Format for ANSWERING REVIEWERS

September 23, 2015

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 20164-Review.doc).

Title: High levels of homocysteine downregulate apolipoprotein E expression *via* nuclear factor kappa B

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Name of Journal: *World Journal of Biological Chemistry*

ESPS Manuscript NO: 20164

The manuscript has been improved according to the suggestions of reviewers:

Answer to reviewers:

We thank the reviewers for their pertinent comments which were very helpful for us in order to improve our manuscript.

Reviewer No 1:

1. "Hcy is a potential excitatory neurotransmitter and binds to the NMDA subtype of glutamate receptor. NMDA receptors are expressed in the neural cells as well as in some non-neural cells. However, HEK293 cells do not express these receptors and thus have been widely used to evaluate the function of NMDA receptors after force expression by transfected construct, vioron or mRNA. Based on these facts, authors should explain how Hcy acts on HEK cells and then the MAPK pathways, NFkB factor and eventually downregulated the ApoE promoter".

Answer to this comment:

It is indeed true that homocysteine acts via NMDA receptors and renal NMDA and Group 1 metabotropic glutamate receptors have been associated with hyperhomocysteinemia-induced glomerulosclerosis [1]. Moreover, in the case of neurodegenerative conditions, increased HCy may exert its cytotoxic effects via its derivatives homocysteic acid and cysteine sulfinic acid, which are also endogenous agonists of NMDA receptors [2]. A question arises regarding the mechanism through which HCy can exert its effects in a cell line like HEK 293, which is apparently void of NMDA receptors and has been used as such to functionally characterize the activity of various receptor subtypes overexpressed in this cell line [3]. The piece of evidence of lack of NMDA receptors on HEK-293 cells comes from the fact that CPP, a competitive

NMDA receptor antagonist, did not affect the effects of the non-competitive NMDA inhibitor MK-801 [4]. However, Hcy can exert its effects not only directly by receptor-mediated cell targeting, but also by indirect actions, as through binding to serum proteins, mainly albumin. Up to 70% of the total HCy is in its protein-bound form and there is a strong correlation between albuminemia and total HCy content [5]. Furthermore, homocysteine was found to be among the strongest protein–bound uremic toxins, which leads to reduced removal upon conventional hemodialysis [6] and thus poor prognosis in atherosclerosis-associated renal complications. Thus, an indirect action could explain the high, non-physiological concentrations at which effects were noticed in our experiments, as well as in reports of other groups.

Additionally, it was shown that Hcy could act as a ligand [7, 8] or a competitive inhibitor [9, 10] for various amino acid transport systems in both laboratory animals and humans, not only in renal cells, but also other cell types.

In the end, it is worthwhile to mention that in HEK-293, the same cell line employed in our experiments, HCy was able to induce leptin resistance via ER-mediated stress, as shown by others [11].

Therefore, despite the lack of NMDA receptors, it could be envisioned that Hcy may exert its effects in HEK-293 cells by certain non-receptor, indirect actions.

This explanation was added into the manuscript at the Discussion section.

References for the answer:

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3. Hayes D, Wiessner M, Rauen T, McBean GJ. Transport of l-[14c]cystine and l-[14c]cysteine by subtypes of high affinity glutamate transporters over-expressed in hek cells. *Neurochem Int*. 2005;46:585-594
4. Nishimura M, Sato K, Okada T, Schloss P, Shimada S, Tohyama M. Mk-801 blocks monoamine transporters expressed in hek cells. *FEBS Lett*. 1998;423:376-380
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6. Jourde-Chiche N, Dou L, Cerini C, Dignat-George F, Vanholder R, Brunet P. Protein-bound toxins--update 2009. *Semin Dial*. 2009;22:334-339
7. Zalups RK, Ahmad S. Homocysteine and the renal epithelial transport and toxicity of inorganic mercury: Role of basolateral transporter organic anion transporter 1. *J Am Soc Nephrol*. 2004;15:2023-2031
8. Wang Y, Zalups RK, Barfuss DW. Potential mechanisms involved in the absorptive transport of cadmium in isolated perfused rabbit renal proximal tubules. *Toxicol Lett*. 2010;193:61-68
9. Brunini TM, Yaqoob MM, Novaes Malagris LE, Ellory JC, Mann GE, Mendes Ribeiro AC. Increased nitric oxide synthesis in uraemic platelets is dependent on l-arginine transport via system y(+)l. *Pflugers Arch*. 2003;445:547-550
10. Kimmich GA, Randles J, Wilson J. Na(+)-coupled alanine transport in llc-pk1 cells. *Am J Physiol*.

1994;267:C1119-1129
11. Hosoi T, Sasaki M, Miyahara T, Hashimoto C, Matsuo S, Yoshii M, Ozawa K. Endoplasmic reticulum stress induces leptin resistance. *Mol Pharmacol*. 2008;74:1610-1619

2. "Introduction should be provided with information relevant to the studies rather than systematically reviewing the background. Some irrelevant paragraphs should be removed, e.g., the last three sentences in the 1st paragraph on page 4; the 2nd and 3rd paragraphs"

Answer to this comment: The reviewer is right, and the Introduction has been abridged correspondingly in the revised manuscript.

3. "Some concepts or English writing should be clarified. Examples are listed below. 1) In the result section of abstract, transfection method does not need to be explained, but the evidence how MAPK activates NFkB et al should be described. 2) In the 3rd sentence of methods of abstract, "plasmids encoding … to drive" does not make sense because first, DNA sequence can encode mRNA for polypeptide and thus be included or constructed in a plasmid; second, promoter sequences instead of a plasmid can be used to drive a reporter gene. 3) In Material and Methods (p. 7), Information of EuroClone is unclear with MI which should be fully spelled out. 4) Source information of some critical materials should be provided, such as, Hcy, expression constructs of DD Ikb, IKKb, and synthetic (likely fused) p65-p50. 5) Information of experimental repeats should be provided. 6) Figures should be prepared consistently, e.g., Fig 1 should have error bars and Fig 2 should be presented in the same format, i.e., bar graph. 7) Statistic method should be provided"

We answer to these items point by point:

1) In the revised manuscript, the transfection method was omitted and the evidence how MAPK activates NF-kB is described.
2) the sentence was revised accordingly in the revised manuscript.
3) Information on EuroClone has been revised (Milan, Italy).
4) The information is available in the text, at the corresponding sections (Materials and Methods for Hcy, Plasmids for the various constructs).
5) The information was added under "Statistics" in Methods section.
6) Figure 1 is a representative experiments and Figure 2 was modified correspondingly
7) Statistic method is provided under "Statistics" in Methods section.

4. "What is Hcy concentration in Hyperhomocysteinemia? Is it comparable to the concentrations used in cultured cells here? Fig 1B is one western blot. Is this only result? If so, this experiment should be repeated at least six times. In addition, longer exposure

of apoE blot to X-ray film should be shown to exclude the possibility that apoE promoter is completely silenced. Hcy concentrations used for RT-PCR and protein assay should be the same.

Answer: As mentioned in the Discussion, "In mild and intermediate HHCy, Hcy plasma levels are found in the domain of 16-100 μM, while in severe HHCy, Hcy values are higher than 100 μM". It is true that in our experiments, slightly higher concentrations were needed. Since HCy exerts an indirect action of Hcy on HEK-293 cells cultured *in vitro*, these concentrations could be expected as being necessary to achieve a measurable effect. It is worth to mention that the Hcy concentrations we used are still in the experimental range of other studies in the literature. HEK-293 cells are not a main source of apoE, they express a minimal amount of apoE, better detected at mRNA than protein level and thus assessing modulation of apoE expression by Western blot experiments is demanding.

5. "Luciferase activity in transient transfected cells is normalized to β-galactosidase as described in the methods. However, information of the β-galactosidase in transfection is missed. Importantly, the promoter that drives the β-galactosidase expression may be regulated by Hcy and this possibility should be excluded. This is particularly critical when Hcy treatment is overnight. This concept should be applied to Fig 2 and Fig 3. This means that whether Mapk pathway acts on the promoter driving β-galactosidase expression and thus change relative luciferase activity should be addressed. The best model is the stable transfectants in which total cellular DNA can be used to normalize luciferase activity and no second report".

Answer to this comment: The reviewer is right, we added in the Methods section the information regarding the co-transfection of β-galactosidase expression vector, for normalization purposes. Hcy effect on CMV promoter that controls β-galactosidase expression was not significant, since the enzymatic activity did not considerably change in the treated cells compared with the control. Thus, we assume that this is a reliable way of normalization and the results are self-consistent.

Reviewer No 2:

1. "The manuscript needs to be careful edited for English language and presentation".

Answer to this comment: Misspellings and other minor errors were corrected. A Certificate of English provided by an authorized translator is included.

2. "The figure legends need to be expanded to provide some details".

Answer to this comment: The reviewer is right, and figure legends were included.

3. "The figures need to include information about statistical significance".

Answer to this comment: Statistical significance was mentioned where needed.

Thank you again for publishing our manuscript in the *World Journal of Biological Chemistry*
Sincerely yours,

Anca V Gafencu