

Lyon, April 27th, 2015

Dear Editor,

Please find enclosed the revision of our manuscript **NO: 16771** entitled:

Novel and unexpected roles of four carbons molecules in the control of metabolism

Authors: Sabrina Chriett, Luciano Pirola

Which we previously submitted to the *World Journal of Biological Chemistry* (ESPS Manuscript NO: 16771)

Name of journal: World Journal of Biological Chemistry

Thank you for your email indicating that the reviewers and editors of our above mentioned manuscript will consider a publication of it after a major revision. According to the reviewers and your requests, we are now submitting a revised manuscript that has addressed all the questions and concerns of all the reviewers in detail, and also corresponds to the formatting and presentation standards of the World Journal of Biological Chemistry. We are very grateful to the editors and reviewers for their careful review, comments and helpful suggestions.

Beside this response letter we are submitting a “MARKED COPY” manuscript file containing the changes made as well as a “CLEAN COPY” manuscript file, which should be considered as our final submission. The “clean copy” has been further reviewed by an English native speaker colleague, to improve the English language presentation of the manuscript.

Based on the suggestions, we have made several changes and additions to the manuscript. Such amendments are also presented in the detailed point-by-point responses below.

General editorial comments:

1. The title, as suggested by reviewer **02615858**, has been changed to “Novel and unexpected roles of butyrate and butyrate derivatives in the control of metabolism” to reflect the content of the review more appropriately.
2. English syntax and grammar and have been improved, and the manuscript has been reviewed by an American colleague who is an English native speaker.
3. Format has been updated accordingly to the journal’s guidelines

We believe that we have addressed all the comments in a satisfactory manner. We hope that you and the reviewers will now consider our revised manuscript as acceptable for publication in *World Journal of Biological Chemistry*. Thank you for your time and efforts in re-reviewing our manuscript.

Sincerely yours, L Pirola and S Chriett

Answers to the comments of reviewer #02681548

The manuscript submitted by Sabrina Chriett and Luciano Pirola is highly interesting, very well organized and written in a quite attractive way. Congratulations! The review is accepted for publication in its present form.

Response: We thank the reviewer for his positive commentary and for taking the time to review our manuscript. All minor requests are addressed as follows.

1. Introduction: 2nd phrase "Obesity predisposes (...) certain cancers". Please add a reference in this statement.

Obesity has clearly been proven to increase the risks of certain cancers, particularly colorectal cancer as discussed by *Gunter, M.J. and al. (2006) Obesity and colorectal cancer: epidemiology, mechanisms and candidate genes, Journal of Nutritional Biochemistry*. This reference has now been added.

2. Page 9: 3rd paragraph G-protein coupled receptors is the first mention. Add GPCRs abbreviation here. Remove the mention without abbreviation from the next paragraph.

GPCRs abbreviation was added on the first mention, and only the abbreviation is used after the first mention.

3. Page 10: 3rd paragraph "Moreover, (...) by skeletal muscle." Please add a reference in this statement.

The reference of *Roberts LD and al.*, regarding Baiba production by skeletal muscle and availability in the organism has appropriately placed within the manuscript. (Roberts, L. D., Bostrom, P., O'Sullivan, J. F., Schinzel, R. T., Lewis, G. D., Dejam, A., Lee, Y. K., Palma, M. J., Calhoun, S., Georgiadi, A., Chen, M. H., Ramachandran, V. S., Larson, M. G., Bouchard, C., Rankinen, T., Souza, A. L., Clish, C. B., Wang, T. J., Estall, J. L., Soukas, A. A., Cowan, C. A., Spiegelman, B. M., and Gerszten, R. E. (2014) beta-Aminoisobutyric acid induces browning of white fat and hepatic beta-oxidation and is inversely correlated with cardiometabolic risk factors. *Cell Metab.* **19**, 96-108. doi: 110.1016/j.cmet.2013.1012.1003.)

Answers to the comments of reviewer #02618027

1. This manuscript needs to be edited by a native English speaker. There are many errors in grammar and syntax throughout.

We accept this criticism as we are not English mother tongue. Firstly, we amended the manuscript to eliminate syntax and grammar errors. As final proofreading, the manuscript has been reviewed by an American visiting professor from Utah, whom we acknowledge in the appropriate section.

2. Can the authors comment on 4-PBA's ability to "shift" energy expenditure towards glycolysis and what effects that might play on compensatory mechanisms of energy expenditure by fatty acid oxidation?

To address this reviewer's point, we discuss in the "4-phenylbutyric acid" paragraph (Page 9) recent work demonstrating that 4-PBA is a (pyruvate dehydrogenase kinase) PDK inhibitor, which could be clinically used to treat pyruvate dehydrogenase complex deficiencies – which result in lactic acidosis. We have introduced this new information as follows: **"Recent preclinical research in zebrafish and mouse models demonstrated that 4-PBA is a PDK (pyruvate dehydrogenase kinase) inhibitor (42). PDK, inhibits the pyruvate dehydrogenase complex (PDHC) catalytic activity via inhibitory phosphorylation and has been used to alleviate lactic acidosis caused by PDHC deficiency by restoring the conversion of glycolytic pyruvate into Acetyl-coA"** And, to comment on the double action of 4-PBA on glycolytic and fatty acid oxidation pathways, we close the paragraph as follows: **"Taken together, these studies suggest that 4-PBA favors the glycolytic utilization of glucose, while inhibiting adipogenesis and, by extension, fatty acid oxidation."**

3. Are the authors proposing that short chain fatty acid supplementation occur in conjunction with use of insulin sensitizers, such as peroxisome proliferator-activated receptor gamma (PPAR γ) ligands, as a therapeutic approach in treating type 2 diabetics? How would these two therapeutic strategies interact with each other?

We would not propose the association between rosiglitazone (a PPAR γ agonist) and 4-PBA for the reason that rosiglitazone – at least in France and Europe – has been removed by the market since almost 5 years. We do not mention this possibility in the manuscript, although we describe the mechanism of action of BAIBA whereby BAIBA-induced WAT to BAT transition is dependent on an increase of PPAR γ . Clearly, this can be considered as a physiological condition, being BAIBA an endogenous molecule, and not a pharmacological agonist as rosiglitazone.

4. *What concentration of fermentable dietary fibers would one have to consume for enough butyric acid to be produced biologically to shift cellular metabolic energy generation towards fatty acid oxidation?*

Accordingly to Gao et al (ref 13 in our manuscript), butyrate supplementation at 5% wt/wt within a high-fat diet in a murine model increased adaptive thermogenesis and fatty acid oxidation, yet the detected levels of circulating butyrate were very low (butyrate had to be detected by Mass-Spectrometry based procedures). It is most likely that any metabolic effect of fiber supplementation is not only dictated by dietary fiber intake, but also by the relative amount of butyric bacteria within the gut. Indeed, in Type 2 diabetic patients the gut microbiota is deficient in butyric bacteria. We comment on these points at page 5 of the manuscript as follows: **“Whether the beneficial actions of butyrate are due to a direct action on metabolic tissues will require further investigation, as a clinical study investigating perfusion of short-chain fatty acids – including butyrate - on glucose metabolism in healthy men failed to show beneficial changes of glucose metabolism (24). On the other hand, a decreased population of gut butyric bacteria has been demonstrated in type 2 diabetic patients (8, 9), indicating the prominent role of butyrate in the maintenance of healthy metabolism.”**

5. *Is there anything known about the long terms effects of continued short chain fatty acid supplementation in animal models?*

Long-term supplementation of SCFAs has not been often reported in animal and human studies. Rather, a food supplementation causing a change in the organism's SCFAs concentration has been used, especially in butyrate's case, because of its “bad taste” and un-palatability. For example, a dietary supplementation in indigestible carbohydrates has been shown to induce a higher butyrate production by the gut microbiota, visible in the plasma SCFAs concentration (1). However, medium-term supplementation in food with SCFA has been made, for example in the study of *Gao and al.* (Ref. 7 in the manuscript), which administered 5% butyrate for 16 weeks to rodents. An independent study administering 5% butyrate for 12 weeks showed no toxicity from butyrate in mice (2).

1. Nilsson AC, Ostman EM, Knudsen KE, Holst JJ, Bjorck IM. A cereal-based evening meal rich in indigestible carbohydrates increases plasma butyrate the next morning. *J Nutr.* 2010;140(11):1932-6. doi: 10.3945/jn.110.123604. Epub 2010 Sep 1.
2. Li HP, Chen X, Li MQ. Butyrate alleviates metabolic impairments and protects pancreatic beta cell function in pregnant mice with obesity. *Int J Clin Exp Pathol.* 2013;6(8):1574-84. Print 2013.

6. *Cellular oxidative stress from mitochondria and ER are as important in cellular signaling as cellular damage. Can the authors reflect on any existing literature and comment on how butyric acid and its analogues molecules affect tissue immunity?*

The interaction between the intestinal immune system and gut bacteria and their metabolites, including SCFAs, is indeed a research field that has recently received much attention. We discuss in a new paragraph at page 6 the interplay between SCFAs and regulation of the immune responses in the gut. (*The interplay between SCFAs and the immune system*)

Answers to the comments of reviewer #02615858

This paper briefly reviews the emerging biological role of short fatty acids (SCFAs), mainly butyric acid as well as the related compounds 4-phenylbutyric acid, D-b-hydroxybutyrate, b-hydroxybutyrate and b-aminoisobutyric acid, and summarizes the current knowledge and the mechanisms involved in the regulation of body weight and metabolism exerted by these molecules. The manuscript is well conceived and is of interest, providing useful and current information on this topic.

We thank the reviewer for his interest in our manuscript, for the time taken to review it and for the thorough commentaries and interrogations he/she raised. All the reviewer's points concerning SCFAs production, availability and metabolic pathways have been added in the revised manuscript.

Below is the response to each point:

1. *The title should be modified to avoid uncertainties, and should reflect clearly the content of the review. In this sense, 4-phenylbutyric acid is not a four-carbon molecule.*

Following the reviewer's comment, we have changed the manuscript title. The new title is: **Novel and unexpected roles of butyrate and butyrate derivatives in the control of metabolism**. We believe the new title is more informative and remove the discrepancy of defining 4-PBA as a 4-carbon molecule.

2. *It is not clear in which sense 4-phenylbutyric acid is a derivative of butyric acid, as mentioned in the abstract. Please explain.*

4-phenylbutyric acid is indeed not produced from butyric acid, but a chemically produced molecule of which formula resembles the one of butyric acid. The way of synthesis for 4-phenylbutyric acid has been added in the relevant paragraph (page 8) with a reference to the relative patent. We refer, however, to 4-phenylbutyric acid as a butyrate derivative as within the review we explore the metabolic actions of butyrate, and in this context, we consider 4-phenylbutyric acid as a butyric acid bearing a phenyl group on its carbon 4. Also, 4-phenylbutyric acid and butyrate have a concordant biological action as histone deacetylase inhibitors. Finally, there are a few examples in the literature in which 4-phenylbutyric acid is considered as a butyric acid derivative (3, 4).

3. Singh OV, Vij N, Mogayzel PJ, Jr., Jozwik C, Pollard HB, Zeitlin PL. Pharmacoproteomics of 4-phenylbutyrate-treated IB3-1 cystic fibrosis bronchial epithelial cells. *J Proteome Res.* 2006;5(3):562-71.

4. Svechnikova I, Gray SG, Kundrotiene J, Ponthan F, Kogner P, Ekstrom TJ. Apoptosis

and tumor remission in liver tumor xenografts by 4-phenylbutyrate. Int J Oncol. 2003;22(3):579-88.

3. *Besides alpha-hydroxybutyrate, which other four-carbon molecules have been found to be early predictors of insulin resistance and glucose intolerance? Although this point is stated in the abstract, no other compounds have been quoted within the manuscript.*

This is a clear mistake from our side that we wish to amend. We have amended the mistake in the abstract as follows: **“Conversely, another butyrate-related molecule, α -hydroxybutyrate, has been found to be an early predictor of insulin resistance and glucose intolerance.”**

4. *p.5. The connection between inflammatory response and the development of obesity and associated pathologies should be stressed.*

We agree on the need to discuss more in detail the connection between inflammation and obesity. In the “Butyric acid and short chain fatty acids” section we have now added a sub-paragraph entitled: **“Inflammation and the development of obesity”**.

Specific comments:

5. *The mechanism by which butyrate increases muscle ATP consumption should be explained in detail.*

As demonstrated by Gao and al, (ref 14 in the manuscript) butyrate acts on insulin sensitivity and improves it apparently through different ways of action. At the mice level, butyrate acts on energy expenditure in different organs particularly muscles and brown and white adipose tissues. An increase in APT consumption has been revealed in muscles and seems to be correlated with butyrate being an HDAC inhibitor and inducing gene expression correlated with ATP consumption like PGC1 favoring type I oxidative muscle fibers and mitochondrial biogenesis. These details have been added in the main manuscript

6. *The proposed mechanisms for the increased levels of α -hydroxybutyrate and for its role in insulin resistance and glucose intolerance should be discussed. α -cetobutyrate might not be defined as the final product of methionine and threonine catabolism, and its formation in the cysteine biosynthetic pathway should be mentioned.*

There are indeed multiple pathways leading to the formation of α -hydroxybutyrate, and we inadvertently missed the production of it during the cysteine biosynthesis. We have now amended the relative paragraph to mention this pathway, and we have added the relevant reference (Landaas, S (1975) The formation of 2-hydroxybutyric acid in experimental

animals. Clin Chim Acta 1975, 58:23-32.)

7. The description of the effects of β -aminoisobutyric acid on adipose tissue is somehow puzzling. Please, explain them accurately.

The paragraph has been expanded to explain more in detail the biological effects of BAIBA. At the same time, we point out that the exact mechanism of activation of PPAR α by BAIBA has not yet been elucidated.

8. The writing needs polishing and some text editing; there are frequent awkward sentences and unnecessary repetitions [e.g., p.5, within 2nd paragraph; p.6, 3rd, 4th and 5th paragraphs; p.9, 2nd (last lines), 3rd, 4th, and 5th paragraphs]. Thus, manuscript assembly should be extensively improved; a detailed account of such errors does not seem warranted.

We have amended the suggested points, and we tried to our best to improve the language presentation. Finally, the final version of our revised manuscript has been read and corrected by an American colleague from Utah currently doing a sabbatical in the laboratory.

9. p.7, 4-phenylbutyric acid section, first three lines. It is not clear what it is meant.

The entire paragraph has been heavily revised also to take into account the criticisms of the other reviewers. We believe it is clearer now.

10. p.8, Hydroxybutyrate section, 2nd paragraph, line 4: the sentence "Once in the target tissues, ketone bodies are converted back to Acetyl-coA, through the sequential action of D- β -hydroxybutyrate dehydrogenase ...", is not correct. The described sequential reactions are specific for β -hydroxybutyrate, and not for other ketone bodies. Please, indicate the pathway and metabolites accurately.

To the best of our understanding, if we consider as main ketone bodies acetoacetate, β -hydroxybutyrate (and acetone), the described pathway is correct (see for example, https://www.rose-hulman.edu/~brandt/Chem330/Ketone_bodies.pdf page 22). We now add the note that acetone is a quantitatively minor ketone body which is actually not used by target organs but rather expired, being a volatile molecule.

11. p.10, β -aminoisobutyric acid section, 1st paragraph, line 2. It should be stressed that β -aminoisobutyric acid can be generated only from the split of thymine, and not from other pyrimidines, and as a byproduct from the catabolism mainly of the branched-chain amino acid valine.

To take into account this commentary, we have modified the sentence as follows:

" β -aminoisobutyric acid (BAIBA) was first identified as a catabolite derived from the breakdown of thymine (5) valine, and - to a substantially lower extent - other

branched chain amino acids (6)."

5. Fink K, Henderson RB, Fink RM. α -Aminoisobutyric acid in rat urine following administration of pyrimidines. *J Biol Chem.* 1952;197(1):441-52.
6. Nielsen HR, Borek E, Sjolin KE, Nyholm K. Dual origin of α -aminoisobutyric acid, a thymine catabolite. *Acta Pathol Microbiol Scand A.* 1972;80(5):687-8.

12. p.3, last line. Some of the molecules described in the manuscript do have a pharmacological rather than a physiological action; this point should be addressed.

We agree with this commentary. We have modified the sentence as follows:

"Here we summarize the current knowledge about these molecules, their biosynthesis or chemical way of production and their physiological or pharmacological modes of action."

Minor points:

1. p.8, Hydroxybutyrate section, 2nd paragraph. There is no need to write Acetyl-coA starting with a capital letter, and the abbreviation previously defined for "ketone bodies" should be used within this paragraph.

Amended

2. Why is it an abbreviation used for 4-phenylbutyric acid (4-PBA), for α -hydroxybutyrate (α HB), for β -aminoisobutyric acid (BAIBA), and not for β -hydroxybutyrate? "Type 2 diabetes" and "histone deacetylase" should be referred within the text as defined in p. 3 (T2D) and p.6 (HDAC), respectively. It seems not to be needed an abbreviation for "high fat and high sugar diets" (HFHS), as it is only used once (p.5). The abbreviation for "G-protein coupled receptor" (GPCR) should be given the first time it is mentioned (p.9, 3rd paragraph), and used thereafter. The abbreviations for white adipose tissue (WAT) and brown adipose tissue (BAT) are not needed.

As in the review we discuss both β -hydroxybutyrate and α -hydroxybutyrate, we have preferred to keep an abbreviation for α -hydroxybutyrate (α -HB) but not for β -hydroxybutyrate, mainly because we refer in the text to the biological D stereoisomer (D-b-hydroxybutyrate), and we find the abbreviation D-b-HB cumbersome.

We have amended the use of the abbreviations for T2D, HDAC and others as necessary (sometimes, for narrative purposes, we keep the full wording). We have removed WAT and BAT as appropriately suggested.

3. *Definitions should be given for AMPK (adenosine monophosphate-activated protein kinase;*

p.7, line 2), for PGC-1 α (peroxisome proliferator-activated receptor gamma coactivator 1-alpha; p.10, 2nd paragraph, line 6) and PPAR α (peroxisome proliferator-activated receptor alpha; p.10, 2nd paragraph, line 10).

Amended as requested.

4. *Use “four-carbon” molecule/compound/etc instead of “four carbons”*

Amended (also “four-carbons” was removed from the title).

5. *p.5, last line. “SCFAs” instead of “SCFA”.*

Amended

6. *p.7, first line of 4-phenylbutyric acid section, “4-phenylbutyric acid” instead of “4-phenylbutyruc acid”.*

Amended, also we have very well proofread the manuscript to avoid typos.

7. *p.9, 2nd paragraph, line 7, “Caenorhabditis elegans” instead of “C. elegans”.*

Amended

8. *Please add references to the first two sentences of the Introduction section.*

We have added two relevant references as required also by another reviewer.

9. *References should be formatted according to journal requirements.*

Done. We have also inserted the d.o.i. of each reference as per journal guidelines

10. *Table1, the 2nd and 3rd columns could be renamed as “Putative molecular target or mechanism involved” and “Overall biological response”, respectively. In some of the table cells, the wording is unclear.*

We have renamed the column's captions as suggested, and we have tried to render more clear the meaning of each cell.

Thank you again for your continued interest in our manuscript, which we hope will be eventually accepted for publication..

Sincerely yours, L Pirola and S Chriett

1. Nilsson AC, Ostman EM, Knudsen KE, Holst JJ, Bjorck IM. A cereal-based evening meal rich in indigestible carbohydrates increases plasma butyrate the next morning. *J Nutr*. 2010;140(11):1932-6. doi: 10.3945/jn.110.123604. Epub 2010 Sep 1.
2. Li HP, Chen X, Li MQ. Butyrate alleviates metabolic impairments and protects pancreatic beta cell function in pregnant mice with obesity. *Int J Clin Exp Pathol*. 2013;6(8):1574-84. Print 2013.
3. Singh OV, Vij N, Mogayzel PJ, Jr., Jozwik C, Pollard HB, Zeitlin PL. Pharmacoproteomics of 4-phenylbutyrate-treated IB3-1 cystic fibrosis bronchial epithelial cells. *J Proteome Res*. 2006;5(3):562-71.
4. Svechnikova I, Gray SG, Kundrotiene J, Ponthan F, Kogner P, Ekstrom TJ. Apoptosis and tumor remission in liver tumor xenografts by 4-phenylbutyrate. *Int J Oncol*. 2003;22(3):579-88.
5. Fink K, Henderson RB, Fink RM. -Aminoisobutyric acid in rat urine following administration of pyrimidines. *J Biol Chem*. 1952;197(1):441-52.
6. Nielsen HR, Borek E, Sjolín KE, Nyholm K. Dual origin of -aminoisobutyric acid, a thymine catabolite. *Acta Pathol Microbiol Scand A*. 1972;80(5):687-8.

Dear Dr. Fang-Fang,

we thank you for your email and for the provisional acceptance of our manuscript by the Journal Editor Dr. Song-Qin Liu.

Following the Editor's remarks, I have now changed the manuscript title into **"Essential roles of four-carbon backbone chemicals in the control of metabolism"**.

Also, the second suggestion (in the "conclusions" paragraph) has been taken into account.

Please find in attachment the revised manuscript. The changes are in red.

Sincerely yours , Luciano Pirola