Author’s response to reviews

Title: Cobalt Chloride Compromises Transepithelial Barrier Properties of CaCo-2 BBBe Human Gastrointestinal Epithelial Cell Layers

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Replies to Reviewers/Revisions to the Manuscript

Revising this manuscript was oftentimes a difficult exercise to perform because of the combined volume of comments by the four different reviewers, and the fact that sometimes comments/suggestions/requirements of one reviewer were actually in conflict with those of another reviewer. Please trust that we did our very best to accede to the comments of the reviewers, or in those instances where we disagreed, carefully and clearly explained our reasoning.

Reviewer 1

1. The issue of the (original) inset to Figure 1 was also raised by Reviewer 3. Our resolution was to leave the inset remain in Figure 1 BUT to clarify things significantly. We are now labeling the inset as Figure 1A, and the physiology data (Rt, Jm) as Figure 1B. We like the inset here because it is crucial to show early on that cobalt does in fact elevate HIF1a in CACO-2, preferably before we show any physiological effects of the cobalt (Figure 1B).
2. The legend to Figure 2 has been rewritten and now states clearly that this is immunoblot density data. Representative immunoblots are now included as supplementary data.

3. Although it is somewhat almost a philosophical point, we do not ascribe to the popular notion that increases or decreases in Claudin-X or Claudin-Y can tell one definitive information about the tightening or induced leakiness of junctional seals. We subscribe to the simpler point that this tight junctional protein data - with occludin decreasing and claudin-5 increasing – is proof positive that cobalt is inducing changes in the epithelial barrier at the level of the tight junctional complexes. We believe that the situation in the tight junctional complexes is far more complex than many researchers give credit to. One should not e.g. say that an increase in claudin-2 will translate to leakiness or a increase in claudins -1 or -3 will induce tightening. It is rather the hetero-and homotypic interactions of these proteins that matter regarding permeability, and this can’t be deduced from changes in abundance. We now make this point in the revised Discussion.

4. We could not find any detailed studies of cobalt transport in polarized intestinal epithelia or CACO-2 cells specifically. We therefore can’t state definitively that cobalt transport across the CACO-2 basal-lateral membrane is greater than for the apical membrane, but this would be a reasonable (and likely simplest) explanation for our data showing a predominantly basal-lateral – based effect of cobalt in this system. This likely explanation is now stated in the revised Discussion.

5. We have now made Figure 4 part of Figure 1. As the new Figure 1C we now provide the time course of the effect of cobalt on Rt and Jm as part of the same figure in which the primary finding is first presented.

6. We prefer to show the primary Hif1a blot in Figure 1, where the effect of cobalt on Rt and Jm is first presented, based on the rationale we gave above in item 1.

7. An RNAi directed against HIF1a would certainly be an excellent way to ascribe the cobalt effects to HIF1a. It is something that we intend pursuing not simply regarding cobalt action, but also in terms of the effects of steady-state HIF1a and HIF1 levels on barrier function. However due to the requirement for HIF1a complexing with HIF1b to produce the functional entity – HIF1 – as regards transcriptional regulation, we do feel
that the HIF1b-knockdown data of Figure 9 (new Figure 7) makes a reasoned and reasonable first step to ascribing the reported effects of cobalt here to the HIF1 complex.

8. We respectfully disagree with the reviewer about the position of our Figure 7. This figure establishes a characteristic of the cobalt (HIF)-induced leak – namely that it possesses sieving characteristics, allowing passage of smaller molecules (mannitol) much more readily than larger molecules (PEG). This is further evidence that we are talking about modified tight junctions here. However the more fundamental point to make in this manuscript is that there IS a leak being induced by HIF1 upregulation. This is why the other figures (concentration dependence, time course, etc) precede this.

9. The reviewer raises a very interesting point, namely is the dramatic magnitude of the Rt decrease in 3-day cell layers (compared to the small decrease in 7-day cell layers) a response to lower endogenous (starting) levels of HIF1a in 7-day cell layers, and/or a greater relative effect of CoCl2 on elevating HIF1a levels. We are choosing not to pursue this experiment in this manuscript because it may yield a very open-ended outcome, where one result would generate yet another experiment, etc, because of HIF1 levels possibly being only part of the story here. It is just as likely that we are dealing with a different tight junctional complex at 3-day vs 7-day, due to progressively increased differentiation. But we are certainly now mentioning the need to pursue these exact studies in our next manuscript, in the revised Discussion re this figure.

10. In a perfect world we would certainly have used a HIF1a knockdown here. However it was not available to us in the cell line model (Caco-2) that we were using. Dr. Sean Colgan did have a Caco-2 HIF1b knockdown which he made available to us. We reasoned that in a knockdown of HIF1b that was dramatic enough to make HIF1b limiting for the formation of HIF1 complex, we may have a situation where elevation of HIF1a levels (through CoCl2) would not have as great an effect on barrier function. This in fact proved to be the case, as the HIF1b knockdown showed a much attenuated response to CoCl2. It helped to make our claim that the CoCl2 effects were being mediated here by HIF1. The figure undeniably makes this point. Along with the CPX and DMOG effects, it clearly shows that HIF1 is the likeliest explanation of events here. At the reviewer’s suggestion, we are revising this figure along the same lines as we revised Figure 1. We now describe in the revised Discussion how a near complete knockdown of HIF1b may result in sharply reduced - but still substantial - effects by cobalt on barrier function. This could occur through a secondary effect of cobalt that is fully independent of HIF1a degradation, such as cobalt action on inhibition of the
microsomal cytochrome P450 system and protein turnover in the cell (Chetty et al., 1979).

Reviewer 2

1. At the suggestion of this reviewer and the others, the data presentation has been extensively reworked and made clearer, as described above.

2. The reviewer says that our in vivo justification is limited because of our use of partially differentiated (7 day) cell monolayers. First, the reviewer should consider that a 7 day CACO-2 cell layer (we seed at near confluent cell density by the way) has attained almost 100% of the barrier function a fully differentiated (21-day) CACO-2 cell layer (see Table 2 of Valenzano et al [2015]). But second, in making this statement, the reviewer overlooks that the intestine in vivo has a crypt-villus axis wherein a variety of differentiation states exist. Even if our current model at 7 days is not fully differentiated, it most certainly does have in vivo relevance.

3. We made a serious error in our Methods section, by copy/pasting our Western immunoblot methodology from an earlier publication where we did in fact perform claudin analyses on cell layers grown in flasks. In this current study on cobalt and HIF1 we exclusively harvested cell layers from the very filters (Millicell PCFs) used in the physiology studies. The reviewer was very correct to point out the fact that to do otherwise would be to have our conclusions regarding HIF1a, HIF1b and tight junction proteins on very different biological material from that used in the physiology studies. We deeply regret this error, and have corrected our Methods section accordingly.

4. The reviewer points out that the use of other stressors could reveal valuable information here, as would also analyses of other gene targets of HIF1. We completely agree and describe this in the revised Discussion. These are also areas where our future research will be directed. It is also true that we do not show levels of actual HIF1 complex (in the nucleus). We now describe this fact as well. We do however capably show that three different prolyl hydroxylase inhibitors are having similar effects on barrier function, and HIF1a levels – the rate limiting component of the HIF1 complex – are being increased.
Based on years of published data from many labs, it is reasonable to conclude that HIF1 complex levels are being affected here.

Reviewer 3

1. As described above, our original Methods description regarding cell lysates and Western immunoblots, incorrectly stated our use of cell layers harvested from culture flasks. We have now corrected this careless mistake. We used in this study ONLY cell layers harvested off the very same permeable filters (Millicell PCFs) that were used in our physiology studies. We completely agree that to do otherwise would be to observe HIF1a and tight junction protein levels in very different biological material from that used in the actual barrier function studies.

2. We agree that our reported INcrease in claudin-5 coinciding with a Decrease in resistance is seemingly heretical. However we feel strongly that epithelial tight junction researchers have vastly oversimplified the molecular situation by claiming e.g. that increased claudin-2 or decreased claudin-5 would result in leakiness. It totally ignores the reality of heterotypic interactions among claudins in producing barrier properties. Accordingly it is the RATIO of claudins to each other that might be far more important. We now add this concept to the revised Discussion and talk about the seeming oxymoron of decreased resistance and increased claudin-5. In addition we emphasize that in this study we have used exclusively whole cell lysates, not membrane fractions. This means that we are looking at cytosolic and as membrane-associated (junctional) claudin-5. This also must be considered in interpreting our findings.

3. We absolutely agree that immunofluorescence localization of claudins would be a very nice addition to our Western data. We will definitely include this in our future work. We are not doing it here simply because of the realities of biomedical medical with limited budgets and limited staffing.

4. We seed our Millicell (akin to Transwells) filter units with a cell number that achieves 50% confluence. A “7 day” culture is actually a cell layer that is 7 days from seeding (and approximately 6 days post confluent). We clarify this point in our revised Methods section. Note that a 7-day cell layer (using our methodology) manifests almost maximal
transepithelial electrical resistance (Valenzano et al, 2015), even though other differentiated features (SGLT transporters; sucrose/isomaltase) require a full 21 days for maximal expression. We now highlight and clarify this point in the revised manuscript.

5. Our measurement of resistance involves direct current pulses of 5-10 uamps/cm². We now describe our measurements in better detail in the revised Methods section.

6. We did not provide the actual data regarding cell viability because it was a clear case of a negative result/no effect. There was simply no statistically significant effect by the CoCl² (125 or 250 uM) regarding cell viability. We make this point even clearer in the revised Results. However, we failed to provide sufficient detail concerning the viability assay (LDH release) that we used, and we now do this in the revised Methods section.

7. We absolutely agree with the reviewer’s suggestion regarding improved labeling and subsectioning of Figure 1 (and Figure 9). This has now been revised accordingly.

8. We are now including sample original Western blots (we run several Western blots on fully independent/different cell lysates for each experiment reported to have confidence in experiment outcomes) in Supplemental data files. We normalize our protein bands of interest (HIF1α, claudin-5, etc.) based on a total protein stain (Memcode) of the immunoblot after chemiluminescent immunodetection of the specific protein of interest. This point is now made clear in the revised Methods section.

9. We simply reported our claudin data (Figure 2) as actual band densities for the control and cobalt-treated conditions. This approach shows an identical outcome to a derivatized approach where we would report the controls as 100%. It would not change the picture at all.

10. Our purpose regarding tight junctional protein data is only to show that cobalt treatment had a clear effect upon tight junctional proteins and thus was having molecular effects on the tight junction complex. Doing so ascribes the reported resistance and mannitol permeability effects to changes in the tight junction complex. The reported statistically significant decrease in occludin and increase in claudin-5 satisfactorily make this point. Our intent was not to provide an exhaustive description of tight junctional protein
changes as a result of cobalt treatment. But in our future work we will provide a more expansive description of tight junction protein changes. To avoid any confusion to the reader, we are removing mention (in Methods) of the non-reported junctional proteins.

11. At the suggestion of Reviewer #1, we have moved Figure 4 to become a part of Figure 1. Given this changed status, we would prefer to show the data in the simpler format that it now is in, rather than separate graphs for control and cobalt-treated cell layers.

12. Given that we are now showing the time course of the cobalt effect in Figure 1, we have no objection to including the concentration effect in a revised Figure 1 as well.

13. There was no specific reason why the comparison of mannitol, lactulose and PEG fluxes were performed after 72 hrs of cobalt exposure, rather than our standard 48 hrs. However it is fortuitous, because the presence of only a barely significant increase in PEG flux after 72 hrs (see the time course showing greater leak at 72 hrs than 48 hrs) is further evidence that the induced leak is likely a junctional phenomenon. The fact that mannitol (mw 182) leak is 300% of control whereas PEG manifests a barely significant 20% increase, indicates that pronounced molecular sieving is still being exerted by this induced leak. This feature is now highlighted in the revised Discussion.

14. As described in our answer to comment #4, 3 day, 7 day and 21 day refer to the number of days after cells were seeded onto filters. This is approximately 2, 6 and 20 days post confluence, and this is now highlighted in the revised text. Unfortunately, we have not examined the effect of Cobalt on cell proliferation, and this fact and consideration is now added to the discussion of this experiment.

15. As now explained in the revised Results section, the reduced Co++ effect in the HIF-1b cell layers was observable for 3-day cultures but not 7-day cultures.

16. We have changed “48 hr” to “48 hrs” in the Results.

17. We have changed “11)” to “1)” in the Discussion.
18. We now use the abbreviation SEM, not SE, for standard error of the mean, throughout.

Reviewer 4

1. We have attempted to remove any and all vernacular jargon from the manuscript, following this reviewer’s suggestion.

2. Our original submission lacked a listing of abbreviations used, and this oversight has been corrected.

3. “mw” has been changed to Da throughout.

4. Order of presentation has been revised considerably.

5. CACO-2 has been changed to CaCo-2 BBe throughout at the reviewer’s suggestion.

6. The electrophysiological measurements were performed in culture medium. The measurements were made in a custom designed polycarbonate chamber fitted with agar-filled (1M NaCl) Teflon tubing for current passing and voltage-measuring electrodes. This improved description has been added. The phrase “additional nutraceuticals…” was added in error and has been removed.

7. We chose the tight junctional proteins occludin, claudin-5 and claudin-7 because we had excellent (commercial) antisera for all three. Our overall purpose regarding tight junctional protein data was only to show that cobalt treatment had a clear effect upon tight junctional proteins and thus was having molecular effects on the tight junction complex. Doing so ascribes the reported resistance and mannitol permeability effects to changes in the tight junction complex. The reported statistically significant decrease in occludin and increase in claudin-5 satisfactorily make this point. Our intent was not to provide an exhaustive description of tight junctional protein changes as a result of cobalt treatment. But in our future work we will provide a more expansive description of tight
junction protein changes. In the revised Discussion we emphasize this rationale to the reader. To avoid any confusion to the reader, we are removing mention (in Methods) of the non-reported junctional proteins.

8. Our statement of a 2 minute incubation was totally in error. It was a 2 HOUR incubation. We are grateful to the reviewer for catching this mistake, now corrected.

9. As described above in replies to comments from Reviewers 1 and 3, our original Methods description regarding cell lysates and Western immunoblots incorrectly stated our use of cell layers harvested from culture flasks. We have now corrected this careless mistake. We used in this study ONLY cell layers harvested off the very same permeable filters (Millicell PCFs) that were used in our physiology studies. We completely agree that to do otherwise would be to observe HIF1a and tight junction protein levels in very different biological material from that used in the actual barrier function studies, not to mention restricting cobalt access to the basal-lateral cell surface.

10. Images of blots are being included now in Supplemental Data. Our loading controls are based on total protein stains (Memcode) of the entire immunoblot. Densitometric results are corrected (normalized) based on this. These steps are now stipulated in the revised Methods.

11. The reason that we don’t show the viability assay data is that there was really nothing to see. Truly, there was no statistically significant difference with the cobalt treatments. In our experience journals have always kicked out such figures as a “negative result.” We are providing more information on the cell viability assay in the revised Methods section.

12. The reviewer is correct. We are switching terminology to 7-days-post-seeding. As we described to Reviewer 3, we seed our filter units at 50% of confluent density. So 7-days-post-seeding is actually 6-days-post-confluent. We explain this carefully now in the revised Methods.

13. Concerning the observation that “CaCo-2 are variable in their differentiation” and we used different states of differentiation in our study, I assure the reviewer that we knew this, it was intentional, and we USED this variability. Our goal in this work was simply
to show that HIF1a elevation leads to leakiness. Once we realized that the magnitude of this effect was influenced by CaCo-2 degree of differentiation, we simply capitalized on this to help to establish our central point.

14. There were other similar criticisms of our Figures, and the reviewer will see that this has been addressed in those earlier comments and in the revised manuscript.

15. We are changing the ordinate of Figure 1 to the simpler “Percent of Control Resistance,” at the reviewer’s suggestion. The fact that it is normalized is explained in the legend, and need not be on the axis label. We are strongly inclined to show absolute value data wherever possible. However we repeat all experiments at least once to verify findings. We then will combine data from separate experiments – which in time can be several weeks apart. It is very possible for example in the case of Resistance values, that there can be significant variations as much as 30% in absolute values from one experiment to the next (due to unintended variability in seeding density, technician handling, etc.), even though the effect of a drug (e.g. cobalt) can be very similar – on a relative basis. Such circumstances call for expression of data as a normalized percent of control. This is why there is sometimes variability in presentation – but wherever possible, we prefer absolute values.

16. There is nothing inherently wrong with presenting the data in this fashion. It accurately makes its point, as in this case that cobalt significantly changed occludin and claudin-5 levels in these cells. As explained above, we normalized between experiments but still retaining a presentation as close to absolute values as possible. Innate variability of band densities across different experiments (arising from different lysates, different gels and blots, and different chemiluminescent detections (exposures) obligatorily requires some normalization across experiments.

17. We use a 0.1 mM concentration for each of our radiolabeled probes, by adding unlabeled probe to the radiolabeled probe. We do this to avoid any non-specific binding. It also allows for easier cross comparisons among different probe molecules. For this reason of using identical concentrations of all three probes, we are actually ok to use our unit of pmols/min/cm². But we now more clearly state in the revised Methods that identical 0.1 mM concentrations are used for all three probes (mannitol, lactulose, polyethylene glycol).
18. This is a time course of the effect of cobalt on barrier function (Rt, Jm). It encompasses a period of 70 minutes, not hours or days. As such one would not even expect a dramatic change in control barrier function (and there wasn’t!). Control Rt did decline somewhat (and control Jm increased somewhat) but it was not nearly to the degree that cobalt-treated cell layers changed. This figure has only one purpose – to show that cobalt-treated cell layers became leaky over a relatively short timespan (70 minutes). Normalizing the Rt and Jm changes to time-matched controls effectively shows this.

19. The same (0.1mM) concentrations were used for all flux studies (see #17 above).

20. We have changed to a lower case “d” as the reviewer requested. Yes, the 3-day-post-seeding cell layers do form a confluent monolayer. Also, we now report absolute values of 3-day and 21-day cell layer resistances in the revised Results section. In the revised Discussion we point out that the mechanisms underlying this greater effect by cobalt on less differentiated cell layers could vary from a mundane explanation due to greater cobalt influx in 3-day cell layers to a more intriguing differentiation-dependent action of HIF1 on transcription of junctional proteins and/or modifiers. At this stage, one can merely speculate across a very wide range of possibilities.

21. We agree completely, and have now properly labeled the inset as Figure 9A (old numbering).