Author's response to reviews

Title: Pharmacological reversal of endothelin-1 mediated constriction of the spiral modiolar artery: a potential new treatment for sudden sensorineural hearing loss

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Author's response to reviews: see over
Responses to Hiroaki Shimokawa

We would like to thank the reviewer for his helpful comment. To assist the reviewer in identifying the manuscript modifications, they have been highlighted as blue text in the revised version of the manuscript.

Major Compulsory Revisions

1. The reviewer states that the authors also should use diseased (spiral modiolar artery) SMA taken from an ischemic stroke model. Otherwise, the clinical importance of this study is unclear.

We agree that examination of a diseased SMA model would increase the clinical relevance of this study. However, before a systematic investigation of pathophysiological mechanisms can be undertaken, it is necessary to establish the normal (i.e., non-pathological) control mechanisms that regulate SMA tone. Our data show that Rho-kinase signalling is the predominant mechanism controlling SMA tone under non-pathological conditions. Classical calcium-mobilizing mechanisms, for example adrenergic, have minimal effects in the SMA. Endothelin-1 (ET-1) is a potent activator of the RhoA/Rho-kinase pathway, and is, based on the present study, a potential physiological regulator of SMA tone. Current literature supports the premise that the extent of Rho/Rho-kinase signalling may be quite different between normal and diseased blood vessels [1,2]. ET-1 synthesis and RhoA/Rho-kinase signalling are both enhanced during inflammation, hypoxia and elevated oxLDL levels [1,3,4], and could therefore be altered under a disease state. We believe, however, that the important next step of investigating a disease model represents a separate and independent study.

An allusion to this pathological potential can be found in background, paragraph 3.

2. Change the term “vasospasm” to “vasoconstriction” throughout manuscript.

According to the reviewer’s suggestion, we have changed the term vasospasm to vasoconstriction throughout the manuscript.

3. It is unclear why the authors used ET-1 in this study.

ET-1 is known to stimulate cerebral artery vasospasm and is linked to cerebrovascular disease [5,6]. Strikingly, sudden sensorineural hearing loss bears several similarities with cerebrovascular disease, including sudden onset, the possibility of rapid resolution and unilateral occurrence.

Based on these similar characteristics, we hypothesized that ET-1 possesses pathological potential in the SMA. ET-1 is a strong Rho-kinase-activating agonist in the SMA, which induces a long-lasting constriction that outlasts the stimulus. It is also likely that ET-1 synthesis is enhanced under pathological conditions (e.g., hypoxia, inflammation, elevated ox-LDL) [3,4].

We believe that this rationale has been clarified in background (paragraph 3).
4. **The authors should provide evidence that Rho-kinase activity in SMA is actually inhibited by the Rho-kinase inhibitors (e.g. the extent of phosphorylation of myosin binding subunit by the Rho-kinase inhibitors (e.g. the extent of phosphorylation of myosin binding subunit by Western blotting).**

Because of its very small size (60µm diameter and a length of 3mm), western blotting SMA protein is technically challenging. We attempted western blots using pooled SMA lysates (20 vessels), but were not able to successfully detect proteins of interest (i.e., phosphorylated myosin). It would be extremely labour and time intensive process to optimize the necessary conditions for western blotting SMA protein. The $IC_{50}$ for Y-27632, fasudil and Hydroxyfasudil Rho-kinase inhibition is documented to be 0.8, 1.9 and 5.1 µM [7-11]. Therefore, the concentrations used in the present study are within the specific and effective range. Since Y-27632 and fasudil are structurally unique, but were observed to have similar functional effects, the concern of non-specific effects of the inhibitors is minimized.

A statement related to structural uniqueness of the inhibitors and how this can minimize the concern of non-specific effects now appears in the discussion, paragraph 3.

5. **The addition of the data with dbcAMP has diluted the importance of the study. The authors should either delete this portion or add more supporting data showing the effect of dbcAMP on abnormal contraction of diseased SMA as well as intracellular cAMP data.**

We disagree that including the data concerning cAMP diluted the importance of the study.

We have previously shown that CGRP is able to reverse ET-1 induced constrictions in the SMA via an increase in vascular smooth muscle cAMP [12]. CGRP is present in perivascular nerves of the SMA and therefore is a potential endogenous vasodilator of the SMA.

Our data employing dbcAMP highlights that the RhoA/Rho-kinase signalling mechanism leading to vasoconstriction is not an “absolute” mechanism, rather it is a constricting mechanism in competition with other vasodilator pathways. In the present study, stimulation of cAMP-activated signalling pathways antagonizes ET-1-mediated vasoconstriction. Thus, clinical reversal of ET-1-induced constriction is not limited to Rho-kinase inhibition.

The rationale for using dbcAMP now appears in the introduction, paragraph 4 and is discussed in the discussion, paragraph 5.

**Minor Essential Revisions**

- Rhokinase was changed to Rho-kinase throughout the text
- Fasudil was purchased by Tocris Cookson and modified to Hydroxyfasudil by Dr. Duy Hua, Dept of Chemistry, Kansas State University.
- We verified that fasudil and hydroxyfasudil are selective inhibitors and included four more references in the manuscript [7-11].

Reference List


Responses to Kazuo Obara

We would like to thank the reviewer for his helpful comments. To assist the reviewer in identifying the manuscript modifications, they have been highlighted as blue text in the revised version of the manuscript.

Mayor Compulsory Revisions

1. In Fig1A, inhibitory effect of fasudil is not clear. Because time course of increase in diameter by fasudil looks like that of spontaneous relaxation in response to ET-1. Authors should provide a typical tracing of ET-1-induced constriction of SMA.

As requested, a representative tracing of ET-1 induced constriction was included in Figure 1.

2. It is not clear why dbcAMP is used in this study. Please explain.

We have previously shown that CGRP is able to reverse ET-1 induced constrictions in the SMA via an increase in vascular smooth muscle cAMP [1]. CGRP is present in perivascular nerves of the SMA and therefore is a potential endogenous vasodilator of the SMA.

Our data employing dbcAMP highlights that the RhoA/Rho kinase signalling mechanism leading to vasoconstriction is not an “absolute” mechanism, rather it is a constricting mechanism in competition with other vasodilator pathways. In the present study, stimulation of cAMP-activated signalling pathways antagonizes ET-1-mediated vasoconstriction. Thus, clinical reversal of ET-1-induced constriction is not limited to Rho kinase inhibition.

We have added the necessary rationale in the introduction, paragraph 4, and put the data into context in the discussion, paragraph 5.

3. Authors show that there is difference among IC50s of Y-27632, fasudil and hydroxy-fasudil for ET-1-induced vasoconstriction in SMA. In most vascular smooth muscle, IC50 of fasudil for ET-1-induced contraction is almost the same as that of hydroxy-fasudil. Please comment.

The difference in the determined IC50 values of fasudil and hydroxy-fasudil is most likely due to variability between species and/or the different vascular bed under investigation. This is consistent with observations previously published where it has been shown that the IC50 values of fasudil and hydroxy-fasudil were different in canine basilar and swine coronary arteries [2,3]. The IC50 also depended, in part, on the agonist used.

4. It is obscure why and how long vessel segments were maintained at 4°C after fluo-4 loading. Authors showed that experiments were started 20 min after loading with fluo-4. Please explain.

Several vessel segments were incubated with Fluo-4 under conditions of continuous gentle shaking. Following the incubation, all of the vessel segments were washed...
and then 1 vessel segment was selected for experimentation by visual inspection. To avoid hypoxic conditions while the best vessel segment was selected, all vessels were maintained at 4°C during this time. A standardized timeframe of 20 minutes was maintained for the election of an appropriate vessel and its transfer to the experimental setup. To clarify this method, we state that the vessel segments were washed with PSS and maintained at 4°C for 20 minutes prior to experimentation at 37°C (page 5, last paragraph).

5. Rho kinase is considered to increase Ca^{2+}-sensitivity of contractile apparatus in vascular smooth muscle through not only an inactivation of MLCP but also activations of MLCK and CPI-17. Have authors measured activity of MLCP and/or MBS phosphorylation during ET-1-induced contraction of SMA?

Because of its very small size (60µm diameter and a length of 3mm), SMA lysate-based assays (i.e., western blots, enzyme activity assays) are technically challenging. For example, we have attempted western blots using pooled SMA lysates (20 vessels), but were not able to successfully detect proteins of interest (i.e., phosphorylated myosin). It would be extremely time and labour intensive process to isolate enough tissue and optimize the necessary conditions for such assays.

Minor Essential Revisions
Minor essential revisions were made according to the reviewer’s comments (highlighted as blue text in the revised version of the manuscript)

Reference List

