Author's response to reviews

Title: Enhanced expression of ROCK2 in left atrial myocytes of mitral regurgitation: A potential mechanism of myolysis

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Author's response to reviews: see over
Enclosed please find the revised manuscript entitled “Enhanced expression of ROCK in left atrial myocytes of mitral regurgitation: A potential mechanism of myolysis” (MS: 9770158511447013 R1). We have revised our manuscript according to the reviewers’ comments. We provide point-by-point responses to the reviewers’ comments. We have underlined all the changes in the revised manuscript. Thank you.

Yours sincerely,

Mien-Cheng Chen, M.D.
Specific responses to the first reviewer’s comments:

(1) Authors defined the role of caspase-3 in apoptosis execution only. However, the evidence on the role of caspase-3 in activation of ROCK proteins by removing the ROCK autoinhibitory C-terminal domain is missing. Because this process occurs in early phase of apoptosis, and considering the fact that activation of ROCK might be caspase-independent, this matter must be clarified. I believe addressing this issue would significantly improve the obtained data on colocalization between ROCK2 and caspase-3. The data of colocalization between these molecules missing in the abstract. The explanation for results of colocalization (The paragraph 1: page 16, lines 233-239) must be rewritten, since those results don’t represent the “correlation analysis”, but the existence of potential protein:protein interaction between these two molecules. Also, the last sentence of this paragraph should be explained as confirmation of caspase-dependent activation of ROCK2 in myocytes of patients with mitral regurgitation.

Responses: The role of caspase-3 in activation of ROCK proteins by removing the ROCK autoinhibitory C-terminal domain is better to be illustrated in cell model or animal model with tissues in different stages of apoptosis. This study was conducted in MR patients with heart failure (late stage); therefore, it is difficult to confirm activation of ROCK proteins via removing the ROCK autoinhibitory C-terminal domain by caspase-3 in early phase of apoptosis in these patients. This is a limitation of this study. However, quantitative co-localization analysis is a frequently used advanced digital imaging tool to examine the aggregation of antigens of interest in immunofluorescence images obtained using confocal microscopes (Acta Histochem Cytochem 2007;40:101–111; Mol Biol Cell 2006;17:5038-5052.).

We have revised our manuscript according to this comment in the revised abstract and page 18, para 1 of the revised manuscript.

(i) Abstract: Immunofluorescence study revealed significant co-localization and juxtaposition of ROCK2 and cleaved caspase-3 in the left atrial myocytes both in the MR AF group (Pearson's coefficient = 0.74 ± 0.03) and the MR sinus group (Pearson's coefficient = 0.73 ± 0.02).

(ii) “Immunofluorescence study revealed a significant co-localization and juxtaposition of the expression of ROCK2 and the expression of cleaved caspase-3 in the left atrial myocytes both in the MR AF group (Pearson's coefficient = 0.74 ± 0.03) and the MR sinus group (Pearson's coefficient = 0.73 ± 0.02) (Figure 5), indicating the existence of potential interaction between ROCK2 and cleaved caspase-3 and confirmation of
caspase-dependent activation of ROCK2 in the left atrial myocytes of MR patients, and this interaction might be involved in the pathogenesis of left atrial myolysis in MR patients. Similarly, Immunofluorescence study revealed a significant co-localization and juxtaposition of the expression of ROCK1 and the expression of cleaved caspase-3 in the left atrial myocytes both in the MR AF group (Pearson's coefficient = 0.65 ± 0.03) and the MR sinus group (Pearson's coefficient = 0.65 ± 0.03)."

(2) In the results section, expression of ROCK2 protein is expressed as “integrated intensity”. This phrase is confusing. I suggest that this term would be replaced with “expression” in abstract and results and the calculation would be explained under the Table 3. Also, authors have chosen to study the activity of ROCK2 expressed as a relative ratio of the phosphorylated and total myosin-binding subunit of myosin light chain phosphatase. However, this information is missing in results section (the only explanation is under the Table 3). Considering the fact that ROCK2 has the other targets, this information must be clearly stated in results section. The result of ROCK2 activity is missing in the abstract.

Responses: We have revised our manuscript according to this comment in the revised abstract and page 16, para 2 of the revised manuscript.

Revised abstract: “Moreover, the ratio of phosphorylated myosin-binding subunit of myosin light chain phosphatase (pMBS)/total MBS of left atrial tissues was significantly higher in the MR AF group (p < 0.04) and the MR sinus group (p < 0.04) compared with the normal control group.”

“The ROCK activity is expressed as a relative blot density ratio (pMBS sample density/tMBS sample density) [27]. The ratio of pMBS/tMBS of left atrial tissues was significantly higher in the MR AF group compared with the normal control group (0.30 ± 0.11 vs. 0.02 ± 0.01, p < 0.04) (Figure 7). Similarly, the ratio of pMBS/tMBS of left atrial tissues was significantly higher in the MR sinus group compared with the normal control group (0.10 ± 0.03 vs. 0.02 ± 0.01, p < 0.04).”

(3) Since the number of patients was small, the conclusions must be rewritten. In the abstract, “..significantly associated…” should be replaced with “might be involved”.

Responses: We have revised our manuscript according to this comment in the conclusion of the revised manuscript.

“Conclusions: The enhanced expression of ROCK2 might be involved in the myolysis of the left atrial myocytes of MR patients.”

(4) In the introduction section, authors stated that “ROCKs play a crucial role in
apoptosis” (page 6, line 44). Since the both propaoptotic and antiapoptotic roles of RhoA/ROCK have been reported in cardiomyocytes (review article of Surma and Shi, Future Cardiology, 2011), this part of introduction needs to be rewriting, with more precise explanation of molecular mechanisms involved in ROCK2 mediated roles in apoptosis.

**Responses:** We have revised our manuscript according to this comment in page 5, para 1 of the revised manuscript.

“ROCKs may play a role either as a proapoptotic or anti-apoptotic regulator [18,19]. The ROCKs mediated apoptosis could be either caspase 3-dependent or caspase 3-independent cleavage of ROCKs [19]. However, exactly how ROCK regulates an apoptotic response is not completely understood in many instances, and is likely different depending on the cell type and the apoptotic stimulus [20]. There are two isoforms of ROCKs, ROCK1 and ROCK2 [21,22]. ROCK2 is distributed mostly in the heart and brain. However, ROCK1 is mainly expressed in the lung, liver, spleen, kidney and testis. Similarly, caspase-3, a key mediator of apoptosis, has a significant role in myocyte apoptosis and is a therapeutic target in heart failure [23-26]. However, the relationship between ROCKs and caspase-3 in the atrial myocytes of heart failure patients due to severe MR remains unknown. Additionally, the relationship between ROCKs and myolysis in the left atrial myocytes of MR patients remain unclear. We hypothesized that there was a positive correlation between myolysis and the expression levels of ROCKs in the left atrial myocytes of heart failure patients due to severe MR. Accordingly, the present study investigated the expression levels of ROCKs and caspase-3 in the left atrial myocytes of heart failure patients due to severe MR.”

(5) Since the definition of apoptosis is a well-known fact, it is not necessary to explain it (background: page 5, line 32). Please delete “…. Programmed cell death pathway activation” and “….is a morphologically distinct form of death in most physiological cells” (page 5, line 34).

**Responses:** We have revised our manuscript according to this comment in page 4, para 2 of the revised manuscript.

“Atrial myocardial stretch caused by volume and pressure overload due to significant MR may cause apoptosis. A previous study revealed that apoptosis occurs in the atrial myocytes of patients with mitral valve diseases [7]. Apoptosis plays a critical role in myocyte loss [8].”

(6) In Table 1, p value obtained for dyslipidemia didn’t match the value in results section (page 12, line 156).
Responses: We have revised our manuscript according to this comment in the revised Table 1 of the revised manuscript.

(7) Delete “… although the difference did not reach statistical significance” page 14 line 194.
Responses: We have revised our manuscript according to this comment and deleted that sentence.

(8) The word “a crucial” (Discussion: page 17, line 255) should be replaced with “important”.
Responses: We have revised our manuscript according to this comment in page 19, para 2 of the revised manuscript.
“ROCKs play an important role in many cellular functions, including proliferation, migration, adhesion, contraction, gene expression, and apoptosis [18-22].”

Thank you for your comments.
Specific responses to the second reviewer’s comments:

(1) ROCK1 is also expressed in atrial tissue. It is not known the relative contribution of ROCK activity by ROCK1 and ROCK2 in atrial myocytes (as well as in many major tissues). It is therefore important to examine expression of ROCK1 as well. In addition ROCK1 is a substrate of caspase 3 and can be activated by caspase 3. The increased ROCK activity measured in MR tissues may be a consequence of ROCK1 activation by caspase 3.

Responses: We have revised our manuscript according to this comment and also studied the expression of ROCK1 in this study (page 15, para 2, page 16, para 1 and page 18, para 1 of the revised manuscript).

“The expression of ROCK1 in left atrial myocytes of the MR AF patients was significantly higher than the expression of ROCK1 of the normal control subjects (2033899.5 ± 172865.3 vs. 1132555.5 ± 42027.4, p = 0.016) (Figure 4). Of note, the expression of ROCK1 in the myolytic left atrial myocytes of the MR AF patients was significantly higher than the expression of ROCK1 in the myolytic left atrial myocytes of the normal control subjects (2173527.6 ± 159745.3 vs. 978383.3 ± 116680.7, p = 0.010). However, the expression of ROCK1 in the non-myolytic left atrial myocytes of the MR AF patients did not significantly differ from the expression of ROCK1 in the non-myolytic left atrial myocytes of the normal control subjects (1303403.2 ± 257033.5 vs. 1248082.3 ± 50698.5, p = 0.815). Interestingly, the expression of ROCK1 in left atrial myocytes of the MR AF patients was significantly higher than that of the MR sinus patients (2033899.5 ± 172865.3 vs. 1514957.0 ± 231591.3, p = 0.041) (Figure 4). The expression of ROCK1 in the myolytic left atrial myocytes of the MR AF patients was higher than that of the MR sinus patients (2173527.6 ± 159745.3 vs. 1804745.3 ± 302136.4, p = 0.091). The expression of ROCK1 in left atrial myocytes of the MR sinus patients was higher than the expression of ROCK1 of the normal control subjects (1514957.0 ± 231591.3 vs. 1132555.5 ± 42027.4, p = 0.398). The expression of ROCK1 in the myolytic left atrial myocytes of the MR sinus patients was higher than the expression of ROCK1 in the myolytic left atrial myocytes of the normal control subjects (1804745.3 ± 302136.4 vs. 978383.3 ± 116680.7, p = 0.091). Of note, correlation analysis demonstrated that there was a direct relationship between the expression of ROCK1 in the myolytic left atrial myocytes and left atrial diameter in the MR patients (p = 0.057; r = 0.422).”

“Similarly, Immunofluorescence study revealed a significant co-localization and juxtaposition of the expression of ROCK1 and the expression of cleaved caspase-3 in the left atrial myocytes both in the MR AF group (Pearson’s coefficient = 0.65 ± 0.03) and the MR sinus group (Pearson’s coefficient = 0.65 ± 0.03).”
(2) Immunohistology analysis shows an increase in ROCK2 protein expression and cleaved caspase 3 expression. As an antibody may have non-specific reaction especially with histology analysis, this data should be confirmed by Western blotting for ROCK2 and cleaved caspase 3 and also at RNA levels for ROCK2.

**Responses:** We have revised our manuscript according to this comment in page 14, para 1, lines 14-19, and page 15, para 1, page 17, para 1, lines 11-19, page 18, para 1, line 1 of the revised manuscript. The protein expression levels by immunoblotting were consistent with those by immunohistochemistry. Therefore, it is not absolutely necessary to study RNA levels of these proteins.

“The expression of ROCK2 protein (normalized to GAPDH) by immunoblotting in left atrial tissues of the MR AF patients (n = 6) was significantly higher than the expression of ROCK2 of the normal control subjects (n = 3) (2.32 ± 0.18 vs. 0.53 ± 0.16, p = 0.020). The expression of ROCK2 protein by immunoblotting in left atrial tissues of the MR sinus patients (n = 4) was higher than the expression of ROCK2 of the normal control subjects (n = 3) (0.85 ± 0.10 vs. 0.53 ± 0.16, p = 0.157). The expression of ROCK2 protein by immunoblotting in left atrial tissues of the MR AF patients was significantly higher than the expression of ROCK2 of the MR sinus patients (p = 0.011).”

“The expression of cleaved caspase-3 protein (normalized to GAPDH) by immunoblotting in left atrial tissues of the MR AF patients (n = 6) was significantly higher than the expression of cleaved caspase-3 of the normal control subjects (n = 3) (0.20 ± 0.04 vs. 0.04 ± 0.02, p = 0.039). The expression of cleaved caspase-3 protein by immunoblotting in left atrial tissues of the MR sinus patients (n = 4) was higher than the expression of cleaved caspase-3 of the normal control subjects (n=3) (0.09 ± 0.02 vs. 0.04 ± 0.02, p = 0.157). The expression of cleaved caspase-3 protein by immunoblotting in left atrial tissues of the MR AF patients was higher than the expression of cleaved caspase-3 of the MR sinus patients (p = 0.055).”

Thank you for your comments.
Specific responses to the third reviewer’s comments:

(1) The authors should have examined ROCK1, even if ROCK1 may not be expressed.

**Responses:** We have revised our manuscript according to this comment and also studied the expression of ROCK1 in this study (page 15, para 2, page 16, para 1 and page 18, para 1 of the revised manuscript).

“The expression of ROCK1 in left atrial myocytes of the MR AF patients was significantly higher than the expression of ROCK1 of the normal control subjects (2033899.5 ± 172865.3 vs. 1132555.5 ± 42027.4, p = 0.016) (Figure 4). Of note, the expression of ROCK1 in the myolytic left atrial myocytes of the MR AF patients was significantly higher than the expression of ROCK1 in the myolytic left atrial myocytes of the normal control subjects (2173527.6 ± 159745.3 vs. 978383.3 ± 116680.7, p = 0.010). However, the expression of ROCK1 in the non-myolytic left atrial myocytes of the MR AF patients did not significantly differ from the expression of ROCK1 in the non-myolytic left atrial myocytes of the normal control subjects (1303403.2 ± 257033.5 vs. 1248082.3 ± 50698.5, p = 0.815). Interestingly, the expression of ROCK1 in left atrial myocytes of the MR AF patients was significantly higher than that of the MR sinus patients (2033899.5 ± 172865.3 vs. 1514957.0 ± 231591.3, p = 0.041) (Figure 4). The expression of ROCK1 in the myolytic left atrial myocytes of the MR AF patients was higher than that of the MR sinus patients (2173527.6 ± 159745.3 vs. 1804745.3 ± 302136.4, p = 0.091). The expression of ROCK1 in left atrial myocytes of the MR sinus patients was higher than the expression of ROCK1 of the normal control subjects (1514957.0 ± 231591.3 vs. 1132555.5 ± 42027.4, p = 0.398). The expression of ROCK1 in the myolytic left atrial myocytes of the MR sinus patients was higher than the expression of ROCK1 in the myolytic left atrial myocytes of the normal control subjects (1804745.3 ± 302136.4 vs. 978383.3 ± 116680.7, p = 0.091). Of note, correlation analysis demonstrated that there was a direct relationship between the expression of ROCK1 in the myolytic left atrial myocytes and left atrial diameter in the MR patients (p = 0.057; r = 0.422).”

“Similarly, Immunofluorescence study revealed a significant co-localization and juxtaposition of the expression of ROCK1 and the expression of cleaved caspase-3 in the left atrial myocytes both in the MR AF group (Pearson's coefficient = 0.65 ± 0.03) and the MR sinus group (Pearson's coefficient = 0.65 ± 0.03).”

(2) Tables 2 and 3. They are not suitable to tables. They should be in graphs with demonstrable pictures.

**Responses:** We have revised our manuscript according to this comment and add
revised Figures 1, 3 and 4 to replace Tables 2 and 3.

(3) Figure 2. It seems that all tissues were stained in each ROCK2 and Caspase 3. Were there immuno-negative cells?

**Responses:** There was no immune-negative cell by immunohistochemistry in this study.

(4) Figure 3. The authors should show myolytic and non-myolytic myocytes also in demonstrable pictures

**Responses:** In revised Figure 2, the normal tissue section showed non-myolytic myocytes. However, most of the myocytes in the tissue sections of MR sinus patients and MR AF patients showed myolysis (arrows: central part of myocytes showing absence of staining with F actin).

Thank you for your comments.