

February 10, 2012

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 8646-review.doc).

Title: Localization and vasopressin regulation of the Na⁺-K⁺-2Cl⁻ cotransporter (NKCC2) in the distal colonic epithelium

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Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 8646

The manuscript has been improved according to the suggestions of reviewers:

1 Format has been updated

2 Revision has been made according to the suggestions of the reviewer

(1) Review #1

1. Please ascertain that all abbreviations are defined on their first usage throughout the text.

[Response: Done.](#)

2. Animals and tissue preparations: Anesthesia and final disposition of the experimental animals is not reported.

[Response: Done. Revised Manuscript P5 Line 10](#)

3. Statistical Analyses please justify the selection of the employed statistical tests, and define the level of significance. Did the authors employ a post hoc test for multiple comparisons? For each study, were

numbers of measurements pre-specified? For instance, for ANOVA, one should normally pre-specify an effect size and the number of required subjects and/or measurements for an alpha value of 0.05 and a power of 0.80. Presentation of the data as mean +/- SD might be preferable.

Response: Done. I did the ANOVA analysis and post the Tukey's post hoc method of multiple comparisons.

Revised Manuscript P8 Line 7-10

4. Results, Paragraph 1: "...consistent with observations in rat and human colonic tissues." Please provide appropriate references.

Response: Done. Revised Manuscript P9 Line12

5. In Figure 1: The number of animals used for the localization experiment is unclear. Figures 2-4: Please specify the number of mice and total number of measurements for each one of the compared variables.

Response: Done. In yellow highlighted contents of figure legends

6. Throughout the manuscript: "Not shown data" should be presented.

Response: Done. We have added the data in the result.Fig2B, b, F, I, J

7. Discussion, first sentence of first paragraph: please provide appropriate references for "previous studies."

Response: Done. Revised Manuscript P12 Line3

(2) Review#2

1. Fig. 3 clearly confirms that dDAVP increases membrane trafficking of NKCC2. Western blot of NKCC2 and GAPDH was performed in the different gels? Since the molecular weights of these two proteins are different, the same gels should be used for the detection. The authors showed whole and membrane NKCC2 expressions by Western blot. Low-density membrane fraction, which predominantly contains intracellular vesicles, would be useful to compare the expression in the high-density membrane fraction, which predominantly contains plasma membrane.

Response: In our experiment GAPDH and NKCC2 were performed on the same gel. We agreed the reviewer's suggestion about the separation of intracellular vesicles and plasma membrane. In this experiment, we isolated colonic mucosa and extracted the membrane protein from control and

dDAVP-treated mice. Western blot showed that the abundance of NKCC2 in the membrane was significantly higher in the dDAVP-treated mice than in the control, as shown in Fig 3 B and 3C, confirming the immunofluorescence data.

2. NKCC2 is present in the thick ascending limbs of the kidney, which lacks water channels. In contrast, NKCC1 is present in the collecting ducts. The author mentioned that NKCC1 and NKCC2 are present in the different types of cells. However, immunohistochemistry did not reveal that the two isoforms are present in the different types of cells. Heterogeneity of the cells is very strong in the kidney. How about the colon? Is the presence of NKCC2 and AQP2 in the different cells?

Response: As the Fig1B and Fig1 C shown, we saw the spatial distributions of NKCC1 and NKCC2 differed in the mouse colon. NKCC2 was located in the apical membrane of the mouse colonic surface epithelia, whereas NKCC1 was found in the lower crypt epithelia of the mouse distal colon. The present study mainly aimed to investigate the expression of NKCC2 in the mouse distal colonic epithelium and to clarify whether NKCC2 was also regulated by vasopressin, as it is in the kidney. It is difficult to identify the heterogeneity of the colon which is not totally same as the kidney. As you mentioned of the existing relationship between AQP2 and NKCC2, we didn't do the experiment of the double labeling. We referred to the literatures ^[1-2] which showed that AQP2 protein also was localized to the apical membrane of surface absorptive epithelial cells. Whether the two proteins located in the same cell should be investigated further.

[1] Pedro G, L. Pablo C, Carlos P. V, Francisco V. Sepúlveda Aquaporin-2, a regulated water channel, is expressed in apical membranes of rat distal colon epithelium. *Am J Physiol Gastrointest Liver Physiol* 2001; 281:G856-G863.

[2] Cristia E, Amat C, Naftalin RJ, Moreto M. Role of vasopressin in rat distal colon function. *J Physiol* 2007; 578: 413-24.

Minor comments

3. Page 8, The antibody against NKCC2 is not sc-21547.

Response: Done. The information of the antibody was corrected. Revised Manuscript P8 Line 1

4. To investigate the differences among more than three groups, multiple comparison should be performed after ANOVA. ANOVA can be used only for the detection of heterogeneity among the groups.

Response: Thank you for your suggestions. I did the ANOVA analysis and post the Tukey's post hoc method of multiple comparisons. Revised Manuscript P8 Line 7-10

(3) Review#3

Base on figure 1b, we can not conclude that NKCC1 is not expressed in the apical pole due to the resolution. The staining seems to be both subapical and apical. Dapi staining should be visible in Fig 1 d and H. The reader is not able to know if Fig 1F show apical or cells staining. Provide a better image. The figure 2 show an increase of NKCC2 in the subapical area. In order to conclude to an increase of NKCC2 into the apical membrane, authors should perform gold particles labeling NCKK2 quantification. GAPDH is a cytosolic protein marker. The western blot of the membrane fraction should be performed with a membrane protein marker such as b-actin or other proteins cited in the literature. The Western blot should show more than 3 tissue samples by condition. Accordingly to the Fig. 1, the mRNA in the kidney is more abundant than the mRNA in the colon. This difference is not apparent in the western blot where all lines were loaded with 20 ug of protein. Mastropaolo et al. (Regul pept 2013) showed that mouse colon has V1R receptor. The author should perform their short-circuit assay with dDAVP in the condition that will only affect V2R. They should confirm the effect with V1 and V2 antagonist, which are commercially available. An increase of calcium via V1R activation may result in the reduction of endocytosis of NKCC2 and subsequently an increase in NKCC2 in the apical pole.

Response: Thank you for your suggestions. We changed two better images as new Fig1B and Fig1F. We didn't do Dapi staining because Fig1D and H are the negative control.

Thank you for your suggestions. We agreed the reviewer's suggestions. Immunogold Labeling Electron Microscopy is a powerful technique for the localization and quantification of antigens in cells and tissues. It will take some time for us to establish the method, and we have not yet performed the experiment now. In this experiment, we isolated colonic mucosa and extracted the membrane protein from control and dDAVP-treated mice. Western blot showed that the abundance of NKCC2 in the membrane was significantly higher in the dDAVP-treated mice than in the control, as shown in Fig 3 B and 3C, confirming the immunofluorescence data.

β -actin is cytosolic protein marker. Moreover it is only expressed in the cytoplasm and cytoskeleton. (See the website <http://www.uniprot.org/uniprot/Q58ID9>) To date, to our knowledge it is a controversial issue about the choice of internal control. GAPDH is not only expressed in the cytoplasm but also located in the plasma membrane. (see the website <http://www.uniprot.org/uniprot/P04406>) Yes, We did the western blot in more than 3 tissue sample.

The protein level of total NKCC2 in the kidney is not apparent higher than that of in the colon although the mRNA level is more abundant than the mRNA in the colon. We think the possible reason is the differential

regulation of post-transcript in differential tissue. Moreover, we think mRNA level of NKCC2 may not exactly represent the protein level. The absence of mRNA-protein correlation for a subset of investigated genes suggests that the relation between mRNA and protein is not strictly linear, but has a more intrinsic and complex dependence, deviating from the classical view referred to as the molecular dogma. Different regulation mechanisms (such as synthesis and degradation rates), acting on both the synthesized mRNA and the synthesized protein, affect the amount of the two molecules differentially. [1]

[1] de Sousa Abreu R, Penalva LO, Marcotte EM, Vogel C. Global signatures of protein and mRNA expression levels *Mol Biosyst*. 2009; 5(12):1512-26. doi: 10.1039/b908315d

Thank you for your suggestions and we agreed with your recommendation. The present study mainly aimed to investigate the expression of NKCC2 in the mouse distal colonic epithelium and to clarify whether NKCC2 was also regulated by vasopressin, as it is in the kidney. Whether the V1 receptor is involved in the process of vasopressin regulation and physiologic mechanism of it are very interesting and need to be investigated further. This subject is important and will be studied in detail in the future.

Minor: 1) Verify to cite the correct references example; the number 16 is wrong. 2) In Materiel and method, add a reference for the previously described method 3) in fluorescence image Analysis specify the statistical method in addition to the software. 4) Can not found Boogo company manufacturer instruction on the web. Please describe the method with more details. 5) Fig 2D, authors should add arrows to determined punctated vesicles.

Response: 1) Done. P16 Line19-21 2) Done. P5 Line16 3) Done P6 Line12-14. 4) You can find the manufacturer instruction from the following website. <http://www.bgswkj.com/index.php/Product/search> 5) Done.

3 References and typesetting were corrected

Thank you again for publishing our manuscript in the *World Journal of Gastroenterology*.

Sincerely yours,

A handwritten signature in black ink, reading "Zhou Deshan". The signature is written in a cursive, flowing style. The first name "Zhou" is written with a large, stylized 'Z' and 'h'. The last name "Deshan" follows in a similar cursive style. The signature is contained within a thin black rectangular border.

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