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PIKE-A Association with Hepatic Insulin Receptor Enhances its Kinase Activity

Chi Bun Chan, Xia Liu, Kunyan He, Qi Qi, Dae Young Jung, Jason K. Kim, Keqiang Ye

Corresponding author: Keqiang Ye, Emory University

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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.)

1st Editorial Decision 02 March 2011

Thank you for the submission of your manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, while the referees agree that the study is potentially interesting, they do raise a number of concerns and point out that the findings need to be strengthened and that the inconsistencies within the study and with your previous publication need to be clarified.

Both referees 1 and 3 point out that the relation of the findings to your previously published data remains unclear and that tissue-specific rescue experiments should be performed to address this issue. While we think that the generation of liver and fat PIKE-A KO animals (as suggested by referee 3) is a good approach, it would probably take longer than 3 months, whereas adenovirus infection could be performed within the time allowed for revision. Referee 1 adds that it should be excluded that AMPK signaling is affected in the liver of LPKO mice. Referees 1 and 2 further indicate that impaired insulin signaling in LPKO liver should be confirmed by examining lipid synthesis/expression of lipogenic genes and referee 1 adds that the inhibitory effect on Akt signaling could be strengthened by investigating FOXO1 activity. While we agree with referee 2 that it would be interesting to determine the role of PIKE-A in diabetes, we also feel that addressing this issue would generate an entire new data set that would likely not fit into the EMBO reports short format. Addressing this point is therefore -while certainly welcome- not essential for publication of the manuscript in EMBO reports.

Given these evaluations and the constructive referee comments, I would like to give you the

opportunity to revise your manuscript, with the understanding that the referee concerns must be fully addressed and their suggestions (as detailed above and in their reports) taken on board. Most importantly, all inconsistencies need to be addressed and the relation of the current data to your previous findings needs to be clarified.

I look forward to seeing a revised version of your manuscript when it is ready.

Yours sincerely

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

In the paper from Chan et al. the authors describe the phenotype of the liver specific PIKE KO mice. They show that these mice have impaired insulin sensitivity in the liver, as a result of deficient insulin signaling in this tissue, secondary to the lack of effects of PIKE on IRTK; This manuscript complements somehow a previous study from the same authors showing that full body PIKE -/- mice are insulin sensitive and protected from diabetes, secondary to increased AMPK activity. The results presented in this paper are not demonstrative enough, and present several inconsistencies within the results and with the previous published data. This reviewer has the following concerns:

1. The authors have performed glucose production experiments in the PIKEA-/- full KO mice, but not in LPKO. Gluconeogenesis experiments should be performed in these mice. Pyruvate tolerance test could be easily performed.

2. There are some conceptual mistakes in the design and interpretation of some experiments. The authors show increased glycemia in fasted mice, but no differences are observed in fed mice. This seems inconsistent with the hypothesis that the effects are mediated by a deficient insulin signaling. Indeed, insulin inhibits glucose production in liver, during the fed state. No relevant insulin signaling exists during fasting, therefore no differences on glycemia should be observed between +/+ and -/- mice.

3. This reviewer is confused when comparing data of this manuscript with the previous published PIKE full KO. The authors should better demonstrate that the differences are mediated by the effects of PIKE on AMPK in WAT and muscle, and not in liver. What happens to AMPK signaling in liver of the LPKO mice?

4. The authors claim that decreased gluconeogenesis is the result of decreased AKT signaling in LPKO mice. First, why AKT activity is inhibited in liver, and actually increased in WAT and muscle of the PIKE full -/- mice? Second, the anti-gluconeogenic effects of AKT are mediated by phosphorylation of FOXO1. Analysis of FOXO pathway would further demonstrate the hypothesis.

5. In addition to block gluconeogenesis, insulin is also a lipogenic hormone in this tissue. Decreased lipid synthesis, fatty acid accumulation, and expression or activity of enzymes implicated in this pathway would be demonstrative of impaired insulin signaling in livers of LPKO.

6. It is difficult to compare the experiments in this manuscript with the published paper f the full KO, because the age of mice is very different (old 8-9 months mice versus young 2-3 months mice)

7. To unequivocally demonstrate the distinct effects of PIKE in liver and peripheral tissues, rescue of expression of PIKE in livers of PIKE full KO mice using adenoviral infection could be used.

8. Numbers of mice used in some experiments is not enough to render the results statistically significant. Three or four mice are not sufficient.

Referee #2:

Summary
The current study by Chan et al. provides data on the role of phosphoinositide 3-kinase enhancer A (PIKE-A) in hepatic insulin receptor signaling and glucose homeostasis. In particular, the authors show that genetic ablation of PIKE-A in liver promotes hyperglycemia, hyperinsulinemia and glucose intolerance. These phenotypes could be attributed to impaired hepatic insulin receptor signaling upon PIKE-A knockout, mediated by decreased insulin receptor autophosphorylation. The authors conclude that PIKE-A represents a critical regulator of systemic glucose homeostasis and hepatic insulin sensitivity.

General comments
Given the increasing prevalence of obesity, type 2 diabetes and other components of the Metabolic Syndrome, the exploration of mechanisms in systemic and tissue-specific insulin sensitivity/resistance clearly represents an important area in biomedical research. In this respect, the current manuscript by Chan et al. provides interesting new insights into the role of PIKE-A in hepatic insulin signaling and downstream metabolic consequences. Overall, the presented studies are well designed and structured and the conclusions are supported by the experimental results. While the function of PIKE-A under healthy, "wild-type" conditions are convincingly demonstrated by the authors, the current manuscript falls short in the following two aspects:
a) With respect to the proposed role of PIKE-A in the development of type 2 diabetes, it remains unclear whether PIKE-A expression/activity is dysregulated under diabetic conditions (e.g. in models for diet-induced obesity and genetic diabetes). A thorough expression analysis of hepatic PIKE-A in selected mouse models would help to clarify this issue.
b) What is the functional contribution of PIKE-A to established insulin resistance/hyperglycemia under diabetic conditions? The authors should provide data on the metabolic phenotype of liver-specific PIKE-A knockout mice under high fat diet feeding conditions, and explore the consequences of acute PIKE-A knockdown (e.g. adenoviral shRNA expression) in genetic models of obesity/diabetes (e.g. db/db mice).
Clarification of these points would substantially strengthen the case for publication. Minor points are listed below.

Specific comments
1. Figure 1B: Do these blots show representative results (n=?)
2. Figure 2: Please provide a more comprehensive characterization of metabolic parameters in LPKO mice (e.g. including hepatic and serum TG, FFA, cholesterol, serum ketone bodies). Are there signs of hepatic steatosis in KO mice?
3. Figure 3B: Please include expression data on other relevant, insulin-dependent pathways (e.g. expression levels of lipogenic genes FAS etc.).
4. Figure 4: Is the interaction between PIKE-A and the IR altered in livers of diabetic animals?

Referee #3:

I think this is a well-written and conducted study. The data presented is compelling in demonstrating that phosphoinositide 3 kinase (PIKE-A) is directly modulating the hepatic insulin receptor. This has been conducted using a tissue specific mouse model and a number of in vitro models. The animal model shows that these mice are insulin resistant in the liver and exhibit whole body glucose intolerance.
My major concern/ question from this study is in relation to the whole body knockout that this group have previously published. The whole body knockout has enhanced insulin sensitivity and is protected from diet induced obesity and insulin resistance due to enhancement of peripheral augmentation of insulin signaling in muscle and adipose tissue (AMPK driven). I think it would be nice to see if the insulin resistance of this animal could be rescued by generating liver +/- fat +/- muscle PIKE-A knockout animals. Otherwise I am happy with the standard of this paper.

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1st Revision - authors' response 28 April 2011

Thank you very much for your email on 2nd March regarding the decision on our manuscript "PIKE-A association with hepatic insulin receptor enhances its kinase activity (EMBOR-2011-34753V1)". We are pleased to know that the referees are "happy with the standard of this paper" in which our study "provides interesting new insights into the role of PIKE-A in hepatic insulin signaling" and "are well designed and structured" that "the conclusions are supported by the experimental results". We have revised our manuscripts with additional data to fully address the concerns of the reviewers. Specific amendments are as follow:

Reviewer 1:

1. The reviewer suggested performing the pyruvate tolerance test in LPKO mice.

Pyruvate tolerance test (PTT) has been performed as recommended. As shown in Fig 2G and H, LPKO mice displayed higher hepatic glucose production during the PTT, which is in line with our conclusion that glucose production is elevated in PIKE-null liver.

2. The reviewer commented that "insulin should inhibit glucose production in liver during the fed state. No relevant insulin signaling exists during fasting, therefore no differences on glycemia should be observed between +/+ and -/- mice".

We agree that insulin is a critical hormonal in controlling hepatic glucose production, especially during fed status. In the simplest model, LPKO mice, which are insulin resistant in liver, should display hyperglycemia in fed status. However, hepatic glucose production (HGP) is not controlled by insulin solely. It is a complicated system that the concentrations of nutrients like glucose, fatty acid, amino acids, etc, also play a significant role in this process (Nordlie and Foster 1999 Ann Rev Nutr 19: 379-406). Presumably, the high concentrations of these gluconeogenic substrates under fed state may provide compensations to the hepatic insulin resistance in LPKO to control HGP, resulting in normoglycemic status. When the LPKO mice are fasted, the availability of these gluconeogenic substrates is absent; the insulin resistant phenotype of LPKO mice thus becomes more prominent. In addition, the glucose uptake of muscle and fat is reduced during fasting, which further worsens the hyperglycemic status of LPKO mice under fasting.

It is noteworthy that impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) are key diagnostic definition of "prediabetes", which is partially caused by enhanced HGP (Buysschaert and Bergman 2011 Med Clin N Am 95: 289-297; Ferrannini, Gastaldelli and Lozzo 2011 2011 Med Clin N Am 95: 327-339). Moreover, our in vitro and in vivo data demonstrated that LPKO mice display hepatic insulin resistance that is caused by the diminished IR kinase activity. Therefore, our data strongly suggest the critical role of PIKE-A in IR activation to control HGP.

3. The reviewer suggested studying the AMPK signaling in the liver of LPKO mice as to demonstrate the differences of phenotypes observed between PIKE full KO and LPKO are mediated by the effects of PIKE on AMPK in WAT and muscle and not in liver.

We have examined the phosphorylation and expression of AMPK and ACC in LPKO liver. While the expression and phosphorylation of AMPK are normal, ACC expression and phosphorylation are decreased in LPKO liver (Fig 3D), which is caused by a reduced transcription of ACC as demonstrated by the RT-PCR result (Fig 3B).

4. The reviewer questioned why AKT activity is inhibited in liver but increased in WAT and muscle of the PIKE full -/- mice.

We have demonstrated that AMPK activity is increased in the muscle and fat of whole body PIKE -/- mice in our previous publication (Chan et al 2010 Diabetes 59: 883-893). Hepatic AMPK activity, however, is not changed in PIKE-null liver (Fig 3D). On the other hand, IR phosphorylation is reduced in PIKE-null liver (Fig 3A) but not in PIKE-null muscle and WAT. It is this differential AMPK and IR regulation that might explain the differences of Akt activity in various PIKE-null tissues. In PIKE -/- muscle and WAT, the high AMPK activity enhances the IRS/PI3K phosphorylation, leading to the upregulation of Akt activities. However, the AMPK activity is not

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enhanced in LPKO liver (Fig 3D) and the liver sample from whole body PIKE-/- mice (data not shown). Instead, the IR activation is diminished in PIKE-null liver, thus resulted in a reduced Akt activity in PIKE-null liver. The differential regulatory ability of PIKE on AMPK activity in different tissues remains unknown but we are now conducting an independent project to study this mechanism.

5. The reviewer suggested analyzing the FOXO1 pathway in LPKO liver.

As suggested, we have determined the expression and phosphorylation of FoxO1 in various tissues of LPKO mice after insulin stimulation. Fitting with our data that Akt activation is reduced in LPKO liver after insulin injection, FoxO1 phosphorylation, but not expression, is also diminished in LPKO liver (Fig 3A, 9th and 10th panels). On the other hand, no significant change in FoxO1 activation after insulin injection in fat and muscle of LPKO mice were observed.

6. The reviewer requested to examine the lipogenic role of insulin in LPKO liver.

We have examined the expression of insulin-regulated lipogenic enzymes fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) in LPKO liver and found that both ACC and FAS expressions were significantly decreased (Fig 3B), which provides further evidences that insulin signaling in LPKO liver is impaired. In addition, we have determined the triglyceride (TG), free fatty acid (FAA) and cholesterol content in LPKO liver (Table 1). Both TG and FAA concentrations are lower in LPKO liver, which further suggest the insulin-regulated hepatic lipogenesis is also affected when PIKE is ablated.

7. The reviewer complained that it is difficult to compare the experiments in this manuscript and the previous published paper as the age of mice used is different between the two studies.

The objectives of the two studies are different that our previous report on whole body PIKE -/- mice aims at studying the role of PIKE in obesity development. To achieve the aim, we examined the mice that have been fed with HFD for ~20 weeks. After such treatment, the age of mice will be ~8-9 months. Therefore, mice of similar ages fed with chow diet should be used as a scientifically matched control. In this manuscript, we aim at demonstrating the insulin receptor-enhancing role of PIKE in liver. As a result, the use of mice ~8-9 month is not necessary. Thus, it would be inappropriate to compare the results from two independent studies with distinct research aims that using animals of different strains (whole body PIKE -/- vs LPKO) and ages.

8. The reviewer suggested rescuing the expression of PIKE in livers of LPKO mice to unequivocally demonstrate the distinct effects of PIKE in liver and peripheral tissues.

As suggested, we have expressed PIKE-A in liver of LPKO mice using adenovirus infection. After adenovirus infection, PIKE-A expression in liver is upregulated in PIKE -/- liver (Fig 1D). These hepatic PIKE-A expressed PIKE -/- mice display ameliorated fasting hyperglycemia, hepatic glucose output and insulin responsiveness (Fig 1E to I), which strongly suggests the role of PIKE in controlling gluconeogenesis.

Reviewer 2:

1. The reviewer suggested performing a thorough expression analysis of hepatic PIKE-A in selected mouse models to clarify whether PIKE-A expression/activity is dysregulated under diabetic conditions. S/he also suggested exploring the consequences of acute PIKE-A knockdown in genetic models of obesity/diabetes. In addition, the reviewer questioned if PIKE-A/IR interaction is altered in the liver of diabetic animals.

We have examined the expression pattern of hepatic PIKE-A in obese mice caused by diet (HFD) composition or genetic mutation (ob/ob) (Chan et al. 2010 Diabetes 59: 883-893). Unfortunately, the expression of PIKE-A does not change significantly in these conditions. While we agree that it would be interesting and informative to further study the role of PIKE-A in diabetes development, the data generated from the suggested studies, however, are more suitable for an independent manuscript. Moreover, including these new data in the current manuscript would not fit into the EMBO reports' short format. Therefore, we consent to the comments of the editor that

addressing these questions is not essential for publication of the current manuscript in EMBO reports.

2. The reviewer suggested providing the metabolic phenotype of liver-specific PIKE-A knockout mice under high fat diet feeding conditions.

As suggested, we have fed the LPKO mice with HFD for 20 weeks and examined their metabolic phenotypes. As shown in Fig 4, we found that both Fl/Fl and LPKO mice developed obesity and severe diabetes after HFD feeding. Moreover, both genotypes display comparable lipid content in serum and liver. Presumably, the accumulation of fat during over-nutrition results in severe insulin resistance in muscle and fat, which overwhelms the role of liver in causing the diabetic phenotypes in LPKO mice.

3. The reviewer questioned the n number of the blot shown in Fig 1B.

The immunoblot shown in Fig 1B is the representative result of three independent experiments. We have included this information in the "Methods" section.

4. The reviewer requested to provide a more comprehensive characterization of metabolic parameters in LPKO mice and asked if there are any sign of hepatic steatosis in KO mice.

We have measured the serum ketone body, triglyceride, free fatty acid and cholesterol content in blood and liver of LPKO mice (Table 1). In all, LPKO mice have lower serum triglyceride and hepatic lipid content. Since insulin is a lipogenic hormone which triggers lipogenesis in liver, the insulin resistance found in LPKO liver thus renders the lipid synthesis. We have also shown that the insulin-regulated lipogenic enzymes FAS and ACC are down-regulated in LPKO liver (Fig 3B and D), which provides a possible mechanism to explain the low lipid content detected.

5. The reviewer requested to include the expression data on other relevant, insulin-dependent pathway (e.g. FAS).

We have determined the expression level of two insulin-dependent lipogenic enzymes FAS and ACC1 in the liver of LPKO mice using RT-PCR (Fig 3B). We found that both enzymes are down-regulated in PIKE-null sample. We further confirm this findings using immunoblotting analysis in which the amount of ACC1 in LPKO liver is reduced, which is in agreement with the RT-PCR result.

Reviewer 3:

1. The reviewer suggested studying if the insulin resistance of LPKO mice could be rescued by generating liver+/-fat+/-muscle PIKE-A knockout mice.

We accede with the idea that the availability of liver+/-fat+/-muscle PIKE knockout mice will be helpful to resolve the tissue-specific role of PIKE in regulating glucose metabolism. However, generating sufficient number of suggested mutant animals will take about a year. Therefore, we think this study should be included in another study which is independent to the current manuscript. Indeed, we are now generating the muscle-specific PIKE-A knockout mice and hopefully we can present the data in near future.

With the above amendments, I hope our manuscript is now suitable for publication in EMBO reports.

2nd Editorial Decision 10 May 2011

Thank you for the submission of your revised manuscript to our offices. We have now received the enclosed report from referee 1 who was asked to assess it. The referee still has one suggestion that I would like you to address before we can proceed with the official acceptance of your manuscript. S/he indicates that the data on AMPK activity in the full PIKE KO mice should be included in order to directly compare them to the data from the LPKO mice. The referee also feels that it should be discussed in a little more detail that the different phenotypes of the PIKE full KO and LPKO are mediated by effects of PIKE on AMPK in WAT and muscle and not in liver (if this is supported by the data to be included).

I also have to tell you that the manuscript text with more than 32,700 characters is too long and needs to be shortened. We can accept a maximum of 30.000 characters. I noticed that you have separated the Results and Discussion section now. EMBO reports has recently decided that Results and Discussion should always be combined and I would therefore like to ask you to recombine both sections. This may also help in shortening the text as it may eliminate redundancy that is inevitable when discussing the same experiments twice. I also noticed that you deleted the definition of the error bars in all of the main figure legends. Error bars need to be defined in the legends so please re-include this information. In order to further shorten the main text the "histology" section in the materials and methods could be moved to the supplementary information. Please note that the materials and methods can otherwise not be shortened any further.

I hope you agree with the suggestions of how to revise the manuscript. If you have any further questions do not hesitate to contact me.

I look forward to seeing a new revised version of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORT:

Referee #1:

The authors have addressed the comments and critics of this reviewer in an appropriate manner. Still this reviewer would like, however that the authors show, at least preliminary data concerning the differences observed between AMPK activity in the full -/-, compared to liver specific -/- mice.It merits at least a thorough discussion.

2nd Revision - authors' response 11 May 2011

Thank you very much for your email on 5/10/11 regarding further suggestions on our manuscript “PIKE-A association with hepatic insulin receptor enhances its kinase activity (EMBOR-2011-34753V2). In response to these suggestions, we have made the following amendments in the revised manuscript:

Reviewer:

1. The referee suggested including data on AMPK activity in the full PIKE KO mice.

   As suggested, the AMPK and ACC phosphorylations and expressions in whole body PIKE KO liver have been included in Fig 3D, which are put together with the AMPK and ACC data of LPKO mice for direct comparison. As shown in the data, AMPK phosphorylations are not altered in both full PIKE knockout and LPKO mice.

2. The referee suggested discussing the different phenotypes of the PIKE full KO and LPKO are mediated by the effects of PIKE on AMPK in WAT and muscle but not in liver.

   A discussion on the role of AMPK on the phenotypic differences between whole body PIKE KO and LPKO mice has been included in p8.

Editorial Office:

1. The editor suggested shortening the article length to a maximum of 30000 characters

   We have deleted some of the text, which reduces the total characters (with space) to 27778 in the revised manuscript.

2. The editor suggested combing the results and discussion.

   As suggested, the two sections have been combined.

3. The editor suggested including the definition of error bar in the figure legends

   Statements defining the error bars in each figure have been included in the figure legends.

4. The editor suggested moving the "histology" section in the materials and methods to the supplemental information.

   As suggested, the experiment procedures for histology studies have been moved to the supplemental information.

I hope our manuscript is now suitable for publication in EMBO Reports with the above revisions.

3rd Editorial Decision 16 May 2011

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

Yours sincerely,

Editor
EMBO Reports