Author's response to reviews

Title: Increased vascular endothelin type B and angiotensin type 1 receptors in patients with ischemic heart disease

Authors:

Ivan Dimitrijevic (ivan.dimitrijevic@med.lu.se)
Marie-Louise Edvinsson Edvinsson (MarieLouise.L.Edvinsson@skane.se)
Qingwen Chen (cherqw@hotmail.com)
Malin Malmsjö (malin.malmsjo@med.lu.se)
Per-Ola Kimblad (Per-Ola.Kimblad@skane.se)
Lars Edvinsson (lars.edvinsson@med.lu.se)

Version: 4 Date: 24 April 2009

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

Thank You for the letter regarding the manuscript MS: 5456790712334545 – “Increased vascular endothelin type B and angiotensin type 1 receptors in patients with ischemic heart disease” by Dimitrijevic et al. We are grateful for the positive comments and that you consider this manuscript almost clear for publication in BMC Cardiovascular disorders.

We thank the reviewers for the positive comments that “the observations are of interest”.

We have addressed each comment raised by the reviewers carefully, performed new experiments that are included (in vitro pharmacology and Western blot). We believe that the manuscript is significantly improved.

We look forward to your response and are keen to accommodate any subsequent suggestions that may facilitate the publication of our manuscript.

Sincerely,

Ivan Dimitrijevic, MD
Reviewer 1 comments:

Comment:

“This manuscript states that they used immunohistochemistry and immunobloting to show the receptor expression. However, no representative pictures are given in this manuscript”

Response:

We have reviewed the pictures and modified them the best we can. The vessels are small and hence difficult to have excellent pictures. We have now performed Western blot to verify the observations at the protein level. In addition, we have done a series of functional myograph experiments on isolated vessels which also supports the original study data.

Comment:

In Figure 3A, the error bars are too big; there is actually no significant difference between two groups.

Reply:

We are sorry for being unclear: The error bars are presented as standard deviation and not standard deviation of the mean; hence, the statistics is ascertained. This is now clarified in the text.

Reviewer 2 comments:

Major comments:

1) The specificity of the antibodies has to be demonstrated either by performing western blots or by pre-absorption experiments with the respective antigens

Reply;

Thank you for addressing this problem. We have now (a) added Western blot to verify the protein (see above comment). (b) In addition, a thorough investigation of experiments involving the included antibodies has been done and this is summarized below providing ample support for their specificity.
The AT1-receptor antibody (sc-1173) and the AT2-receptor antibody (sc-9040), Santa Cruz Bio-technology, Santa Cruz, CA) are rabbit polyclonal anti-body of human origin. The AT1 receptor has no-cross-reactivity with the AT2 receptors and the AT2 antibody receptor has no cross-reactivity with the AT1 receptor. The supplier has characterized specification, and the antibodies were successfully used in several studies as well as controlled for specificity. The used Endothelin antibodies have also been included in several studies as well (pertinent references are given below and some included in the manuscript).

SC 1173;


SC 9040


IBL, 16207


**SC-21194**


**Comment:**

2) *The quantification based on immunofluorescence measurements should be confirmed by western blot analysis. The analysis of mRNA expression would further add valuable information.*

**Reply:**

The suggested Western blot analysis has now been performed on novel vessels and the data together with statistical analysis is included in the manuscript.

**Comment:**

*It is not clear in which terms the groups of patients with angina pectoris and patients undergoing CABG differ. Did all patients in the CABG group have a history of myocardial infarction? What was the time interval between myocardial infarction and CABG? Do the patient groups differ in the clinical degree of severity of heart failure (NYHA classification)? At which time point were NT pro-BNP levels obtained?*

**Reply:**

CABG patients differed from angina pectoris patients by having;

I. Three vessel heart disease concluded by coronary angiography.
II. Verified history of aggravating stable angina pectoris.

III. Cardiac ischemia being responsible for the heart failure (this is clarified in table I).

The CABG patients had no prior history of myocardial infarction (MI) and the ECG showed no signs of new or old myocardial injuries. Preoperative laboratory investigation (the day before CABG surgery) including NT pro-BNP. Clarification is included under the methods section “study groups” part.

Comment:

*Were the levels of ET- and AT-receptor expression influenced by drug treatment especially with ACE-inhibitors or AT-blockers?*

Reply;

This is an interesting question that really has no reply. We are not aware of data in the literature that specially address this issue. The effects of these confounding factors on the endothelin and angiotensin II receptor expression can't be concluded from the present study; we have now included this potential confounding error in a new paragraph called study limitations.

Minor comments:

Comment

*Reference 11 does not relate to ETB-receptors.*

Reply;

This error has now been corrected.

Comment

*The authors should include more citations of previous work in the field (for example: Kobayashi et al., 2000; Babaei et al., 2000; Pernow et al., 2000; Böhm et al., 2002; Strachan et al., 2000; Spieker et al., 2001; Cowburn et al., 1999).*

Reply;
We have read the suggested articles and included them in the text where appropriate.

Reviewer 3 comments:

Major comments:

The authors are encouraged to conduct functional experiments with S6c (sarafotoxin- ETB receptor antagonist) and human angiotensin II and include the data in the paper. This will improve the quality of paper significantly.

Reply;

At the outset of the study we were considering to perform such a study. However, due to the small size of the arterioles in the biopsies this was not possible. However, to accommodate the suggestion of the reviewer we have obtained somewhat larger subcutaneous arteries and performed In vitro pharmacology study on these. The data came out nicely and is now included in the manuscript.

Minor revisions:

1- page 4,line 6: the continuous interaction between endothelin-1 and angiotensin II is important for the control of vessel tone, and changes.......  

Reply

Amended

2-page 4, line 20: Endothelin A and B receptors located on vascular smooth muscle cells are both mitogenic,.....

Reply

Amended

3- page 4, line 22 (last sentence at the bottom of the page 3): ......which contribute to the development of ischemic cardiovascular disease.

Reply

Amended
4- page 5, line 9: ......that regulates cell growth, differentiation and fibrosis, and.........

Reply

This has now been adjusted

5- page 5, line 13: To the best of our knowledge, this is the first in vitro study using the peripheral vascular tissue samples isolated from patients.

Reply

The sentence is now changed

6- page 5, line 14: The peripheral vasculature is contributing significantly to total peripheral resistance.

Reply

The sentence is now changed

7- page 6, line 21: get rid of the extra "dot" (.) after "surgery.

Reply

Done

8- Why the plasma concentrations of ET-1, angiotensin-II, Adrenalin (or noradrenalin) were not measured ?

Reply

Although we did not measure plasma ET-1, angiotensin-II, or Adrenalin in this study, other studies have studied the concentration of these peptides under different conditions such as cardiovascular disorders. **ET-1** binds to receptors close to the site of its release rather than a circulating hormone. ET-1 assay results based just on plasma concentrations says little about the ET-1 biosynthesis. Since ET-1 binding to residual proteins in plasma, purification is mandatory if samples contain low ET-1 concentrations but according to some manufacturers of
commercially available assays the samples can be handled without prior purification. However, reliable ET-1 quantification is hampered by technical difficulties such as cross-reactivity of assay antibodies against other endothelins because of strong sequence homologies. Use of beta-blockers, angiotensin receptor inhibitors and ACE inhibitors certainly may modulate the concentrations of angiotensin II and noradrenalin. We have addressed these restrictions in the novel section on study limitations.

9-page 10, line 12: According to the data presented in table 1, the BNP is not significantly increased in "Angina" group.

Reply
This has now been changed.

10- In general, the authors should also discuss the increase in BP and BNP in connection to the increased levels of receptor proteins (ETB and AT1). How do these value correlate with the clinical condition and the extent of receptor upregulation?

Reply
We have in part responded to this question above (ref 2). It is difficult to know and only future experiments will with certainty provide the answered. However, we have recently analysed about 60 arteries from the CABG patients for responses to S6c. It turned out that there were significantly more ETB receptor responses in the hypertensives as compared to the normotensive patients (Nilsson et al, Human Hypertension 2008). If this is due to the hypertension or the medication against hypertension is still unclear. We have added a note on this in discussion.