Dynamic glucose uptake, storage and release by human microvascular endothelial cells

Samaneh Yazdani, Philip Bilan, Javier Jaldin-Fincati, Janice Pang, Felicia Ceban, Ekambir Saran, John Brumell, Spencer Freeman, and Amira Klip

Corresponding author(s): Amira Klip, Hospital for Sick Children

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|------------------------|------------|
| Submission Date        | 2022-04-30 |
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Transaction Report:

(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.)
May 26, 2022

1st Editorial Decision

RE: Manuscript #E22-04-0146
TITLE: Dynamic glucose uptake, storage and release by human microvascular endothelial cells

Dear Amira,

Thank you for submitting your manuscript, "Dynamic glucose uptake, storage and release by human microvascular endothelial cells", to be considered for publication in Molecular Biology of the Cell. I asked two colleagues who are experts in the field to review the manuscript, and their comments are appended verbatim below. I agree with both reviewers that the manuscript is very well done, addresses an extremely important question in the biology of glucose metabolism, and thus is of high potential interest to readers of MBoC. However, both reviewers have identified a few concerns that need to be addressed. Both reviewers agree that the data presented using NBD-glucose introduce more confusion and distraction than clarity in the manuscript, and that they should either be removed outright or substantially reduced. Reviewer #1 would ideally like to see further testing of the model with genetic knockout or knock down studies to support the pharmacological approaches, but I agree with this reviewer that such experiments are not necessary for the paper to be accepted (but would be welcome if you have them!). Reviewer #2 has additional concerns related to the use of epithelial monolayers on plastic rather than on filters - neither the reviewer nor I are familiar enough with endothelial cell cultures to know whether this is standard practice for transepithelial studies using endothelial cells. The reviewer also raises concerns about the concentration of glucose used in the study that might require a few additional experiments to address.

While I cannot accept the manuscript in its current form, I believe that the experiments suggested by Reviewer #2 and the additional modifications to the manuscript suggested by both reviewers will be achievable within a relatively short period of time. I therefore look forward to receiving a suitably revised manuscript that addresses all of the reviewer comments. When you resubmit, please include a response to the reviewers that details how you have addressed each of the concerns, and if possible please indicate in the manuscript where changes were made (e.g. by use of a different colored font). Based on the reviewers' comments, I should be able to make a quick decision on the revised manuscript without having to return the manuscript to the reviewers.

Thank you for submitting your nice work to MBoC!

Sincerely yours,

Mickey

Michael S. Marks, Ph.D.
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Klip,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments 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.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us at mboc@ascb.org.

Revised manuscripts are assigned to the original Monitoring Editor whenever possible. However, special circumstances may preclude this. Also, revised manuscripts are often sent out for re-review, usually to the original reviewers when possible. The Monitoring Editor may solicit additional reviews if it is deemed necessary to render a completely informed decision.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures.
Reviewer #1 (Remarks to the Author):

The objective of this study is to characterize the transport of glucose across endothelia, a critical step in the delivery of glucose from the blood to the interstitial space for utilization by tissues. Despite the physiologic importance of this process, very little is known about the mechanism. The challenge is for endothelial cells to efficiently allow glucose to be transported across the cell, through the cytosol, without being metabolized by glycolysis. The authors present data that support a model in which glucose is phosphorylated to glucose-6-phosphate, transported in the ER, dephosphorylated and released from the ER for transport out of the cells -- analogous to the model for release of glucose from liver cells following the breakdown of glycogen of synthesis of glucose-6-phosphate by gluconeogenesis. Here rather than following glucose then follow 2-deoxy-glucose and they present data supporting the hypothesis that glucose for secretion moves through glycogen intermediate.

The data are strong, clearly presented and the story is nicely told. It is an excellent study and I have little to recommend. Although I'd like to see more testing of the model (because it is so important and exciting), perhaps using protein knockdowns to supplement the inhibitor studies, I appreciate this is a very important first step towards describing new biology. My one recommendation would be to greatly shorten the description of the NBD-glucose data and to mostly cut discussion of NBD-glucose results in the discussion section. As note by the authors, there have been a series of recent papers challenging the use of NBD-glucose to study glucose transport.

Reviewer #2 (Remarks to the Author):

In this study Yazdani et al study the uptake and metabolism of glucose analogs in endothelial cells. They find that the majority of 2DG once taken up into cells is phosphorylated or stored as glycogen. A smaller proportion of the tracer can be released from the cells as free 2DG. Using a series of inhibitors they show that this release mechanism is mainly mediated via the ER glucose 6 phosphatase system. Separately they show that another analog, 2-NBDG, is mainly taken up via fluid phase endocytosis and not by transporters. This is a very important area of research that addresses fundamental questions. The study is well written and the description of the literature is very well balanced.

I have a number of technical issues with the study.

1. The NBDG part of the story is rather distracting from the main story and simply casts doubt on the use of this analog as a true glucose analog. While I do not wish to detract from the importance of this observation, I do believe it would be better placed in a separate technical note that speaks to this issue. I should add that many people in the transport field including this referee have long believed this analog to be inappropriate for use as a glucose analog and so l urge the authors to pursue this course of action.

2. The use of monolayers in the form used seems strange to this referee. Surely if you wish to examine the movement of a solute across a cellular barrier one would have to study this specific process. Then this would require establishing polarised cultures on filter membranes where one could truly assess unidirectional movement across a polarised monolayer? I am by no means an endothelial cell biologist and so maybe I am missing something here but such an experimental system would on face value seem to be superior.

3. I am concerned about performing these kinds of studies using 50 uM 2DG as the only source and concentration of glucose that the cells are exposed to. My concern is that this will/may profoundly modulate the size of intracellular metabolite pools and this could in turn substantially change the fluxes. For example, it is conceivable that at physiological glucose concentrations (~5
mM) a large proportion of glucose taken up on the blood side may remain 'free' inside the cell and efflux on the other side without any intermediate steps. I think this is a serious concern and deserves further attention. I could imagine that some very simple experiments in polarised monolayers just using cold 5 mM glucose on one side and measures of its efflux on the other side could resolve these issues.
June 28, 2022

RE: Manuscript #E22-04-0146
TITLE: Dynamic glucose uptake, storage and release by human microvascular endothelial cells

Dear Dr. Marks,

Thank you for your supportive letter of May 26th, encouraging us to submit a duly revised version of our manuscript upon thorough revision according to the Reviewers’ comments.

We appreciate the collective view that our study “addresses an extremely important question in the biology of glucose metabolism, and thus is of high potential interest to readers of MBoC”.

We agree with the comments made by the reviewers and have attended to them to a large degree, as follows:

1. **NBD-glucose findings.** We understand that the NBD-glucose findings constituted a story within a story, and we have taken to heart the suggestion to trim their description and avoid interrupting the flow of the process illustrated by 3H-2-deoxyglucose. However, we feel it is important to highlight to the field the problem with the fluorescent probe, and moreover we have turned it into a useful probe to reveal an unrecognized endocytic uptake and release of glucose.

   We have therefore eliminated some panels, and several others were re-assigned to the Supplementary section. As well, we removed a section of text from the Results and incorporated it in brief in the Discussion. We kept some key panels in the Results but moved them towards the end of this section so as not to interrupt the flow of the study. We sincerely hope you understand our view that the 2-NBD-glucose results serve both as a technical/alert to the community, and more importantly that they were helpful in revealing the endocytic route of glucose traffic, which would have not been known otherwise. We have emphasized this in the Abstract and throughout the text. As well, we have added a schematic at the end of the manuscript to illustrates the contributions of the 2-DG and 2-NBD-glucose studies to our understanding of glucose transport and processing by endothelial cells.

2. **Glucose concentration in the uptake solution.** The reviewer brought up an important point and we have indeed attended to it experimentally. We have compared the nmol/min/mg protein of hexose taken up when the pulse of [3H]-2-deoxyglucose is in the presence of 50 uM 2-deoxyglucose only (our standard condition) or in the presence of 5 mM glucose. The new results clearly show that the cells take up more nmol/min/mg protein of hexose in the latter conditions, and importantly regarding the Reviewer’s question, the net amount of intracellular hexose increased, but the proportion of ‘free’ (non-phosphorylated) glucose inside the cells was only somewhat higher than in the tracer pulse condition. Moreover, the efflux nmol/min/mg protein also increased, consistent with the existence of a higher intracellular...
hexose pool. We present the new findings and discuss them in the text (pages 6-7 for uptake, 7-8 for efflux and 10 for phosphorylation proportion).

It is important to note, however that tracer conditions are typically used in the field to measure glucose uptake in order to remain within initial rates of influx, as the physiological glucose concentration (5 mM) is several times higher than the Km of any of the GLUTs and hence not within the linear range of the uptake vs. concentration curve.

3. Genetic knockout or knockdown models. We agree that it would be ideal to have knockout models to assess the participation of specific GLUTs or other molecules in the uptake and efflux of glucose from endothelial cells. While we spent a lot of time and effort towards achieving sufficient efficiency of knockdown, these being primary cells proved to be an impossible task. We tried several strategies to introduce the siRNA’s targeting GLUTs, but the efficiency of knockdown was well below 50%. This is insufficient for studies of uptake and efflux in cell monolayers. In fact, the use of fluorescent 2NBD-glucose was in part intended to give us a single-cell strategy to study uptake and efflux, but this reagent turned out not to be a reporter of the transporter-mediated uptake/efflux route. We appreciate that you see the value of our use of inhibitors as a very important first step towards describing a new biology. We hope that in the future we can implement CRISPR strategies that do not alter the primary endothelial cell phenotype. Of note, transient transfections allowed to study the localization of GLUT1 and GLUT3 in single cells, using HaloTag novel constructs that we generated for this purpose (new fig. S1) that should become a resource in the field.

4. Cells on plastic, glass or filters (Note, we use endothelial cells throughout, the word epithelial must have arisen in the review comments by mistake). We agree with Reviewer #2 that use of filters would have been desirable and spent quite a bit of time trying to optimize the system. Of 4 successful experiments with cells seeded on transwell filters, 2 had frank efflux into the upper and lower chambers, 1 showed efflux into the top chamber and some efflux into the lower chamber, and 1 showed efflux only into the upper chamber. The variability may be explained by ‘clogging’ of the filters with the tight monolayer, or other methodological reasons. This variability precluded us from using filters as our routine system of study.

We appreciate your interest and that of the Reviewers, and we hope that the changes in organization and balance of the findings, along with the new results, make the revised manuscript acceptable for publication in MBoC.

The duly revised manuscript shows the new results or considerations in red font,

Sincerely,

Amira Klip, Ph.D.
RESPONSE TO REVIEWERS

REVIEWER #1:

The data are strong, clearly presented and the story is nicely told. It is an excellent study and I have little to recommend.

We appreciate the Reviewer’s positive comments on our study, and have attended to his/her specific comments as follows:

Although I’d like to see more testing of the model (because it is so important and exciting), perhaps using protein knockdowns to supplement the inhibitor studies, I appreciate this is a very important first step towards describing new biology.

We agree that it would be ideal to have knockout models to assess the participation of specific GLUTs or other molecules in the uptake and efflux of glucose from endothelial cells. While we spent a lot of time and effort towards achieving sufficient efficiency of knockdown, these being primary cells proved to be an impossible task. We tried several strategies to introduce the siRNA’s targeting GLUTs, but the efficiency of knockdown was well below 50%. This is insufficient for studies of uptake and efflux in cell monolayers. In fact, the fluorescent 2NBD-glucose was in part intended to give us a single-cell strategy to study uptake and efflux, but this reagent turned out not to be a reporter of the transporter-mediated uptake/efflux route. We appreciate that you see the value of our use of inhibitors as a very important first step towards describing a new biology. We hope that in the future we can implement CRISPR strategies that do not alter the primary endothelial cell phenotype.

My one recommendation would be to greatly shorten the description of the NBD-glucose data and to mostly cut discussion of NBD-glucose results in the discussion section.

We understand that the NBD-glucose findings constituted something like a story within a story, and we have taken to heart your suggestion to trim their description and avoid interrupting the flow of the process illustrated through the use of [3H]-2-deoxyglucose. Attending to your point, we removed some panels, and several other panels were re-assigned to the Supplementary section. As well, we removed a section of text from the Results and incorporated it in brief in the Discussion. We did keep some key panels in the Results. This was done because we felt it was important to highlight to the field the problem with the fluorescent probe, and in doing so we have identified its route of entry, which was unknown until now. Accordingly, we turned NBD-glucose into a useful probe to reveal an unrecognized endocytic uptake and release of glucose. This is now graphically presented in the schematic of the new Fig. 7.

We sincerely appreciate your thoughtful comments and hope that the changes incurred in the revised version attend satisfactorily to your concerns.
REVIEWER #2:

This is a very important area of research that addresses fundamental questions. The study is well written and the description of the literature is very well balanced.

We appreciate the Reviewer’s positive comments on our study and have attended to his/her specific comments as follows:

The NBDG part of the story is rather distracting from the main story and simply casts doubt on the use of this analog as a true glucose analog. While I do not wish to detract from the importance of this observation, I do believe it would be better placed in a separate technical note that speaks to this issue. I should add that many people in the transport field including this referee have long believed this analog to be inappropriate for use as a glucose analog and so I urge the authors to pursue this course of action.

We understand your point and we have struggled ourselves on how to better present the salient findings. In spite of the recent studies expressing doubt that NBD-glucose is efficiently transported via GLUTs, the probe remains widely used as a bona fide glucose analog. Therefore, we felt it was important to highlight to the field the specific issues that are problematic about the fluorescent probe; importantly as well, we have identified its route of entry, which was unknown until now. Accordingly, we turned NBD-glucose into a useful probe to reveal an unrecognized endocytic uptake and release of glucose. This is now graphically presented in the schematic of the new Fig. 7. Specifically attending to your comments, we removed some panels on NBD-glucose uptake, and a few other panels were re-assigned to the Supplementary section. As well, we removed a section of text from the Results and incorporated it in brief in the Discussion. We kept some key panels in the Results but moved them towards the end of this section so as not to interrupt the flow of the study. We hope that you appreciate the value of doing so, and that the trimming and reorganization of the manuscript meet with your approval.

The use of monolayers in the form used seems strange to this referee. Surely if you wish to examine the movement of a solute across a cellular barrier one would have to study this specific process. Then this would require establishing polarised cultures on filter membranes where one could truly assess unidirectional movement across a polarised monolayer? I am by no means an endothelial cell biologist and so maybe I am missing something here but such an experimental system would on face value seem to be superior.

We agree that use of filters would have been preferable to plastic, and we had spent time and effort trying to optimize the measurements on monolayers seeded on filters. Regrettably, the system proved too variable. Of 4 successful experiments with cells seeded on transwell filters, 2 had frank efflux into the upper and lower chambers, 1 showed efflux into the top chamber and some efflux into the lower chamber, and 1 showed efflux only into the upper chamber. The variability may be
explained by ‘clogging’ of the filters with the tight monolayer, or other methodological reasons. This variability precluded us from using filters as our routine system of study.

*I am concerned about performing these kinds of studies using 50 uM 2DG as the only source and concentration of glucose that the cells are exposed to. My concern is that this will/may profoundly modulate the size of intracellular metabolite pools and this could in turn substantially change the fluxes. For example, it is conceivable that at physiological glucose concentrations (~5 mM) a large proportion of glucose taken up on the blood side may remain 'free' inside the cell and efflux on the other side without any intermediate steps. I think this is a serious concern and deserves further attention. I could imagine that some very simple experiments in polarised monolayers just using cold 5 mM glucose on one side and measures of its efflux on the other side could resolve these issues.*

You bring up an important point and accordingly we compared the nmol of hexose taken up when the pulse of [3H]-2-deoxyglucose is in the presence of 50 uM 2-deoxyglucose or 5 mM glucose. The results show that the cells take up more nmol/min/mg protein of hexose in the latter conditions, and as you anticipated, the net amount of intracellular hexose increased. Nonetheless, but the proportion of ‘free’ (non-phosphorylated) glucose inside the cells was only somewhat higher than in the tracer pulse condition, evincing the high capacity of hexokinase. Moreover, the efflux nmol/min/mg protein also increased, consistent with the existence of a higher intracellular hexose pool. *We present the new findings and discuss them in the text (pages 6-7, 7-8 and 10 for uptake, efflux and phosphorylation proportion, respectively).*

Of note, as indicated in the revised text, tracer conditions are typically used in the field to measure glucose uptake in order to remain within initial rates of influx, as the physiological glucose concentration (5 mM) is several times higher than the Km of any of the GLUTs and hence not within the linear range of the uptake vs. concentration curve.

We sincerely appreciate your thoughtful comments and hope that our new results, reorganization of the manuscript and text changes incurred in the revised version attend satisfactorily to your concerns.
Dear Amira,

Thank you for submitting your revised manuscript, "Dynamic glucose uptake, storage and release by human microvascular endothelial cells", to be considered for publication in Molecular Biology of the Cell. Because the original reviewers requested few revisions, I felt that it was not necessary to send the revised manuscript back to them for re-review, and thus read the manuscript and your response to their reviews myself. In my view, you have responded in a largely satisfactory manner to the concerns raised by the reviewers. I particularly like that you have now moved the data using 2-NBD-glucose as a glucose analogue to the end of the results; this relieves the concerns of both reviewers that the data interrupted the flow of the paper and left it somewhat "buried" for the casual reader, and I think improves the emphasis for each part of the paper individually. The new added data also improve the manuscript substantially.

There remain a few confusing statements and other minor issues in the text that need attending to, and they are described in detail below. With these corrections I would be happy to accept your manuscript for publication in MBoC. Congratulations on a job well done, and thank you for submitting your nice work to MBoC!

Sincerely yours,

Mickey

Michael Marks
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Klip,

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 editor comments 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).

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In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL): Link Not Available

Authors of Articles and Brief Communications whose manuscripts have returned for minor revision ("revise only") are encouraged to create a short video abstract to accompany their article when it is published. These video abstracts, known as Science Sketches, are up to 2 minutes long and will be published on YouTube and then embedded in the article abstract. Science Sketch Editors on the MBoC Editorial Board will provide guidance as you prepare your video. Information about how to prepare and submit a video abstract is available at www.molbiolcell.org/science-sketches. Please contact mboc@ascb.org if you are interested in creating a Science Sketch.

Thank you for submitting your manuscript to Molecular Biology of the Cell. 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

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Editor's comments:

Minor Concerns:

- Page 3, 5th line from the bottom: what is meant by "Moreover, GLUT1 has been observed to be enriched release, i.e. facing the flow of blood"? Is "enriched release" a typo, or missing something?

- Page 4, paragraph 2, line 6: Here you state that "a vesicular route of glucose release was not" documented, but then further below in the same paragraph (and in the results), you describe how 2-NBDG is rapidly released from cells through endosomal recycling. Did you mean something different here?

- In the new uptake experiment described on page 6-7, how were the kinetics determined - i.e., at what time points were measurements of [3H]-2-DG made to obtain the data? The same question applies to the new efflux experiment described on the bottom of page 7 - top of page 8, and the text is missing the value (~26) for the fold increase in glucose mass release in the presence of 5 mM glucose.

- While the data in Suppl. Fig. 1 are fine, it should be mentioned in the text that these transporters are overexpressed relative to the endogenous level, and thus might not reflect the localization of endogenous GLUT1 and GLUT3. It might also be mentioned at the onset that antibodies to the endogenous proteins were not utile for immunofluorescence microscopy imaging.

- At the bottom of page 8 (2nd line from the bottom), the "new" should be removed from the description of the new experiments - they are new to the editor but not to potential readers.

- Bottom of page 9, a reference should be provided regarding the localization of SLC37A4 (note, not SLC374A) and G6PC3 to the ER and the release of free glucose in the ER lumen.

- Middle of page 10: since the high level of G6PC3 in HAMEC was already described in the new section on page 9, the sentence starting with "Compellingly, HAMEC express..." should be replaced with something like "This is consistent with the high expression of G6PC3 in these cells".

- On page 12, please note that Pearson's coefficient does not reflect a percentage of colocalization but rather a measure of how well the signals from each component correlate with each other. Given the insets shown, I suspect the actual percentage of colocalization is much higher than 70%.

- In Supplemental Figure 2A, no labeling for 2-NBDG is shown. Is this an error of omission?

- I like the revised order of the results in which the major findings about glucose uptake and release are presented first, and the data cautioning against the use of 2-NBDG as a glucose analog for uptake and release are discussed last. However, this order was not reflected in the revised discussion. It seems to me that it would make sense to rearrange the text of the discussion to reflect the new order of the results - i.e., discuss the deficiencies of using 2-NBDG as a readout for glucose uptake at the end of the discussion.
RE: Manuscript #E22-04-0146R
TITLE: "Dynamic glucose uptake, storage and release by human microvascular endothelial cells"

Dear Dr. Marks,

Thank you for your very thorough reading of our revised manuscript. The comments you brought up are most pertinent and we truly appreciate your insight and fine attention to detail. Below is a list of how we have attended to all your points:

Response to Editor’s Minor Concerns:

- Page 3, 5th line from the bottom: what is meant by "Moreover, GLUT1 has been observed to be enriched release, i.e. facing the flow of blood"? Is "enriched release" a typo, or missing something?

  We apologize for the error, the sentence has been corrected as follows: "Moreover, GLUT1 has been observed to be enriched luminally, i.e. facing the flow of blood"

- Page 4, paragraph 2, line 6: Here you state that "a vesicular route of glucose release was not" documented, but then further below in the same paragraph (and in the results), you describe how 2-NBDG is rapidly released from cells through endosomal recycling. Did you mean something different here?

  We regret and apologize for our confusing writing. We have now clarified at the end of the preceding paragraph that: ‘Metabolic rerouting or vesicular delivery emerge as possibilities but have not been previously investigated’.
  We have also removed the sentence in paragraph 2 that "a vesicular route of glucose release was not" where we describe our findings which include that 2-NBDG is released through endosomal recycling. Thank you for noticing the prior inconsistency.

- In the new uptake experiment described on page 6-7, how were the kinetics determined - i.e., at what time points were measurements of [3H]-2-DG made to obtain the data? The same question applies to the new efflux experiment described on the bottom of page 7 - top of page 8, and the text is missing the value (~26) for the fold increase in glucose mass release in the presence of 5 mM glucose.

  The duration of the uptake pulse is now indicated at the top of page 7 (30 min). We also clarify that the uptake shown in Fig. 1C was measured at 30 min (page 6).
  For the pulse preceding efflux, the pulse duration (30 min) is now indicated at the bottom of page 7.
  At the top of page 8 we now provide the value of the fold increase (indeed, 26.6, apologies for the omission 😅).

- While the data in Suppl. Fig. 1 are fine, it should be mentioned in the text that these transporters are
overexpressed relative to the endogenous level, and thus might not reflect the localization of endogenous GLUT1 and GLUT3. It might also be mentioned at the onset that antibodies to the endogenous proteins were not utile for immunofluorescence microscopy imaging.

Good points. On page 8 we have now introduced a sentence regarding the unsuitability of the anti-GLUT3 antibodies for immunofluorescence:
‘Because antibodies to GLUT3 are not reliable for immunofluorescence, we instead generated tagged versions of GLUT1 and GLUT3’
and we acknowledge the overexpression incurred in transfecting Halotag GLUTs:
‘While acknowledging the overexpressed levels, these results suggest that both transporters could be positioned’

- At the bottom of page 8 (2nd line from the bottom), the "new" should be removed from the description of the new experiments - they are new to the editor but not to potential readers.

The word has been removed

- Bottom of page 9, a reference should be provided regarding the localization of SLC37A4 (note, not SLC374A) and G6PC3 to the ER and the release of free glucose in the ER lumen.

Two references are now provided describing the localization of the complex to the ER membrane, one using fractionation and the other referring to a number of sources evaluating this localization. This is now at the top of page 10, with a clearer sentence regarding the function of the translocase and phosphatase, as follows:
‘This is inferred from the known localization of SLC37A4 and G6PC3 to the ER membrane (Vorhaben and Campbell, 1979; van Schaftingen and Gerin, 2002) where, at least in hepatocytes, they translocate glucose-6-phosphate and hydrolyze the phosphate bond to release the free glucose to the ER lumen.’

- Middle of page 10: since the high level of G6PC3 in HAMEC was already described in the new section on page 9, the sentence starting with "Compellingly, HAMEC express..." should be replaced with something like "This is consistent with the high expression of G6PC3 in these cells".

The sentences have been removed or corrected as suggested (page 10), to read: ‘This is consistent with these cells exhibiting high expression of G6PC3’

- On page 12, please note that Pearson's coefficient does not reflect a percentage of colocalization but rather a measure of how well the signals from each component correlate with each other. Given the insets shown, I suspect the actual percentage of colocalization is much higher than 70%.
We agree, the revised sentence now reads simply: ‘resulting in a high coefficient of colocalization determined using Pearson’s analysis (Fig 5A).’

- In Supplemental Figure 2A, no labeling for 2-NBDG is shown. Is this an error of omission?
We chose to show two different strategies to determine that the SRB or 2-NBDG puncta (illustrated in Fig. 5 to strongly colocalize) are of endosomal nature. SRB is shown as intracellular with regards to WGA, and 2-NBDG is shown surrounded by FM4-64 when the latter is allowed to internalize intercalated in the plasma membrane and endosomal membranes.

- I like the revised order of the results in which the major findings about glucose uptake and release are presented first, and the data cautioning against the use of 2-NBDG as a glucose analog for uptake and release are discussed last. However, this order was not reflected in the revised discussion. It seems to me that it would make sense to rearrange the text of the discussion to reflect the new order of the results - i.e., discuss the deficiencies of using 2-NBDG as a readout for glucose uptake at the end of the discussion.

Thank you. We have now moved the section entitled “Surprising differences between 2-DG and 2-NBDG uptake” to the end of the Discussion (page 21). The title of the section and amended first sentence are shown in red as well reading:
‘Finally, our study points out important differences in the behaviour of 2-DG and 2-NBDG’.

Once again, our sincere thanks for your insight and attention to detail. We trust that the revisions have made the manuscript acceptable for publication in MBoC.

Sincerely,

Amira Klip, PhD, FRSC
Senior Scientist
Cell Biology Program, Research Institute
The Hospital for Sick Children, Toronto
Professor of Paediatrics, Biochemistry and Physiology
The University of Toronto
3rd Editorial Decision

July 14, 2022

RE: Manuscript #E22-04-0146RR
TITLE: "Dynamic glucose uptake, storage and release by human microvascular endothelial cells"

Dear Amira,

Thank you very much for quickly revising your manuscript, "Dynamic glucose uptake, storage and release by human microvascular endothelial cells". I am pleased to officially accept your manuscript for publication in Molecular Biology of the Cell. Congratulations on a nice piece of work!

Sincerely,

Mickey

Michael Marks
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Klip:

Congratulations on the acceptance of your manuscript.

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