Glucagon signalling in the dorsal vagal complex is sufficient and necessary for high-protein feeding to regulate glucose homeostasis in vivo

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Review timeline:

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Barbara Pauly

1st Editorial Decision          20 May 2015

Thank you very much for the submission of your research manuscript to our editorial office and for your patience while we were waiting to hear back from the referees. I would like to apologize for the unusual delay in getting back to you with a decision on your study for which we have just now received the full set of reviews.

As the detailed reports are pasted below I would prefer not to repeat them here. In essence and while all reviewers agree on the potential interest of the findings and are, in principle, supportive of publication of the study in our journal, both referee 2 and 3 raise a point about the relative contributions of fat versus carbohydrate content of the animals' diet to the observed effects and make suggestions on how to address this issue.

Overall, and given the reviewers' constructive comments, I would like to give you the opportunity to revise your manuscript, with the understanding that the main concerns of the referees should be addressed. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that therefore, acceptance or rejection of the manuscript will depend on the
completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions, but given the rather minor nature of the issues that need to be addressed, I do not anticipate any problems meeting this deadline. I look forward to seeing a revised form of your manuscript when it is ready. Should you in the meantime have any questions, please do not hesitate to contact me.

REFEREE REPORTS:

Referee #1:

This is timely and important work by an expert team which has been successfully pushing the limits of our understanding of endocrine control of metabolism over the recent years. They here report that new glucagon biology findings showing that glucagon signalling in the dorsal vagal complex (DVC) of the brain is sufficient to lower glucose production by activating a Gcgr-PKA-ERK-KATP channel signalling cascade in the DVC. They also demonstrate that direct blockade of DVC Gcgr signalling negates the acute ability of high- vs. low-protein feeding to lower plasma glucose concentration, indicating that the elevated circulating glucagon during high-protein feeding acts in the brain to lower plasma glucose concentration. The authors herewith prove that the full physiological role of glucagon has not been understood yet and suggest an intriguing new model where brain glucagon signalling contributes to glucose homeostasis during dietary protein intake. The experiments have been well conceptualised and were performed with lots of attention to details and cutting edge methodological approach. The conclusions are solidly based on the novel results. I actually have no reservations and support rapid publication of this translationally relevant contribution to a fast moving field.

Referee #2:

In this paper, the authors continue their exploration of the role of brain glucagon detection in the regulation of endogenous glucose production. In a previous publication, this group presented evidence suggesting that glucagon detection in the mediobasal hypothalamus lowers glucose production from the liver. In this manuscript, they explore the effect of glucagon sensing in another brain glucoregulatory region: the dorso-vagal complex in the brainstem (DVC). They use various combinations of pharmacological interventions to characterize the effect of DVC glucagon on endogenous glucose production and identify mechanistical contributors to this effect.

The paper is built around (what the authors believe is an unresolved question) the fact that postprandial glucose increase following the ingestion of a HP meal is lower than that seen following the ingestion of a LP meal. As stated in the introduction, there is an abundant literature supporting that this effect is mediated both by the lower carbohydrate content of HP diets and the insulinotropic effect of proteins. Although the authors provide evidence that central glucagon signaling regulates glucose production, the data supporting the role of this pathway in the glycemic response to a HP meal are less convincing. In particular, in the experiment presented on Figure 4, the lack of effect of GRA alone when given with the LP meal could be attributed to the high carbohydrate content of the meal compared to the HP meal. The inclusion of a group ingesting a HP meal containing the same amount of carb as the LP meal could help answer this question. In one of the paper cited in this manuscript (Claessens M 2009), it appears that the postprandial glycemic response to various HP loads with equal carbohydrate contents is strongly correlated with the postprandial insulin to glucagon ratio and independent of the postprandial glucagon AUC. Also, in this experiment, we need to see the full kinetics of plasma insulin and glucagon.

Proteins also affect incretin levels, and the potential contributions of CCK, GLP-1 and PY in the observed effects are not mentioned.

It is not clear why the authors decided to test the role of DVC glucagon detection in the glycemic response to HP diets instead of hypothalamic glucagon detection, given their previously published work. Do the authors think that MBH glucagon detection likewise contributes to the glycemic effect of HP diets? Why not test whole brain glucagon detection then, using intracerebroventricular
injections? At a minimum, the authors should discuss the respective roles of these 2 different sensing areas (completely redundant or complementary to some extent?).

The authors should include the composition of the diets they are using and specify the Protein to Carbohydrate ratios.

Referee #3:

It is an interesting study which well aligns with the authors' recent work suggesting that glucagon works in the brain to regulate blood glucose levels. In general, the hypothesis is novel, and the experiments were sophisticatedly designed and well carried out. I appreciate that these physiological experiments required a lot of different skills by nature, and the authors made a big effort towards this accomplishment. I have a few suggestions/questions for the authors to consider, which might help improve the quality of this manuscript:

1. Was the dietary effect in reducing postprandial blood glucose in Figure 1 due to the higher amount of protein (65.4% vs. 21.5%) or lower amount of carbohydrate (21.3% vs. 65.3%)? Can it be the case that the elevation in glucagon release was a result of the reduced uptake of dietary carbohydrate in the high-protein diet?

2. Glucagon infusion in the DVC is not limited to this small region. Similarly, glucagon receptor antagonist infusion in the DVC is not limited to this region. So the authors may change the interpretation a little bit, regarding whether DVC's glucagon signaling is sufficient for the glucose-lowering effect of high-protein diet.

Correspondence - authors
21 May 2015

Thank you for sending a very encouraging decision letter. I have discussed the editorial and reviewers' comments in details with my lab. We anticipate a re-submission date in about 2 weeks to address the remaining minor (or main) concerns from Reviewer #3 and #2 via textual changes. Of note, although Reviewer #2 suggested an additional experimental group (carbohydrate-matched diet) may be needed, Reviewer #3 simply asked us to comment on the issue revolving the relative contributions of protein vs. carbohydrate content of the animals' diet to our observed effects. And that Reviewer #1 did not have such concern at all. We strongly believe this issue could be dealt with via textual changes.

Correspondence - editor
21 May 2015

Many thanks for your email. With regard to your question whether the protein vs. carbohydrate-diet issue raised by both referees 2 and 3 would need to be addressed experimentally, I think this is a crucial control and central to the main message of the study. As such, I think it would be important to address this experimentally, rather than just discussing it. Please note that referee 3, although being less clear than referee 2 in what s/he deemed necessary, didn't specifically state that it would be sufficient to just address this issue in words.

Correspondence - authors
22 May 2015

Thank you for your reply. If I may further explain why we do not think it is necessary to perform the requested control study, and why we do not think the requested control study is central to the main
message of the study, please see our detailed response below. Please let me know what you think so we can proceed accordingly.

>>> We understand that Reviewers #2 and #3 both expressed concerns about whether the elevation of plasma glucagon release and the subsequent glucagon action in the brain (which is inhibited by glucagon receptor antagonist infusion in the brain) are due to the lower amount of carbohydrate in the diet rather than the higher protein content. Thus, it was suggested by reviewer #2 that we should perform the refeeding experiments with carbohydrate-matched diets. However, we really feel that previous literature has already addressed this concern and that is why we did not perform such control studies to begin with.

>>> In our submission to EMBO Rep, we cited a paper by Day et al. (1978) which found that, compared to a high-carb diet, a protein-matched low-carb diet did not increase glucagon levels (Figure 1, bottom left panel: group 3 vs. group 1 of the paper in 1978). Therefore, these studies already reported that the reduction of dietary carbohydrates 'does not' stimulate glucagon release into the plasma. More importantly, an equally low-carb diet with increased protein content (group 2 from the Day et al. 1978 paper) potently increased plasma glucagon levels, identical to what we observed in our submission to EMBO Rep (Figure 1). Thus, the ability of a high-protein/low-carbohydrate diet to increase glucagon secretion relies on the increased protein content of the diet, rather than the reduced carbohydrate content, and therefore performing studies with a 'low carb-low protein' control would be really unnecessary since such diet would not have increased plasma glucagon levels to begin with.

>>>> However, if you feel that previous evidence is not enough, we will perform fasting-refeeding studies with a low-carb/low-protein/high-fat diet to exclude the possible effect of reduced carbohydrate content on glucagon release into the plasma and the subsequent brain glucagon action; however, I would reiterate again there would really be no rise in glucagon levels (and thus no effect on DVC glucagon action) to begin with as this has already been clearly demonstrated in the Day et al (1978) study.

>>> Furthermore, Reviewer #2 stated that the Claessens 2009 paper we cited demonstrates that the postprandial glucose AUC correlates with the insulin-to-glucagon ratio, but not with the glucagon AUC, thus weakening our claim of high-protein feeding increasing central glucagon action to regulate glycemia. However, this paper did not measure the insulin-to-glucagon ratio, nor did it make any claims with regard to the correlation of glucose AUC with either insulin or glucagon AUC. 'In fact', the data from this paper suggests that glucagon levels have an inverse correlation with glucose levels, as the most potent reduction of glucose occurs at the same time points as the most potent stimulation of glucagon levels.

Correspondence - editor 28 May 2015

I have now received feedback on your response by referee 2 who raised the carbohydrate- vs. protein-diet issue.

Here is what s/he said:

>>> I agree with the authors that reducing carbohydrate intake per se will likely have a minor impact on blood glucagon levels. However, the main outcome measurement in the experiment presented on Figure 4 is plasma glucose, which, I am sure we will all agree, is mostly determined by carbohydrate intake (see same reference, Day et al. 1978). As seen on Figure 4, the LP and HP meals produce dramatically different raises in blood glucose, and therefore I do not believe that the lack of effect of GRA when combined with the LP meal is convincing, because there could be a ceiling effect. In other words, to convincingly demonstrate their hypothesis, I think the authors need to check that LP/GRA combination does not affect blood glucose levels in a context where the postprandial increase in blood glucose is in the same range as the one measured after the high-protein meal. Therefore I think that the authors need to include a group receiving an isocaloric low carbohydrate low protein meal and show that GRA does not affect postprandial blood glucose with that meal.<<<
Please do let me know what you think about it.

Correspondence - authors
28 May 2015

Thank you for your email.
First, I want to clarify we are in total agreement with this reviewer that the dramatic difference in the glucose rise between HP vs. LP diet is not only due to a blood glucagon rise. Clearly, the reduction in carbohydrate intake from the HP would have led to the drop in blood glucose vs. LP. In fact, our own data in Figure 4a suggest that since there is still a significant difference in the blood glucose level rise after 30 min of refeeding in the HP + GRA and LP + GRA group. However, it is the fact that there is no longer a difference in the blood glucose rise between these 2 groups by time 60 min of refeeding that tell us that there is a redundant glucose-lowering effect that is activated by brain glucagon action secondary to a rise in blood glucagon levels. We can clearly revise the text to further clarify this point.

Second, as to whether a lack of glucose effect of GRA in the LP diet vs. saline in the LP diet may be due to a ceiling effect (Figure 4a), we sincerely do not believe that is the case because in Figure 1f and 1G, we already reported that GRA infusion (given at the same duration and dose as Figure 4a) along in basal conditions (when glucose is at basal levels) did not per se had an effect on glucose production. This tell us that GRA specifically block the action of glucagon when the level of glucagon is elevated (as in the case of HP diet but not the LP diet). Thus, the difference in glucose rise that we see in HP diet saline vs. HP diet GRA (at 60 min) is not due to a non-specific effect of GRA, but rather due to a specific inhibition of brain glucagon action secondary to a rise in blood glucagon levels.

Third, if you feel our second argument (above + the Figure 1f and 1g data) is insufficient, we will perform the suggested group that will receive an isocaloric low carbohydrate low protein and high fat meal + with or without the GRA. We would expect, as in the case of figure 1f and 1g, GRA will not affect postprandial blood glucose levels.

Please let us know what you think so we can proceed immediately and accordingly.

Correspondence - editor
29 May 2015

Many thanks for your email and your patience while I was waiting to hear back from the reviewers about the concern raised by referee 2. I have now received feedback from referee 3 and s/he agrees that the request of reviewer 2 to include the control group is reasonable. I would thus kindly ask you to go ahead and include this control before submitting your revision.

I understand that you feel that discussing this issue would be sufficient, but two reviewers feel that experimental data would be required to address this issue.

Correspondence - authors
29 May 2015

Thanks Barbara for getting back and no problem ... we will get to it immediately and we should be done in a few weeks.
Referees’ comments:

Referee #1: This is timely and important work by an expert team which has been successfully pushing the limits of our understanding of endocrine control of metabolism over the recent years. They here report that new glucagon biology findings showing that glucagon signalling in the dorsal vagal complex (DVC) of the brain is sufficient to lower glucose production by activating a Gcgr-PKA-ERK-KATP channel signalling cascade in the DVC. They also demonstrate that direct blockade of DVC Gcgr signalling negates the acute ability of high- vs. low-protein feeding to lower plasma glucose concentration, indicating that the elevated circulating glucagon during high-protein feeding acts in the brain to lower plasma glucose concentration. The authors herewith prove that the full physiological role of glucagon has not been understood yet and suggest an intriguing new model where brain glucagon signalling contributes to glucose homeostasis during dietary protein intake. The experiments have been well conceptualised and were performed with lots of attention to details and cutting edge methodological approach. The conclusions are solidly based on the novel results. I actually have no reservations and support rapid publication of this translationally relevant contribution to a fast moving field.

>>> We thank the referee for these positive and very encouraging comments.

Referee #2: In this paper, the authors continue their exploration of the role of brain glucagon detection in the regulation of endogenous glucose production. In a previous publication, this group presented evidence suggesting that glucagon detection in the mediobasal hypothalamus lowers glucose production from the liver. In this manuscript, they explore the effect of glucagon sensing in another brain glucoregulatory region: the dorsal vagal complex in the brainstem (DVC). They use various combinations of pharmacological interventions to characterize the effect of DVC glucagon on endogenous glucose production and identify mechanistical contributors to this effect.

The paper is built around (what the authors believe is an unresolved question) the fact that postprandial glucose increase following the ingestion of a HP meal is lower than that seen following the ingestion of a LP meal. As stated in the introduction, there is an abundant literature supporting that this effect is mediated both by the lower carbohydrate content of HP diets and the insulinotropic effect of proteins. Although the authors provide evidence that central glucagon signaling regulates glucose production, the data supporting the role of this pathway in the glycemic response to a HP meal are less convincing. In particular, in the experiment presented on Figure 4, the lack of effect of GRA alone when given with the LP meal could be attributed to the high carbohydrate content of the meal compared to the HP meal. The inclusion of a group ingesting a HP meal containing the same amount of carb as the LP meal could help answer this question.

>>> Thank you for your comment. To directly address whether the lack of effect of GRA in the LP diet group could be due to the high-carbohydrate content of the meal causing a “ceiling effect”, where plasma glucose has already reached a maximal elevation, we have now included an additional control group which was fed an isocaloric, low-carbohydrate, and low-protein
(“LP/LC/HF”) diet during the fasting-refeeding protocol (Supplementary Fig S5) with or without GRA infusion into the DVC. In this LP/LC group, we discovered that there was no “ceiling effect” of glucose since the postprandial glucose rise was lower than that of the LP/HC group (Supplementary Fig S5 and Fig 4). In addition, the infusion of GRA vs. vehicle into the DVC ‘did not’ increase plasma glucose levels (Supplementary Fig S5), similarly as observed in the original LP/HC diet group (Fig 4). Thus, the lack of effect of GRA seen in the LP group can be attributed to the lack of protein-stimulated glucagon action, rather than to the high dietary carbohydrate content and high plasma glucose levels.

In one of the paper cited in this manuscript (Claessens M 2009), it appears that the postprandial glycemic response to various HP loads with equal carbohydrate contents is strongly correlated with the postprandial insulin to glucagon ratio and independent of the postprandial glucagon AUC. Also, in this experiment, we need to see the full kinetics of plasma insulin and glucagon.

>>> It is important to clarify that the Claessens et al. (2009) study did not measure the insulin-to-glucagon ratio, nor did it make any claims with regard to the correlation of glucose AUC with either insulin or glucagon AUC. In fact, although no formal calculations were performed, the data from Claessens et al. (2009) suggest that glucagon levels may have an inverse correlation with glucose levels, as the most potent reduction of glucose levels occurs at the same time points as the most potent stimulation of glucagon levels. Lastly, the kinetics of plasma insulin and glucagon was reported in Fig 1C and D.

Proteins also affect incretin levels, and the potential contributions of CCK, GLP-1 and PYY in the observed effects are not mentioned.

>>> We agree with the reviewer that high-protein feeding could potentially stimulate the release of CCK, GLP-1, and PYY (Fromentin G et al., Nutr Res Rev 2012) in our experimental conditions and could, in parallel, regulate glucose homeostasis. We have now revised the discussion accordingly (page 10).

It is not clear why the authors decided to test the role of DVC glucagon detection in the glycemic response to HP diets instead of hypothalamic glucagon detection, given their previously published work. Do the authors think that MBH glucagon detection likewise contributes to the glycemic effect of HP diets? Why not test whole brain glucagon detection then, using intracerebroventricular injections? At a minimum, the authors should discuss the respective roles of these 2 different sensing areas (completely redundant or complementary to some extent?).

>>> We have revised the introduction to further clarify why we targeted the DVC in the present study (page 3-4). We chose to investigate an extra-hypothalamic brain region that is known to regulate glucose homeostasis in order to increase the novelty as well as the physiological relevance of the study, since glucagon entry into the brain would not be restricted to the hypothalamus but would also act in other gluco-regulatory brain centres such as the DVC. Therefore, we first wished to determine if this effect of glucagon is conserved across different brain regions. Now that we have established a
physiological role for DVC glucagon action, it will be important to next examine whether the MBH plays a parallel role during high-protein feeding. Establishing whether these two regions elicit redundant, additive, or synergistic effects on glucose production and glucose homeostasis after high-protein feeding will require future investigations.

The authors should include the composition of the diets they are using and specify the Protein to Carbohydrate ratios.

>>> We kindly direct the referee’s attention to the Materials and Methods section (page 14), which contains the compositions of all diets used in this study.

**Referee #3:** It is an interesting study which well aligns with the authors' recent work suggesting that glucagon works in the brain to regulate blood glucose levels. In general, the hypothesis is novel, and the experiments were sophisticatedly designed and well carried out. I appreciate that these physiological experiments required a lot of different skills by nature, and the authors made a big effort towards this accomplishment.

>>> We thank the referee for her/his comments.

I have a few suggestions/questions for the authors to consider, which might help improve the quality of this manuscript:

Was the dietary effect in reducing postprandial blood glucose in Figure 1 due to the higher amount of protein (65.4% vs. 21.5%) or lower amount of carbohydrate (21.3% vs. 65.3%)?

>>> We agree with this referee that the difference in the glucose rise between HP vs. LP diet is not only due to the effect of high protein intake to stimulate glucagon, but is likely also caused by the reduction in carbohydrate intake from the HP diet compared to the LP diet. In fact, our own data in Fig 4A support this postulation, since there is still a significant difference in the blood glucose level rise after 30 min of refeeding in the HP/GRA vs. LP/GRA group. However, the fact that there is no longer a difference in the blood glucose rise between these two groups by time 60 min of refeeding demonstrates the presence of a redundant glucose-lowering effect that is activated by brain glucagon action secondary to a rise in blood glucagon levels.

To experimentally confirm that this brain glucagon-mediated glucose-lowering effect is due to high protein intake and not to low carbohydrate intake, we have now included an additional control group which received an isocaloric amount of a low-protein, low-carbohydrate (“LP/LC/HF”) diet (Supplementary Fig S5). We found that postprandial glucose levels were lower than those of the LP/HIC group (Fig 1), demonstrating a glucose-lowering role of reduced carbohydrate intake, as expected. However, the presence of a GRA infusion into the DVC did not affect glucose levels in this group, thus indicating that the effect of DVC glucagon action to lower plasma glucose is specific to the presence of high-protein, and not low-carbohydrate intake. Can it be the case that the elevation in glucagon release was a result of the reduced uptake of dietary carbohydrate in the high-protein diet?

>>> In our current submission, we cited a paper by Day et al. (1978) which found that, compared to a high-carbohydrate/low-protein diet, a low-carbohydrate/low-protein diet did not increase glucagon levels (Figure 1,
bottom left panel: group 3 vs. group 1 of the Day et al. 1978 paper). Therefore, this previous study reported that the reduction of dietary carbohydrates does not stimulate glucagon release into the plasma. More importantly, an equally low-carbohydrate diet with increased protein content (group 2 from the Day et al. 1978 paper) potently increased plasma glucagon levels, identical to what we currently observed in our submission (Figure 1). Thus, the ability of a high-protein/low-carbohydrate diet to increase glucagon secretion relies on the increased protein content of the diet, rather than the reduced carbohydrate content.

Glucagon infusion in the DVC is not limited to this small region. Similarly, glucagon receptor antagonist infusion in the DVC is not limited to this region. So the authors may change the interpretation a little bit, regarding whether DVC's glucagon signaling is sufficient for the glucose-lowering effect of high-protein diet. >>> We have demonstrated that our current protocol for intra-DVC infusion of compounds is restricted to only the DVC region that specifically targets the nucleus of the solitary tract within the DVC (Lam CK et al., JBC 2010). We have also shown that our intra-DVC viral injection (which administers a larger volume compared to the glucagon receptor antagonist infusion during refeeding) is mostly contained within the NTS with minor amounts in the DMX, and most importantly, is fully restricted to the DVC region (Filippi BM et al., Diabetes 2014). Therefore, intra-DVC infusion of glucagon receptor antagonist specifically blocks the actions of glucagon signalling within the DVC during high-protein refeeding.

2nd Editorial Decision

Thank you for your patience while we have reviewed your revised manuscript. As you will see from the reports below, the referees are now all positive about its publication in EMBO reports.

Before we can officially accept your paper for publication here I would like to point out that we are in the process of updating the way in which we display additional/supplementary information. In essence, all supplementary figures are now called Expanded View Figures and should be labeled and referenced as Figure EV1, Figure EV2 etc. in the main text of the manuscript. The legends for the EV figures should be incorporated in the main body of the text after the legends for the main figures. Please modify your additional figures accordingly.

You will then receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports.

REFEREE REPORTS:

Referee #2:

The authors responded to my comments.

Referee #3:
I think the authors have addressed my questions. Thus, I support its publication in EMBO Reports.

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2nd Revision - authors’ response 22 July 2015

We have revised the main text and the supplementary material file based on the instructions in the decision letter, and just sent back both of the revised files (EMBOR-2015-40492V3). Thank you and I look forward to receiving your official acceptance letter.

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3rd Editorial Decision 23 July 2015

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.