PEER REVIEW HISTORY

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ARTICLE DETAILS

TITLE (PROVISIONAL)	Identification of vitamin C transporters in the human airways – a cross-sectional in vivo study
AUTHORS	Larsson, Nirina; Rankin, Greg; Bicer, Elif; Roos-Engstrand, Ester; Pourazar, Jamshid; Blomberg, Anders; Mudway, Ian; Behndig, Annelie

VERSION 1 - REVIEW

REVIEWER	carroll cross MD Univ California, Davis, USA
REVIEW RETURNED	25-Nov-2014

GENERAL COMMENTS	The authors have interrogated an important issue, e.g. factors contributing to ascorbate metabolism in respiratory tract airways. There are few data addressing the airway ascorbate transporters and further investigations of the contributions of ascorbate to airway biology are needed. However, there are a number of significant issues the authors need to address in revision, including a strengthening in the Discussion of limitations and some strengthening of their methodologies relating to the reported immunochemistry and ascorbate concentrations. It should be realized that other investigators have found that the use of some of the SVCT antibodies to be quite challenging (if not of questionable quality). COMMENTS 1. Authors have used SVCT 1-2 antibodies from DAKO (Glostrup, Denmark) (which seem to be no longer available) for their flow cytometry analysis and have used SVCT 1 and 2 antibodies from Santa Cruz Biotech for their immunohistochemistry. Have the DAKO SVCT 1-2 antibodies been used by anyone in the literature? If they are not available, it would be hard for others to repeat the work. At least one other investigator has found these antibodies to be less than robust. The issue is compounded by the issue that SVCT 1 does not appear to have been found in leukocytes and blood vessels have been shown to have only SVCT 2 previously. At a minimum, this would require further mention in Discussion. 2. No images of the SVCT stains are shown, limiting readers to judge quality of the immunohistochemistry data. 3. The findings could be considerably strengthened by confirming the protein measurements with mRNA expression data. This would certainly be a limitation. 4. The dehydroascorbate levels in bronchial and alveolar RTFL are

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	suspicious and need to be compared with other values in the literature. Was acid added before the samples were frozen? Did the rather extended processing (freeze-thaw-vortex-extraction) influence the findings? Were recoveries of analytes done? The dehydroascorbate levels seem too high considering the instability of dehydroascorbate. 5. In Abstract it should be made clear that airway vitamin C transporters were interrogated, not “lung”. Abstract should mention that RTLFs were also interrogated.

VERSION 1 – AUTHOR RESPONSE

COMMENTS

1. Authors have used SVCT 1-2 antibodies from DAKO (Glostrup, Denmark) (which seem to be no longer available) for their flow cytometry analysis and have used SVCT 1 and 2 antibodies from Santa Cruz Biotech for their immunohistochemistry. Have the DAKO SVCT 1-2 antibodies been used by anyone in the literature? If they are not available, it would be hard for others to repeat the work. At least one other investigator has found these antibodies to be less than robust. The issue is compounded by the issue that SVCT 1 does not appear to have been found in leukocytes and blood vessels have been shown to have only SVCT 2 previously. At a minimum, this would require further mention in Discussion.

RESPONSE

We used SVCT1-2 and GLUT 4 antibodies that are commercially available from Santa Cruz and GLUT 1-3 antibodies available from R&D systems. This was not correctly stated in the original submission. The method section has now been corrected and we thank the reviewer for making us aware of the incorrectness in the manuscript.

Some important points to also consider is that there is a lack of in vivo human studies on these transporters especially in regards to the lung. Most studies looking at tissue localization have been conducted in animals that can synthesize vitamin C themselves. Much of the data accumulated on expression in particular cells has been conducted using in vitro experiments, however expression of the SVCTs have been shown to change depending on culture conditions and therefore is a factor to consider. There is one paper addressing the distribution of these transporters in rat liver, where they found co-localisation of SVCT1 and 2 with endothelial cells. Of note, in this paper they used the same antibodies as we did. This reference has now been added to the manuscript. A short statement on how the antibodies were selected has also been added to the discussion.

2. No images of the SVCT stains are shown, limiting readers to judge quality of the immunohistochemistry data.

RESPONSE: The images of SVCT were included in the original submission, but came last in the fused pdf-file. The JPG-files are now placed immediately after the main document. We hope this will be better and apologize for this inconvenience.

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3. The findings could be considerably strengthened by confirming the protein measurements with mRNA expression data. This would certainly be a limitation.

RESPONSE: We agree that having mRNA expression data would further have strengthened the findings, but these measurements were not performed. However, as we have demonstrated protein expression of vitamin C transporters, this enabled us to determine localisation within the airway mucosa, as well as detection of the protein, which follows mRNA expressions.

4. The dehydroascorbate levels in bronchial and alveolar RTFL are suspicious and need to be compared with other values in the literature. Was acid added before the samples were frozen? Did the rather extended processing (freeze-thaw-vortex-extraction) influence the findings? Were recoveries of analytes done? The dehydroascorbate levels seem too high considering the instability of dehydroascorbate.

RESPONSE: The concentrations of DHA in the samples were high (>5% of Vitamin C and extremely high in the bronchial lavages), compared with comparative values previously quoted in blood plasma and cell/tissue extracts. We highlighted this observation in the paper and made some attempt to discuss this – page 17. We didn't extend this discussion as we felt it would likely distract away from the major focus of the paper; namely the expression of vitamin C and DHA transporters in the bronchial epithelium of the upper airways.
Specifically, in relation to the reviewer's queries, we did not perform the analysis on lavage samples that were pre-acidified with metaphosphoric acid prior to storage, as we have previously shown that the stability of ascorbate and urate (especially the later) is actually better in the presence of BHT and DES. This was added before the samples were frozen, as outlined in methods section (page 8, line 25 and page 9, lines 1-3) and in greater detail in the supplementary material (page 3, lines 23-25 and page 4, lines 1-9). The samples were subsequently acidified with MPA for deproteination after thawing, as part of the extraction protocol (Online supplement page 4, lines 1-9). This extraction protocol is not protracted and batches of samples (30-60) can be processed within 15-20 minutes at 4oC. The incubation with TECP is longer, 1 hour, but this by definition will not promote ascorbate oxidation. We have previously shown that recoveries of ascorbate, or DHA spiked into lavage fluids, or a simple saline base give recoveries of >95%, at concentration down to the limit of detection.
It is notable perhaps that the proportion of DHA in the BAL fluid fractions was 13.7 and 18.1% in the healthy controls and mild asthmatics respectively, whilst the corresponding values in the bronchial washes were 77.8 and 78.4%. Considering the corresponding proportion of GSSG to GSx in the respective compartments, for the BAL fraction the values were broadly equivalent to DHA, around 13.3 (healthy) and 10.9 (asthmatics)%, but in the bronchial fraction the proportion of GSSG was markedly lower, and similar to the concentrations in the alveolar sample: 10.3 (healthy) and 9.3 (asthmatics)%. Thus, it is only really the bronchial DHA concentrations that stand out. The question the reviewer raises is pertinent; is this real.
Firstly, all the samples were collected concurrently, treated and stored under identical conditions, so it is difficult to simply attribute this difference to storage / processing artefacts, unless one assumes that ascorbate is inherently less stable in bronchial airway lavages. There is some experimental evidence supporting this view, but then conditions likely to favour ascorbate oxidation will also promote the further oxidation and hydrolysis of DHA.
As the lavage samples were treated with DES and BHT immediately after centrifugation to remove cells, there are two possibilities, either that sample oxidation occurred during this early lavage processing, prior to the addition of stabilizers, or that the concentrations are actually real, and that poor sample handling has previously resulted in a loss of DHA from lavage samples. This isn't to be overly critical, as there are few investigations that have attempted to quantify DHA in airway lavage fluids. We routinely do, and find measurable concentrations, but only ever in lavage fluids from non vitamin C synthesising species. In other tissues, where there are known recycling mechanisms our results mirror those of the literature. In fact, in cells/tissues and plasma, in the absence of overt

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oxidative stress we never see measurable concentrations of DHA.
We have performed a fair degree of work to confirm this observation and have previously put together our preliminary observations in a manuscript, which we would be willing to share with the reviewer. Whilst this was reviewed, we felt in light of the reviewer's comments at that time we needed to go away and do more work. Specifically this preliminary work focused only on nasal lavage and we believed it was necessary to obtain equivalent data in from other regions of the lung using a segmental lavage technique. It was also necessary to perform long term storage stability experiments. This is now complete and we see the same pattern of responses with greater DHA concentrations in airway lavages taken from ever more proximal airway segments. Ascorbate is less stable in proximal airway lavage returns and its oxidation can be inhibited through the use of metal chelators, but not azide. We are therefore very confident of the observations and methodologies in this paper, but given that this still requires publication we agree that in the current paper needs to interpret the results with a little more caution. We have therefore made a statement in text that whilst ascorbate oxidation will undoubtedly occur at the surface of the lung, we cannot wholly exclude the possibility that the high concentrations observed in the bronchial wash may be augmented through subsequent oxidation during sample processing and storage.

5. In Abstract it should be made clear that airway vitamin C transporters were interrogated, not "lung". Abstract should mention that RTLFs were also interrogated.

RESPONSE: This has now been corrected.

VERSION 2 – REVIEW

REVIEWER	carroll cross Univ Calif, Davis USA have published papers on vit C
REVIEW RETURNED	27-Feb-2015

GENERAL COMMENTS	authors have paid due diligence to most of the concerns of the reviewer

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