Hydrostatic Pressure Sensing by WNK Kinases

John Humphreys, Liliana Teixeira, Radha Akella, Haixia He, Ashari Kannangara, Kamil Sekulski, John Pleinis, Joanna Liwocha, Jenny Jiou, Kelly Servage, Kim Orth, lukascz joachimiak, Josep Rizo, Melanie Cobb, Chad Brautigam, Aylin Rodan, and Elizabeth Goldsmith

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

RE: Manuscript #E23-03-0113
TITLE: "Hydrostatic pressure sensing by WNK kinases"

Dear Dr. Goldsmith: Your manuscript has been assessed by two experts in the field. Both agree, and I concur that your work is timely and quite interesting. Both had critiques that do need to be addressed before your article would be deemed appropriate for publication. I strongly urge you to address the reviewers comments and submit a revised manuscript. Focus primarily on the suggested minor edits. If time permits either discuss or attempt to address the uniqueness of the pressure response of WNK molecules. Otherwise we look forward to receiving a revised version of your manuscript.

Sincerely,
Valerie Weaver
Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Goldsmith,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

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To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL):
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Thank you for submitting your manuscript to MBoC. Please do not hesitate to contact this office if you have any questions.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office

mbc@ascb.org

Reviewer #1 (Remarks to the Author):

In their paper "Hydrostatic Pressure Sensing by WNK kinases", Humphreys and colleagues investigate how cells transduce the physical stimulus of hydrostatic pressure into a biochemical signaling process, an important type of mechanosensation which is not well understood. The authors implicate WNK kinase 3 as a direct pressure sensing molecule. In cultured cells and an extracted Drosophila tissue, they find that pressure enhances phosphorylation of a WNK substrate. They go on to perform a variety of in vitro assays with purified WNK3 kinase domain, which collectively suggest that pressure tilts the equilibrium away from an auto-inhibited WNK3 dimer towards a monomeric form which can auto-phosphorylate and become active, a mechanism previously implicated in WNK3's osmolarity sensing.

This paper is conceptually very interesting, as it suggests that pressure sensing can directly occur through an intracellular cytoplasmic kinase. One critique is that the effect size observed in vitro with purified WNK3 is quite modest compared to the cell work, suggesting other mechanisms are at play in vivo. However, to their credit, the authors do note this explicitly in the discussion. Moreover, small effects are often important, so I don't think this should preclude consideration of this study for MBoC.

My major concern would be whether the pressure effects on WNK3 are specific to this molecule. I think the study would benefit from a negative control, by examining whether a related kinase domain also features a dimerization equilibrium which is impacted by pressure. As I believe it is unreasonable to ask the authors to repeat the entire study with a new molecule, I'd highlight the direct studies of oligomerization (the gravity SEC vs. pressurized FPLC SEC studies shown in Fig. 4, as well as SEC-MALS, where a striking change is observed), as the most relevant. The authors mention in their introduction that WNK3 is more pressure sensitive than WNK1, but as far as I can tell no actual data is shown for WNK1. Since the WNKs form a fairly unique dimer according to the authors' previous work, one suggestion therefore would be to compare the pressure effects on the oligomeric status of WNK1 vs. WNK3 kinase domain. However, other comparisons could also potentially be informative to address this point, at the authors' discretion.

Additional minor concerns are outlined below:

1) Fig. 1A: Visually, the amount of FLAG signal (total KCC2) appears to increase to a similar degree as phosphorylated KCC2. Do the authors know why? It would be useful to show a loading control.
2) Fig. 1A as well: I do not understand why the pKCC2/GAPDH ratio is plotted, rather than pKCC2 / total KCC2 (FLAG signal). This seems like the relevant comparison.
3) Fig. 1B: As was the case with KCC2, the tSPAK signal visually appears to be increasing to a similar degree as the pSPAK signal. Do the authors know why? It would once again be useful to show a loading control.

Writing / typos:

-In the first section of the results, the authors refer to a "desktop control". What does this mean? "Benchtop", e.g. no increased pressure?

-Legend of figure 4, "gavity" should be "gravity".

Reviewer #2 (Remarks to the Author):

In this manuscript, Humphreys and colleagues explore the impact of hydrostatic pressure on WNK kinase, a well known kinase for its role in cell volume control which they previously showed to be sensitive to osmolarity and cytoplasmic crowding.

Here, they combines assay in cell culture, in vivo (or at least on dissected tissues of Drosophila) as well as various biochemical assays showing the WNK activity is enhanced at high hydrostratic pressure (~190 kPa) mostly due to its dissociation in monomers which are autophosphorylated. These are interesting results and to my knoweldge there has not been many molecular characterisation of the impact of hydrostatic pressure on kinase/enzyme activity.

The data and the demonstration are overall convincing. I only have minor suggestions mostly related to point to include in the discussion of the article.

1. The authors have shown that WNK kinase activity can be modulated with value of 190kPa. It would be good to discuss more thoroughly in which context this might be relevant in vivo which will help the readers to evalute in which context this might be

relevant (even if speculative). To my recall, osteoblasts, which are probably the cells experiencing higher mechanical load in our body, are experiencing pressure around 100 to 300kPa, which suggest that 190kPa pressure might only be experience by cells in very specific context. As a comparison, tumour and experiments with spheroid usually used pressure of the range of few kPa, very far from the value used in this study.

2.Hydrostatic pressure on cells and in vivo seems to have a quite strong impact on the levels of phosphorylation of WNK substrate (2 fold), while all the effect characterise in vitro in isolated proteins seems much milder. How the levels of WNK autophorylation (here a change of 20-30%) translate in the total activity of the kinase ? It would be important to know and document this as this would help to evaluate to which extend the change of activity observed in cell and in vivo can be fully explained by the change of autophosphorylation observed in vitro at high hydrostatic pressure or whether to infer other indirect effects. This was discussed briefly in the discussion but I think this would deserve a more thorough comparison as it is one key point of this article.

FROM THE DESK OF
Elizabeth J. Goldsmith, Ph.D.
Department of Biophysics
Patti L. Brown (Chilton) Professor

August 8, 2023

Dear Editor,

Here we submit our revised manuscript entitled "Hydrostatic Pressure Sensing by WNK kinases," by John M. Humphreys, Liliana R. Teixeira, Radha Akella, Haixia He, Ashari Kannangara, Kamil Sekulski, John Pleinis, Joanna Liwocha, Jenny Jiou, Kim Orth, Lukasz Joachimiak, Josep Rizo, Melanie H. Cobb, Chad A. Brautigam, Aylin R. Rodan, and Elizabeth J. Goldsmith.

The monitoring editor, Valerie Weaver, instructed us: "Focus primarily on the suggested minor edits. If time permits either discuss or attempt to address the uniqueness of the pressure response of WNK molecules." Below please find responses to specific reviewer comments. Changes to the manuscript are in italics.

REVIEWER 1

1. The first reviewer comment concerns whether WNK is unique among kinases in their pressure sensitivity. At an earlier stage in our study, we initiated characterization of MAP kinase pathway components. We found that each kinase has its own features, such as state of phosphorylation, that need to be analyzed in order to evaluate their osmotic or hydrostatic pressure sensitivity.
We did observe small effects in a MAP3K that require much more data to be publishable. We added a sentence in the discussion to open the possibility that other stress-activated kinase cascades may use related molecular mechanisms in stress responses.

"*We look forward also to identification and analysis of other intracellular pressure sensors to determine whether molecular mechanisms related to those observed in WNKs are involved.*"

We agree with the reviewer that biophysical analysis of other kinases is a good idea. We would like to pursue these studies.

2. In Figure 1, the reviewer requested that gel loading controls be used. However, we did not use loading controls. Instead, we report the ratio, in the bar graph, between the fluorescent signal from the antibodies to phosphorylated versus total protein in three (Figure 1A) or ten (Figure 1B) repeats.

3. In Figure 1A, the reviewer noted correctly that the axis was mislabeled. The correct axis is, as suggested, the ratio pKCC2/flag-KCC2, and Figure 1A has been corrected.

4. Figure 1B. See comment 2 above.

5. "desktop control" has been changed to "*benchtop control (no added pressure)*"

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6. “gavity”->”gravity”, thank you.

REVIEWER 2

1. Reviewer 2 suggested that we need to discuss further the potential relevance of the 190 kPa we used to enhance autophosphorylation in WNKs. We stated in the discussion “Here we applied 190 kPa pressure to uWNK3 in cells in culture and in vitro, and 80 kPa to Malpighian tubules. These pressures are higher than the 16 kPa (120 mmHg) typical of systolic blood pressure, but orders of magnitude below pressures used (200 MPa and more) in studies of effects on soluble proteins.” As the suggested by the reviewer, hydrostatic pressure ranges from a few pascals to megapascals in different tissues and cells. Accordingly, we add the comment *“The hydrostatic pressure experienced by tissues varies from pascals to megapascals (Liu et al., 2019). The pressure we used in our study, 190 kPa, is within this range, suggesting potential physiological relevance of direct pressure sensing by WNKs.”*

2. Reviewer 2 also asked for more discussion of autophosphorylation v. substrate phosphorylation activity. We did not directly correlate autophosphorylation and activity in each experiment. Both Figure 1 and Figure 2 show substrate phosphorylation enhancement by hydrostatic pressure Figure 1 in cells and Malpighian tubules, and Figure 2, in vitro. The correlation of activity and phosphorylation in WNKs has been significantly addressed in the original discovery and analysis of WNKs ((Xu *et al.*, 2000);(Xu *et al.*, 2002);(Zagorska *et al.*, 2007);(Thastrup *et al.*, 2012)). Simultaneously measuring autophosphorylation and downstream phosphorylation in vitro is challenging. We would like to make this experiment work, but so far have found that substrate becomes limiting as the autophosphorylation reaction progresses.

Sincerely,

E. Goldsmith

Elizabeth Goldsmith, Ph.D.

Liu, S., Tao, R., Wang, M., Tian, J., Genin, G.M., Lu, T.J., and Xu, F. (2019). Regulation of Cell Behavior by Hydrostatic Pressure. Appl Mech Rev *71*, 0408031-4080313.
Thastrup, J.O., Rafiqi, F.H., Vitari, A.C., Pozo-Guisado, E., Deak, M., Mehellou, Y., and Alessi, D.R. (2012). SPAK/OSR1 regulate NKCC1 and WNK activity: analysis of WNK isoform interactions and activation by T-loop trans-autophosphorylation. Biochem J *441*, 325-337.
Xu, B., English, J.M., Wilsbacher, J.L., Stippec, S., Goldsmith, E.J., and Cobb, M.H. (2000). WNK1, a novel mammalian serine/threonine protein kinase lacking the catalytic lysine in subdomain II. J Biol Chem *275*, 16795-16801.
Xu, B.E., Min, X.S., Stippec, S., Lee, B.H., Goldsmith, E.J., and Cobb, M.H. (2002). Regulation of WNK1 by an autoinhibitory domain and autophosphorylation. Journal of Biological Chemistry *277*, 48456-48462.
Zagorska, A., Pozo-Guisado, E., Boudeau, J., Vitari, A.C., Rafiqi, F.H., Thastrup, J., Deak, M., Campbell, D.G., Morrice, N.A., Prescott, A.R., and Alessi, D.R. (2007). Regulation of activity and localization of the WNK1 protein kinase by hyperosmotic stress. Journal of Cell Biology *176*, 89-100.

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RE: Manuscript #E23-03-0113R
TITLE: "Hydrostatic Pressure Sensing by WNK Kinases"

Dear Prof. Goldsmith:

Thanks for submitting your revised manuscript to MBoC. You'll see that the reviewers now recommend publication and we are almost ready to accept your manuscript pending your addressing one last minor point. In the intervening time since your manuscript was submitted, we implemented the inclusion of Significance Statements for MBoC papers. After reading your Significance Statement, we suggest the following revisions to meet our formatting and stylistic requirements. Please format as three separate points when inputting into the appropriate box in the submission system. Furthermore, we suggest the following revised and shortened text. Please check for accuracy and feel free to make minor adjustments that do not add significantly to the length.

Proposed Significance Statement:

WNK kinases are intracellular protein kinases associated with familial hypertension. They are directly activated by osmotic pressure and inhibited by ions, but the precise mechanisms of activation and inhibition are unknown.

We show the kinase domain of WNK3 is activated by hydrostatic pressure both in vivo and in vitro. Hydrostatic pressure induced modest ~30%-50% enhanced autophosphorylation or substrate phosphorylation. Biophysical characterization exposed a dimer to monomer equilibrium promoted by pressure.

This reveals that an intracellular protein kinase is activated by hydrostatic pressure.

Once you've submitted the manuscript with the revised Significance Statement (no need to update the files) we will accept it without delay.

Sincerely,
Valerie Weaver
Monitoring Editor

Matthew Welch
Editor-in-Chief
Molecular Biology of the Cell

Dear Prof. Goldsmith,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

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To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL):
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Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

RE: Manuscript #E23-03-0113RR
TITLE: "Hydrostatic Pressure Sensing by WNK Kinases"

Dear Prof. Goldsmith:

I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

Sincerely,
Valerie Weaver
Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Goldsmith:

Congratulations on the acceptance of your manuscript! Thank you for choosing to publish your work in Molecular Biology of the Cell (MBoC).

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