Fig 1 A, the %FS of WT diabetic females are almost same as *Hif-1a*+/- diabetic males. Since diabetes itself is a pathological condition and on top of that attenuation of Hif-1a deteriorate cardiac structure and function as claimed by the authors. In this context, please discuss how the apoptotic death of myocytes improves cardiac function in diabetes.”

- We believe that only a possible suggestion for the increased apoptosis in the diabetic *Wt* heart but not in the diabetic *Hif1a*+/- heart is the decreased sensitivity of *Hif1a*+/- cardiac tissue to apoptosis-induction signals as a consequence of HIF-1α partial deficiency. We have changed the discussion accordingly.

“One of the major limitations of the conclusion is that whole body *Hif-1a*+/- mice is used. The remodeling effects in the heart may include neuronal stimulation by HIF-1α. The neuronal effect of HIF-1α is quite established and may contribute to cardiac function. Cardiac specific knock down will be more specific to conclude the direct effect of HIF-1α in the heart.”

- Actually, reviewer 2 assesses: “this manuscript reports new information on the interaction of diabetes and HIF-1 on myocardial gene expression which may have implications for understanding the mechanisms of adaptive remodeling of the myocardium in response to chronic hyperglycemia. In particular, the findings suggest a role of HIF-1 in delaying the onset of diabetes induced cardiac injury. …” We disagree with the reviewer. The first reason why we disagree is that our study represents the first analyzes of the effects of decreased *Hif1a* gene dosage on the diabetic heart similar to gene polymorphisms in humans, which may increase susceptibility to diabetic cardiomyopathy. The second reason is that this is the first study documenting the negative effects of decreased HIF-1α
expression in the diabetic hearts with the complete evaluation of the echocardiographic geometrical and functional parameters of the left ventricle. Although Xue et al. showed that the overexpression of Hif1a gene under the control of the myosin heavy chain promoter normalizes VEGF-A levels and inhibits fibrosis in hearts exposed to diabetes (PMID20566749; our Manuscript reference # 22), they have not evaluated the echocardiographic functional parameters of the mutant heart to provide a more complex analysis.

- We have also concluded the Discussion with a paragraph summarizing the study’s limitations due to the global deletion of Hif1a gene (page 20).

Minor comments:

1. Although authors presume Hif-1#++/- decreases the level of HIF-1#, but is no validation for it.
   - We have newly analyzed the protein levels of HIF-1α. See Western analysis

2. The representative data for collagen 1, in Figure 3 B and E, the representative WB data does not comply with the graphical presentation specifically when comparing a) WT non-DIA and Hif1a+/- DIA (bands are more intense in WT non-DIA than Hif1a+/- DIA but graph shows almost 1.5 fold higher in Hif1a+/- DIA) and b) WT DIA with Hif1a+/- no-DIA (bands are more intense in WT DIA than Hif1a+/- non-DIA but graphical presentation show almost equal levels).
   - The graphs represent the mean data of 3 samples normalized to the load control protein obtained from densitometric analysis of Western blots. Although the bands of collagen are more intense in non-DIA Wt than DIA Hif1a+/−, the total protein load is lower for DIA Hif1a+/−; specific protein amount needs to be normalized the total protein amount per lane. We double checked our results using Ponceau S visualized protein bands for the loading normalization.

3. Figure 3A, Clarify using arrow how the distribution of Cx43 differs between Wt DIA vs. Hif1a+/- DIA.
   - There is no difference in the distribution of Cx43 between Wt DIA vs. Hif1a+/- DIA. The differences are in the phosphorylated form of Cx43, as shown in Figure 3D.

4. Page 6, first line. What is the age of the male and female mice? How you would rule out the effect of hormones in females?
   - Diabetes was induced in mice 8-10 weeks of age by 2 intraperitoneal injections of 100 mg/kg body weight of streptozotocin. We cannot rule out the effect of hormones in females. However, the trends in the echocardiographic evaluation of geometrical and functional parameters of the left ventricle in males and females are the same. This suggests that the hormones in females have not affected the cardiac responses. We have only used males for our subsequent analyses.

5. Page 6, second paragraph, second sentence. Is the blood glucose level is fasting or post prandium?
   - We measured the fasting blood glucose level in the experimental animals. This information is added to the text.
6. Cardiac gene expression profiling. Is it a PCR array? Why authors want to target specifically these genes/ pathways? Please explain in the Introduction part about these genes/ pathways in relation to diabetic cardiomyopathy and elaborate it in your discussion section about why you focus on these genes/ pathways.

- We have used quantitative PCR. These genes were selected based on the literature search in relation to diabetic cardiomyopathy, HIF-1 pathways, and cardiovascular diseases. The explanation was added to the Introduction. The particular link for each gene is explained in the Discussion in detail.

**Reviewer: 2**

"Perhaps the most important limitation of this work is the fact that HIF-1-alpha content isn’t reported. Were there differences in HIF-1-alpha content that might correlate with the amount of mRNA encoding HIF-1 target genes? What were the effects of HIF-1-alpha underexpression and, importantly, hyperglycemia/hypoinsulinemia on HIF-1-alpha protein content? This information is essential to understand the interaction of diabetes and HIF-1-alpha underexpression, especially since the first paragraph of the abstract mentions other studies that have shown hyperglycemia (but not hypoinsulinemia?) to suppress HIF-1-alpha content."

- The other studies (references #15-18) have used streptozotocin-induced diabetes, db/db mice, cell cultures in high glucose, and tissue samples from diabetic patients. Therefore, we have changed the sentence in the Abstract, because it is a diabetic environment not only hyperglycemia, which affects HIF-1α stability and activity.
- The measurement of HIF-1α levels in the heart are added to the Results. See, Western analysis results (page 13-14).

"There is a partial data set for female mice, limited to echocardiography; gene expression, protein contents and apoptosis are not shown for females. To simplify the manuscript, the studies in female mice can be omitted without detracting from the manuscript."

- We believe that the additional data for females further support our results in males. The female data show similar trends, and thus, these data confirm our finding.

"Please state in the abstract and introduction the specific hypothesis this study tested."

- The hypothesis is stated.

"The last several lines of the morphological analysis methods describe measurements of cardiomyocyte morphology and dimensions, yet these results do not appear in this manuscript. Also, the preceding text states that anti-CD34 detects blood vessels, not cardiomyocytes."

- The results describing the cardiomyocyte dimension are in the Result section (page 14): *Quantitative measurements of myocyte width yielded identical values in all groups (data not shown), which confirmed the absence of hypertrophy at this stage.*
- Anti-CD34 antibody stains blood vessels. The staining of blood vessels indicates how the muscle is cut. Myocyte size (minimum transverse diameter) is measured on sections stained with anti-CD34. The cardiomyocytes can be best approximated as rod-shape with an oval cross section. Any errors due to a variation of the section plane are avoided by choosing the minor axis only in cells where a nucleus is present.
"One way ANOVA (Statistical analysis) is not appropriate for this study design. There are two (diabetes, genetic modification) and sometimes 3 (diabetes, genetic modification, gender) independent factors at play. Statistical analyses of these data require 2- or 3-factor ANOVA."

- I want to especially thank the reviewer for this important suggestion. We have re-analyzed our data and changed the text accordingly.

"Does Cx43 phosphorylation affect gap junctions? Please discuss, especially given the decreased phosphorylation in the HIF-1-alpha heterozygous diabetic mice."

- The discussion of the results is further elaborated, page 14 and 18.

"Discussion, fifth paragraph: Why would increased apoptosis be protective? Please elaborate."

- We believe that only a possible suggestion for the increased apoptosis in the diabetic Wt heart but not in the diabetic Hif1a+/− heart is the decreased sensitivity of Hif1a+/− cardiac tissue to apoptosis-induction signals as a consequence of HIF-1α partial deficiency. We have modified the discussion.

"It would be appropriate to conclude the Discussion with a paragraph summarizing the study’s limitations."

- We have concluded the Discussion with a paragraph summarizing the study’s limitations.

"In Table 2, the only statistical comparison is against non-diabetic Wt. The more meaningful comparisons would be HIF-1-alpha heterozygous diabetic vs. WT diabetic, and HIF-1-alpha heterozygous diabetic vs. HIF-1-alpha heterozygous non-diabetic."

- We have re-analyzed our data with 2-way ANOVA with and Tukey’s post-hoc multiple-comparisons test for between-group differences (See Table 2).

"Was the evaluator of the VEGF-A expression (Figure 5) blinded to the treatment?"

- Yes, we have added a statement to the Methods (page 10).

MINOR ESSENTIAL REVISIONS:

- All corrections suggested by reviewer 2 have been made.

"The first sentence of the abstract and second sentence of the introduction mention “left ventricular abnormalities” without further explanation. Are the abnormalities structural? Mechanical? Metabolic? Please specify."

- We have changed the sentence.

"Last sentence of introduction; change “the heart functions” to the more specific “cardiac mechanical function.”"

- We have changed the sentence.
“First sentence of the Western blot methods: Since the protocols are standard ones for the laboratory, please provide a literature citation for more detail.”

- We have included a citation.

“Quantitative real-time PCR: Please indicate why you consider Hprt1 to be the best reference gene for your analysis.”

- The statement has been added (page 9): The expression of this reference gene was unchanged in response to the experimental conditions being investigated.

“Last sentence of results: According to Figure 5B, the reduction in VEGF-A protein content in diabetic vs. non-diabetic mice with HIF-1-alpha underexpression was not statistically significant.”

- We clarify our statement (page 15): Furthermore, the VEGF-A protein levels were significantly decreased in the coronary vessels of diabetic Hif1a+/− and Wt compared to non-diabetic Wt, indicating vascular changes in the diabetic heart.

“In Figure 3, the scale bar is missing from the Coll1 images.”

- The Figure has been corrected.

“Please delete “in the heart” from the y-axis label in Figure 4. As written, the present label implies every apoptotic cell in the heart was detected.”

- The Figure has been corrected.