Jing Yu
Scientific Editor
World Journal of Gastroenterology

January 19th, 2017

Dear Dr. Yu,

Re: Manuscript reference No. 30328

Please find attached a revised version of our manuscript including a revised title “Intestinal permeability: the role of proteases”, which we would like to resubmit for publication as a Review in World Journal of Gastroenterology.

The comments of the reviewers were highly insightful and enabled us to improve the quality of our manuscript. In the following pages our responses to each of the comments of the reviewers can be found.

Revisions in the text are shown using yellow highlights for additions, and strikethrough fonts for deletions. In accordance with the suggestion of the reviewers, we inserted a paragraph on mucosal immunology in relation to intestinal barrier function.

We hope we addressed the comments and suggestions satisfactorily and that the revisions in the manuscript and our accompanying responses are sufficient to make our manuscript suitable for publication in World Journal of Gastroenterology.

We look forward to hearing from you.

Yours sincerely,

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Responses to the comments of Reviewer #1 – No. 3537822

1. The title of gastrointestinal permeability implies that permeability of the whole gut, ie gastric, small intestinal and colonic permeability. However, the review mainly concentrate/discuss colonic permeability, very little on small intestinal permeability and nothing on gastric permeability. Clarify? Furthermore, it is hard to interpret what type of permeability the authors are referring to in the text. Suggest making this clear in the text.

Response: The referee is correct, we adjusted the title accordingly. It is however often impossible to distinguish small intestinal and colonic permeability since many tests (for instance FITC-dextran) are not specific for either of them. If it was clear from the literature we used either small intestinal or colonic permeability, if it was unclear we used 'intestinal permeability'.Therefore we adapted the title to intestinal permeability.

2. No Abstract?

Response: Indeed, the abstract was not included in the main text. It was submitted in a separate box and added to the manuscript as well.

Abstract

The gastrointestinal barrier is -with approximately 400m^2- the human body's largest surface separating the external environment from the internal milieu. The barrier function serves a dual function: permitting the absorption of nutrients, water and electrolytes on the one hand, while limiting host contact with noxious luminal antigens on the other hand. To maintain this selective barrier, junction protein complexes seal the intercellular space between adjacent epithelial cells and regulate the paracellular transport. Increased intestinal permeability is associated with and suggested as a player in the pathophysiology of various gastrointestinal and extra-intestinal diseases such as IBD, celiac disease and type 1 diabetes.
The gastrointestinal tract is exposed to high levels of endogenous and exogenous proteases, both in the lumen and in the mucosa. There is increasing evidence to suggest that a dysregulation of the protease/antiprotease balance in the gut contributes to epithelial damage and increased permeability. Excessive proteolysis leads to direct cleavage of intercellular junction proteins, or indirectly via activation of protease activated receptors. In addition, proteases regulate the activity and availability of cytokines and growth factors, which are also known modulators of intestinal permeability.
This review aims at outlining the mechanisms by which proteases alter the intestinal permeability. More knowledge on the role of proteases in mucosal homeostasis and gastrointestinal barrier function will definitely contribute to the identification of new therapeutic targets for permeability-related diseases.

3. In the introduction the authors mentioned the importance of mucosal immunology, this should be discuss in a little detail in the main text with context to intestinal permeability.

Response: This is a good suggestion of the referee and completes the review. Therefore, we added a paragraph on page 3 of the manuscript in which the most important mediators of mucosal immunology are discussed in relation to intestinal barrier function.

4. Again barrier defect and disease was mentioned briefly in the introduction, a little bit more detail in this space in the main text leading to a therapeutic target in proteases.

Response: We expanded the information on this topic in the introduction (page 2) and the conclusion (page 19). Since this topic has been nicely reviewed previously, we also referred the reader to two reviews by Odenwald & Turner (references 14 and 15).

5. There is a lack of a basic definition of what are "proteases" and "proteinase-activated receptors". Suggest a general section on proteases and the different types, just introducing them to the reader before discussing each class later in the text.

Response: The section 'Proteases and proteinase-activated receptors' has been re-organized and we added a clear definition. For more basic information on proteases and proteinase-activated receptors we also referred to the companion review from our group (Ceuleers et al., Dec 2016, World Journal of Gastroenterology – reference 41).

6. A schematic diagram/cartoon detailing the mechanism of action proteases, PAR, calmodulin, MLCK/ERK1/2 cascade leading to increased intestinal permeability may be helpful to the reader.

Response: A schematic diagram of the factors regulating MLC phosphorylation is included in figure 2 which 2 is an enlargement of figure 1 that shows the receptors and ligands. This has been made more clear.

7. Again, incorporating zonulin into the above cartoon, which includes EGFR/PAR2.

Response: We incorporated zonulin signaling in figure 1.

8. Define zonulin receptor (bottom of pg 7)?

Response: The lack of clarity concerning the zonulin receptor has been tackled.

9. Proteases are also found in plants/food, viruses and fungi. Would this be worth discussing, considering in individuals consuming a high protein diet? Would the endogenous proteases be induced (another area worth discussing is induction of proteases), is bacterial proteases (or bacterial species high in proteases) also induced?

Response: Little is known about the relation between food proteases, high protein diet and the reaction of the endogenous and bacterial proteases regarding intestinal barrier function. We addressed the effect of fungal proteases on prebiotics briefly in the paragraph on luminal proteases (page 18). Also, we discussed the induction of proteases in the new paragraph on mucosal immunology and barrier function (page 3).

10. The authors did discuss the bacterial proteases, it would be useful to have a Table sumarrising the bacterial proteases and their effects.

Response: The bacterial proteases and their effects are included under 'luminal proteases' in table 1.

11. The author briefly mentioned food allergens and intestinal permeability eg kiwifruit and loss of barrier function. More detail in the space is warranted and a Table summarizing it would also be useful.

Response: More information has been added on this topic. The summary can be found under 'luminal proteases' in table 1.

12. The statement "These proteoltyic enzymes cleave proteinase-activated receptors ...." in the conclusion is misleading. Clarify.

Response: This statement has been adjusted. We hope it is clearer in this way.

Responses to the comments of Reviewer #2 – No. 3475710

1. In addition to protease activation, it would be nice to include a paragraph about regulation of protease expression: which ones are induced under what conditions etc

Response: We added a paragraph on mucosal immunology in relation to intestinal barrier function where protease expression is also discussed (page 3). For more basic information on proteases and proteinase-activated receptors we also referred to the companion review from our group (Ceuleers et al., Dec 2016, World Journal of Gastroenterology – reference 41).

2. Description of actomyosin contractility on page 3 should also briefly mention ROCK functions as the RhoA/ROCK pathway also contributes to contractility.

Response: Indeed, this is a good point. It has been added to the text (page 3) and figure 2.

3. Page 5: the authors should add a discussion of why the Par4 inhibitor does not abolish the effect of the CD supernatants, even though this might be speculative. What could be the alternative pathway?

Response: We feel that it is very difficult to speculate on this matter because there is a clear lack of studies investigating the expression of cathepsin G (specific activator of PAR4) and compare them in UC versus CD patients. Other proteases are likely to play a key role in barrier function in CD.

4. Desmoglein-2 is also a substrate of matriptase (Wadhawan, Biochem J, 2012). This should also be discussed as a potential mechanism contributing to regulating barrier functions.

Response: Indeed, but since desmoglein-2 is a desmosomal protein and responsible for cell-cell adhesion and not the barrier function it is more relevant to invasion (cancer) studies and therefore we chose to not include it in this review.

5. Page 7: define 2SD

Response: This has been clarified.

6. Abbreviate tight junction as TJ and use it throughout the text.

Response: This has been adjusted.

7. Page 9: Please clarify: “levels of occludin were elevated in vehicle and DSS-treated MMP9-/- mice”. What is the vehicle? And should vehicle-treated mice not be the appropriate control group?

Response: The elevated occludin levels were observed in MMP9 knockout mice, both in the DSS-treated group as well as in the control group treated with the DSS vehicle (or more precisely water). We adjusted this sentence to make it clearer to the reader.

8. Page 10: what do the authors want to claim with the statement that MUC2 delivers immunoregulatory signals? And what does it have to do with meprin. If not a clear link

between this or other proteases is provided, it should better be deleted for the sake of focus.

Response: Mucus is not only a nonspecific physical barrier, it is also a part of the immunological barrier since the nonattached MUC2 protein promotes oral tolerance towards harmless gut antigens by the induction of immunoregulatory signals such as IL-10 and retinoic acid, which drives the dendritic cells to induce regulatory T cells. Meprin β plays a key role in this mechanism since this protease detaches MUC2 from the goblet cell surface and this induces this tolerance phenomenon. We have clarified this statement in the text on page 14.

9. Page 11: what is really meant by "autocrine effect of TIMP-3"? Please discuss in more detail.

Response: Indeed, not enough information to correctly interpret this statement was provided. We discussed this statement more in detail on page 15.

10. Page 11: "NF?B"

Response: "nuclear factor (NF) kappa B", clarified on page 6.

11. Page 12: when discussing amoeba-derived proteases, recent articles of the Orozco and Chadee groups should be mentioned (e.g. Betanzos et al., 2013, Plos One; Kissoon-Singh et al., 2013, Am J Pathol).

Response: Indeed, these articles were overlooked. They have been added now as reference number 152 and 153.

12. Page 13: "Food allergens"?

Response: We expanded the paragraph on the "food allergens" in relation to intestinal permeability in order to make it clearer to the reader. We also refer the reviewer to the comments towards the remarks of reviewer 1.

13. In figure 2 the proteases affecting MLCK activity and thus MLC phosphorylation should be included.

Response: Figure 2 (the apical junction complex) is actually an enlargement of figure 1 and therefore proteases affecting barrier integrity are included in figure 1. We added a reference to figure 2 in figure 1.

14. The interested reader should be referred to another extensive review on this topic by the Nusrat group (Nava et al., 2013, Tissue Barriers).

Response: Indeed. We added the reference (reference 25).

15. There are many instances where "(cfr. supra)" appears? Are these missing references???

Response: We used cfr supra to indicate "as described above" to the reader. The necessity of each of these references was considered and a few were deleted.