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# Taking control over intracellular fatty acid levels is essential for the analysis of thermogenic function in cultured primary brown and brite/beige adipocytes

Yongguo Li, Tobias Fromme, Sabine Schweizer, Theresa Schoettl and Martin Klingenspor

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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 17 April 2014

Thank you very much for the submission of your research manuscript to our editorial office. We have now received the full set of reviews on your manuscript, which are copied below for your information.

You will see that all reviewers, in principle, appreciate the interest of your findings. However, they also point out several aspects of your study that they do not consider fully conclusive yet and that would need to be further strengthened to support the manuscript's claims. These concerns include, but are not limited to, showing statistical significance for all results based on at least three independent replicates (i.e. biological, not technical, replicates) (referee 1), addressing the issue with compensatory mechanisms being active in the absence of UCP1 (referee 2), addressing the issues with the use of oligomycin and palmitate-BSA (referee 1 and 2, respectively) and placing the study in a historical context (referee 1).

While I would like to invite you to submit a revised version of your manuscript, I would also like to make clear that all concerns will need to be addressed to the satisfaction of the reviewers and that acceptance of the manuscript will depend on a positive outcome of a second round of review. 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. If you feel that this period is insufficient for a successful submission of your revised manuscript I can potentially extend this period slightly. Also, the length of the revised manuscript should not exceed roughly 29,000 characters (including spaces and references). At the moment, your manuscript with more than 38,0000 characters is too long and would need to be shortened, for example by combining the results and discussion section. If you feel that the additional data requested by the reviewers would make the manuscript too long you may consider including some peripheral data in the form of Supplementary information. However, materials and methods essential for the repetition of the key experiments should be described in the main body of the text and may not be displayed as supplemental information only.

We have also started encouraging authors to submit the raw data of biochemical and/or microscopical images to our editorial office. These data will be published online as part of the supplementary information. This is voluntary at the moment, but if you agree that this would be useful for readers I would like to invite you to supply these files when submitting the revised version of your study.

We also welcome the submission of cover suggestions or motifs that might be used by our Graphics Illustrator in designing a cover.

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:

While I strongly agree with the authors that a protocol of how to run a meaningful experiment with cultured adipocytes and the Seahorse equipment is definitely required to prevent further inappropriate work being published, I would nonetheless suggest a major revision of the current manuscript to put it into a more historical context.

The first publications on oxygen consumption measurements in intact brown adipocytes following norepinephrine stimulation were from 1967/68 (see e.g. Reed and Fain, 1967 and 1968; Prusiner et al. 1968, 1968). Thus, mitochondrial respiration "within the natural setting of the cytosol in intact cells" has been studied for any decades. In these early publications, 4 % bovine serum albumin was used in the medium because already at that time problems with released fatty acids were realised. Studies were carried out using Warburg apparatus or Clark-type oxygen electrode systems. Numerous publications followed from many laboratories around the world that made use of these mature brown adipocytes, notably in the pharmacology of signal transduction and respiration.

In 2000, Matthias et al. published an article showing the marked difference in oxygen consumption between brown adipocytes from wild-type and UCP1 knock-out mice, both following NE stimulation and stimulation with oleate. These studies also showed that there was no difference in basal respiration between the two preparations i.e. UCP1 was not active without stimulation. Studies by Shabalina et al. from 2008 further extended these observations on UCP1-KO mice. In all these studies, clear differences were seen between WT and UCP1-KO animals.

In addition, investigations with primary cultures of brown and brite cells by Petrovic et al. in 2008 and 2010, respectively, showed a correlation between UCP1 expression (as induced by rosiglitazone) and NE-stimulated oxygen consumption. Thus, the problems of an absence of UCP1 dependence of stimulated respiration derive from studies performed under conditions defined by the Seahorse company, that have involved an absence of BSA.

The cells are primary cultures of brown and brite adipocytes, not primary brown/brite adipocytes, a term that gives the impression of freshly isolated mature adipocytes. This should be rectified throughout and the term "cultured.... adipocytes" should be included in the title.

There seems to be considerable inter-experiment variation yet remarkably little intra-experiment

 

variation. When the authors state that "each experiment was repeated at least 2 times with similar results", does this actually mean that the standard errors we see are only replicates from one culture? Basal OCR varies between 250 and 100 and ISO-stimulated OCR also varies notably. Does this reflect different densities of cells in different experiments? How many independent cultures have been run for each experiment?

The use of oligomycin to determine the "basal proton leak" prior to stimulation is acceptable when added during basal respiration, even though the residual respiration encompasses more than basal proton leak, since also e.g. ion transport driven by delta psi will be included. However, I am concerned that this is not a good protocol for determination of NE/ISO-stimulated respiration. The problem is that if the cells are respiring on fatty acids, then ATP is required to activate these for combustion and oligomycin will eliminate most of this. Oligomycin does cause inhibition of NE-stimulated respiration under some circumstances. I am aware that pyruvate is present and therefore my concern is perhaps exaggerated, as the cells may elect to use this substrate instead. To allay my concerns, the authors should show parallel traces with and without oligomycin prior to ISO addition in the presence of BSA.

In this context, it may also be necessary to titrate suitable concentrations of both oligomycin and particularly FCCP, since these compounds bind rather well to BSA.

It is striking that the respiration induced by ISO in the absence of BSA reaches a peak extremely slowly, supporting the conclusion that it is not a specific phenomenon. The stimulated rates in the presence of BSA peak faster, as indeed would be expected from known data in oxygen electrode studies where peaks are after ca 1 min, as in e.g. control in Fig. 8A (peak at earliest possible time point).

The OCRs should all be given as pmoles/ min and not be switched to % Basal in some figures.

I do not think the term "non-mitochondrial respiration" as the residual after antimycin A is a good term. Rather, it seems to be a non-biological background or dye phenomenon.

More appropriate and earlier references to fatty acid-induced uncoupling should also be given e.g. Wojtczak and Lehninger, 1961.

The observations with CsA are of interest, although Bernardi's recent findings that the PTP is a dimer of the mitochondrial ATP synthase make it perhaps somewhat harder to understand. The experiment should also be repeated with wild-type cells to see if the magnitude of the response is the same.

Referee #2:

In this manuscript entitled "Taking control over intracellular fatty acid levels is essential for analysis of thermogenic function in brown and brite/beige adipocytes", by using the Seahorse technology, Yogguo Li et al. provide new insights into the contribution of UCP1 and lipids to the uncoupling respiration of both brown and brite/beige adipocytes. The authors suggest that upon beta-adrenergic stimulation, FFA released from lipolysis stimulate UCP1 activation and are also responsible for an even higher unspecific uncoupling effect that would mask the specific uncoupling effect of UCP1. Indeed, they show that the use of BSA as a scavenger of the excess of FFA allows them to unravel the real contribution of UCP1 to the uncoupling respiration. The results presented in this manuscript would suggest that all the literature that so far has used the seahorse technology to determine Ucp1 dependent uncoupling (but not brown fat formation per se, see comments below) following beta-adrenergic-lipolysis stimulation should be revisited in relation to the effective real contribution of UCP1. This scenario is intriguing, however the results presented in this paper at the moment, do not fully support the conclusions of the authors. Therefore, the following points will need to be addressed.

Major comments

 

1- In figure 1 the authors present results showing that there is not difference between WT and UCP1 KO primary adipocytes in term of respiration profiles. They conclude that this is due to the fact that UCP1 is not active under basal condition. In line with this, is the finding that UCP1 KO mice do not show a metabolic phenotype. On the other hand it has been reported that BAT ablation results in a strong metabolic phenotype. This divergence might be because other mechanisms can compensate for the absence of UCP1 in UCP1 KO mice. The authors need to address this issue before concluding that the lack of differences between KO ant WT mice is due to a free fatty acid effects alone. One possible approach would be to repeat the experiment of figure 1 by using knock down approaches (e.g. siRNAs) in primary cells which would be much more short term and would make compensation less of a problem. The problem I see is that in figure 4A the authors show that in the presence of lipase inhibitors there is a basal uncoupling respiration that cannot be due to FFAs released from basal lipolysis. Thus, if we assume that UCP1 is not active, like the authors propose, some other mechanism must be responsible for this basal uncoupling effect.

2- Figure 4C clearly is a key experiment which is problematic for the ultimate conclusion drawn in this paper. By treatment with palmitate-BSA the authors claim to promote uncoupled respiration. This conclusion is not correct as palmitate-BSA promotes also fatty acids beta-oxidation with a consequent increase in oxygen consumption. Treatment with etomoxir could reveal the contribution of uncoupling respiration and FAO to oxygen consumption. There is sufficient evidence that the effect of 200 uM of palmitate-BSA in brown adipocytes can completely be reversed by 100 uM of etomxir, indicating that there is also a block of the oxidation of endogenous fatty acids. Notably the increase in OCR obtained with palmitate-BSA (figure 4C) is equal to that obtained with isoproterenol (fig 4C). This suggests that in the same way palmitate-BSA cannot uncouple the mitochondria membrane at the dose of 200 uM, the fatty acids released from lipolysis upon treatment with ISOP cannot promote unspecific uncoupling respiration.

3- Several points in the paper are massively overstated. For example the term primary brite and primary brown is misleading. The authors use ex vivo differentiated cells from subcutaneous and brown adipose tissue which are not primary brown or brite cells. To make such a statement the authors would need to redo the experiments with primary mature adipocytes isolated from different depots. Alternatively the title and the manuscript have to be changed accordingly. Also the statement in the abstract: "Our study provides the essential guidelines to assess the bioenergetic and thermogenic capabilities of brown and brite/beige adipocytes in cell cultures." and the statement in the discussion: "demonstrate the pitfalls that have repeatedly led to misinterpretation of data in a number of past studies [26-34]". One has to be careful with these kind of statements because of the two different aspects that are studied in brown adipose tissue biology. On the one hand people study adipose tissue browning and the concomitant increase in mitochondria and FA flux. For such studies it does not matter where the uncoupling effect comes from and the conclusions drawn from such studies is perfectly valid since whole BA uncoupling and not only the Ucp1 effect is considered. The paper presented here is relevant for the people studying the specific effect of Ucp1 to BA uncoupling which according to this paper are negligible. In my opinion this is actually the most important finding of the paper as it suggests that BAs do not really need Ucp1 to uncouple but that they can do it by increasing FA flux. The problem I see is whether this holds true for BAs in vivo as well and whether we just see this unspecific effect due to the strong induction of lipolysis by Isop. in cell culture. These aspects need to be taken into consideration in the discussion

Minor comments:

1- In the material and methods section there is no reference to the palmitate-BSA experiments. Also, it is not clear to me how the authors determined the BSA concentration. Do they reflect circulating albumin levels.
2- In figure 8 the authors claim that UCP1 activation is largely dependent on ATGL rather than on HSL. The authors do not discuss this unexpected finding, as HSL is the lipase reported from the literature to be activated upon beta-adrenergic stimulation. One possibility would be that free fatty acids release by ATGL are better activators of UCP1 than those released by HSL due to transport associated mechanisms. The pertinent literature for that effect should be included.

 

Referee #3:

According to the introductory review of the literature, apparently little progress been made to understand the role of fatty acids in the regulation of brown and brite versus white adipocytes thermogenesis for many years The present work sets out to resolve some of the issues.

By comparing wt brown (BAT) and brite adipocytes with UCP1 Ko cells, the authors perform a systematic study of the influence of the source of fatty acids on the respiratory activity of the adipocytes , whether they are externally added or originate from general lipolysis or from hormone induced lipolysis. Contrary to conventional wisdom and evidence , here it is shown , that under "normal" conditions FA induced uncoupling is independent of UCP1 in BAT cells. Further, most of the FA effective in uncoupling originate from general and not from hormone induced lipolysis, as shown by different lipase inhibitors. By employing cyclosporine A it is claimed that Fa induce transition pore opening rather than activate UCP1 in the adipocyte uncoupling. Only when FA are scavenged by BSA, the uncoupling becomes UCP1 dependent.

The difference to the previous work on the uncoupling in adipocytes resides in the more systematic controls, using ko mice and and inhibitors against lipases.

The low FA concentrations obtained by BSA addition are assumed to mimic the in vivo conditions. Also under these conditions the major source of uncoupling FA comes from general and not from hormone induced lipolysis. This again is against the textbook concept of hormonal control of BAT thermogenesis.

It seems paradox that only at low FA concentration UCP1 can be activated in BAT cells. This would contradict the view that FA activate UCP1 by competitively removing nucleotides. The authors do not discuss this crucial question. At any rate this point is unresolved similar as the contradictory evidence reported for over 30 years, using isolated BAT mitochondria , on how UCP1 is regulated by FA and nucleotides.

The present work is sound and well performed but experimentally quite simple, relying only on the measurements of respiration. Therefore the work stays on a more overall metabolic level . All the figures contain recordings of respiration abundantly illustrating the results. The column in the figures repeat the recordings and should be omitted. In some cases only the columns but not the recordings might be presented.

The introduction might be condensed and more focused on the issue of FA mediated thermogenesis.

Although the issue addressed is important, it may be on the borderline of significant general interest to be published as an EMBO report. It may be better suited to a journal more specialized to metabolic issues.

1st Revision - authors' response 12 July 2014

Thanks a lot for your decision letter. We very much appreciate the timely processing as well as the positive feedback and the valuable comments contributed by the three reviewers. We have carefully revised our manuscript according to the suggestions of reviewers. We hereby submit our revised version of MS and our point-to-point response to reviewer’s comments to you.

You will see that all reviewers, in principle, appreciate the interest of your findings. However, they also point out several aspects of your study that they do not consider fully conclusive yet and that would need to be further strengthened to support the manuscript's claims. These concerns include, but are not limited to, showing statistical significance for all results based on at least three independent replicates (i.e. biological, not technical, replicates) (referee 1), addressing the issue with compensatory mechanisms being active in the absence of UCP1 (referee 2), addressing the issues

 

with the use of oligomycin and palmitate-BSA (referee 1 and 2, respectively) and placing the study in a historical context (referee 1).

We have conducted additional experiments and now present data from at least three independent biological replicates, and addressed all the other issues raised by the reviewers. We also have strongly condensed our manuscript to roughly 29,000 characters. You find our detailed responses to the individual points of criticism as follows (highlighted in blue).

Referee #1:

While I strongly agree with the authors that a protocol of how to run a meaningful experiment with cultured adipocytes and the Seahorse equipment is definitely required to prevent further inappropriate work being published, I would nonetheless suggest a major revision of the current manuscript to put it into a more historical context.

We thank reviewer #1 for the positive assessment of our study and greatly appreciate the helpful comments.

**Historical perspective**
The first publications on oxygen consumption measurements in intact brown adipocytes following norepinephrine stimulation were from 1967/68 (see e.g. Reed and Fain, 1967 and 1968; Prusiner et al. 1968, 1968). Thus, mitochondrial respiration "within the natural setting of the cytosol in intact cells" has been studied for any decades. In these early publications, 4 % bovine serum albumin was used in the medium because already at that time problems with released fatty acids were realized. Studies were carried out using Warburg apparatus or Clark-type oxygen electrode systems. Numerous publications followed from many laboratories around the world that made use of these mature brown adipocytes, notably in the pharmacology of signal transduction and respiration.

In 2000, Matthias et al. published an article showing the marked difference in oxygen consumption between brown adipocytes from wild-type and UCP1 knock-out mice, both following NE stimulation and stimulation with oleate. These studies also showed that there was no difference in basal respiration between the two preparations i.e. UCP1 was not active without stimulation. Studies by Shabalina et al. from 2008 further extended these observations on UCP1-KO mice. In all these studies, clear differences were seen between WT and UCP1-KO animals.

In addition, investigations with primary cultures of brown and brite cells by Petrovic et al. in 2008 and 2010, respectively, showed a correlation between UCP1 expression (as induced by rosiglitazone) and NE-stimulated oxygen consumption. Thus, the problems of an absence of UCP1 dependence of stimulated respiration derive from studies performed under conditions defined by the Seahorse company, that have involved an absence of BSA.

We thank the reviewer for this detailed historical perspective. We have now placed our study in this historical context and hopefully acknowledge the pioneers in an appropriate manner. Due to space limitations we have extended the contributions of earlier studies in our supplemental text.

The cells are primary cultures of brown and brite adipocytes, not primary brown/brite adipocytes, a term that gives the impression of freshly isolated mature adipocytes. This should be rectified throughout and the term "cultured.... adipocytes" should be included in the title.

We are grateful to the reviewer for this suggestion. We have modified our terminology from primary brown/brite adipocytes to cultured primary brown/brite adipocytes.

There seems to be considerable inter-experiment variation yet remarkably little intra-experiment variation. When the authors state that "each experiment was repeated at least 2 times with similar results", does this actually mean that the standard errors we see are only replicates from one culture? Basal OCR varies between 250 and 100 and ISO-stimulated OCR also varies notably. Does this reflect different densities of cells in different experiments? How many independent cultures have been run for each experiment?

 

For primary cultures, there is indeed considerable inter-experiment variation. The main cause of this variation is the actual number of differentiated BA obtained per well in each independent experiment, which can be influenced by the viability of precursors isolated from mice and their differentiation efficiency. This variability is the rationale behind presenting the data as % basal respiration in some cases. However, some graphs in our original manuscript were mislabeled. We apologize for the lack of clarity. Basal OCR with 250 is the raw OCR, while OCR with 100 is that has been normalized (basal OCR is set to 100).

In our revised MS, we show raw data traces from one representative experiment and the bar chart summarize the normalized data from 3 independent experiments. For the raw traces mean values were calculated from 8-12 wells (N=1, n=8-12) on the same plate (technical replicates) and the standard deviation of these replicates is indicated by the error bars. For the bar charts the respiration rates in each experiment were expressed as a percentage of the initial respiration. The bars present such normalized data from 3 independent experiments with mean values from 3 biological replicates and the standard deviation of these replicates (N=3).

The use of oligomycin to determine the "basal proton leak" prior to stimulation is acceptable when added during basal respiration, even though the residual respiration encompasses more than basal proton leak, since also e.g. ion transport driven by delta psi will be included. However, I am concerned that this is not a good protocol for determination of NE/ISO-stimulated respiration. The problem is that if the cells are respiring on fatty acids, then ATP is required to activate these for combustion and oligomycin will eliminate most of this. Oligomycin does cause inhibition of NE-stimulated respiration under some circumstances. I am aware that pyruvate is present and therefore my concern is perhaps exaggerated, as the cells may elect to use this substrate instead. To allay my concerns, the authors should show parallel traces with and without oligomycin prior to ISO addition in the presence of BSA.

We thank the reviewer for this comment. We followed the reviewer's suggestion and performed additional experiments with and without oligomycin prior to ISO addition in the presence of 2% BSA. Comparable levels of uncoupled respiration were observed in both conditions (Figure below).

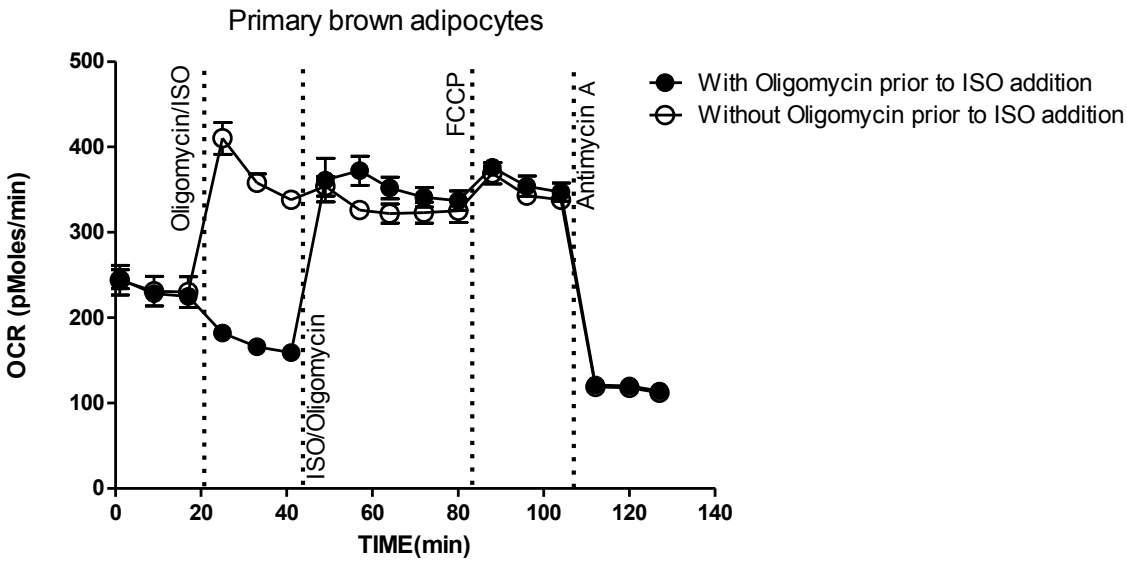


Figure legend: Time course of oxygen consumption rates (OCR) of primary brown adipocytes treated with and without oligomycin prior to isoproterenol (ISO) addition.

In this context, it may also be necessary to titrate suitable concentrations of both oligomycin and particularly FCCP, since these compounds bind rather well to BSA.

 

We are grateful to the reviewer for this comment. As judged from our BSA titration experiments (Supplemental Figure 2), no obvious BSA effect on oligomycin and FCCP induced respiration change was observed, indicating the concentrations we used are suitable.

It is striking that the respiration induced by ISO in the absence of BSA reaches a peak extremely slowly, supporting the conclusion that it is not a specific phenomenon. The stimulated rates in the presence of BSA peak faster, as indeed would be expected from known data in oxygen electrode studies where peaks are after ca 1 min, as in e.g. control in Fig. 8A (peak at earliest possible time point).

We thank the reviewer for supportive this comment, which indicates our conclusion is solid.

The OCRs should all be given as pmoles/ min and not be switched to % Basal in some figures.

Thanks for this advice. However, due to considerable inter-experiment variation in the overall level of respiration (see above response), we prefer to show raw data traces from one representative experiment and the bar chart to summarize the normalized data from 3 independent experiments.

I do not think the term "non-mitochondrial respiration" as the residual after antimycin A is a good term. Rather, it seems to be a non-biological background or dye phenomenon.

Thanks for this valuable comment. We had adapted the terminology from other publications without realizing the problem. The term “non-mitochondrial respiration” implies that OXPHOS may occur in cellular compartments other than mitochondria. To avoid misconception we now use the term “non-mitochondrial oxygen consumption”.

More appropriate and earlier references to fatty acid-induced uncoupling should also be given e.g. Wojtczak and Lehninger, 1961.

We have cited this paper in our revised MS.

The observations with CsA are of interest, although Bernardi's recent findings that the PTP is a dimer of the mitochondrial ATP synthase make it perhaps somewhat harder to understand. The experiment should also be repeated with wild-type cells to see if the magnitude of the response is the same.

We are grateful to the reviewer for this comment. As we mentioned Yehuda-Shnaidman E et al. (2010) also reported that PTP opening was responsible for ISO stimulated uncoupled respiration in white adipocytes. These two lines of evidence suggest that PTP involved in ISO stimulated uncoupled respiration is a common phenomenon among adipocytes.

We have repeated the experiment with wild-type cells and found that the magnitude of the response is not the same between genotypes. The inhibition of CSA on ISO-induced respiration is more pronounced in UCP1 KO cells.

 

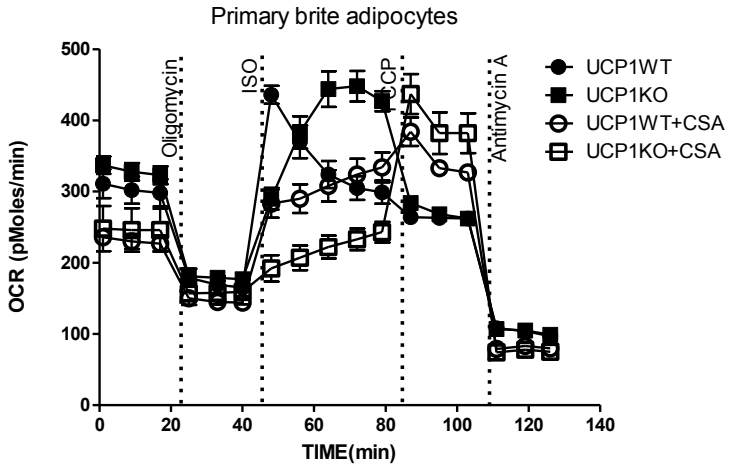


Figure legend: Time course of oxygen consumption rate (OCR) of primary brite adipocytes from UCP1 wildtype (WT) and knockout (KO) mice. Adipocytes were pretreated with the PTP inhibitor cyclosporine A (CSA) (5µg/ml, 72h) or vehicle prior to bioenergetic profiling. The inhibitory effect of CSA on ISO-induced respiration is more pronounced in UCP1 KO cells.

**Referee #2:**

In this manuscript entitled "Taking control over intracellular fatty acid levels is essential for analysis of thermogenic function in brown and brite/beige adipocytes", by using the Seahorse technology, Yongguo Li et al. provide new insights into the contribution of UCP1 and lipids to the uncoupling respiration of both brown and brite/beige adipocytes. The authors suggest that upon beta-adrenergic stimulation, FFA released from lipolysis stimulate UCP1 activation and are also responsible for an even higher unspecific uncoupling effect that would mask the specific uncoupling effect of UCP1. Indeed, they show that the use of BSA as a scavenger of the excess of FFA allows them to unravel the real contribution of UCP1 to the uncoupling respiration. The results presented in this manuscript would suggest that all the literature that so far has used the seahorse technology to determine Ucp1 dependent uncoupling (but not brown fat formation per se, see comments below) following beta-adrenergic-lipolysis stimulation should be revisited in relation to the effective real contribution of UCP1. This scenario is intriguing, however the results presented in this paper at the moment, do not fully support the conclusions of the authors. Therefore, the following points will need to be addressed.

Major comments

1- In figure 1 the authors present results showing that there is not difference between WT and UCP1 KO primary adipocytes in term of respiration profiles. They conclude that this is due to the fact that UCP1 is not active under basal condition. In line with this, is the finding that UCP1 KO mice do not show a metabolic phenotype. On the other hand it has been reported that BAT ablation results in a strong metabolic phenotype. This divergence might be because other mechanisms can compensate for the absence of UCP1 in UCP1 KO mice. The authors need to address this issue before concluding that the lack of differences between KO ant WT mice is due to a free fatty acid effects alone. One possible approach would be to repeat the experiment of figure 1 by using knock down approaches (e.g. siRNAs) in primary cells which would be much more short term and would make compensation less of a problem.

Our data were obtained on primary cultured brown and brite adipocytes differentiated from progenitor cells in the stromal vascular fraction. Such progenitor cells do not express Ucp1. Ucp1 expression in primary cultured brown and brite adipocytes occurs only after induction of differentiation. Brown and brite adipogenesis in cell culture occurs within days so that we think that compensation is less of a problem. As the UCP1 protein is rather stable a siRNA based knockdown would also require several days to ablate the protein.

 

The problem I see is that in figure 4A the authors show that in the presence of lipase inhibitors there is a basal uncoupling respiration that cannot be due to FFAs released from basal lipolysis. Thus, if we assume that UCP1 is not active, like the authors propose, some other mechanism must be responsible for this basal uncoupling effect.

We thank the reviewer for this comment. It should be noted that the inhibition of lipolysis by ATGL and HSL inhibitor is only 95% as reported by Zechner et al. The residue of lipolysis may responsible for this minor uncoupled respiration induced by ISO treatment.

2- Figure 4C clearly is a key experiment which is problematic for the ultimate conclusion drawn in this paper. By treatment with palmitate-BSA the authors claim to promote uncoupled respiration. This conclusion is not correct as palmitate-BSA promotes also fatty acids beta-oxidation with a consequent increase in oxygen consumption. Treatment with etomoxir could reveal the contribution of uncoupling respiration and FAO to oxygen consumption. There is sufficient evidence that the effect of 200 uM of palmitate-BSA in brown adipocytes can completely be reversed by 100 uM of etomxir, indicating that there is also a block of the oxidation of endogenous fatty acids. Notably the increase in OCR obtained with palmitate-BSA (figure 4C) is equal to that obtained with isoproterenol (fig 4C). This suggests that in the same way palmitate-BSA cannot uncouple the mitochondria membrane at the dose of 200 uM, the fatty acids released from lipolysis upon treatment with ISOP cannot promote unspecific uncoupling respiration.

According to the respiratory control theory of mitochondria, when substrates are supplied, the rate of oxygen consumption depends mainly on the supply of ADP+Pi. Of note, in our experimental setup, we first treat the cells with oligomycin which inhibit the ATP synthase and certainly will block the coupled respiration. In this context, the increased oxygen consumption will be only contributed by uncoupling.

To further allay the reviewer's concern, we followed the reviewer's suggestion to determine whether etomoxir, which inhibits the entry of fatty acids into the mitochondria by blocking the activity of carnitine palmitoyl transferase 1 (CPT1) and thus blocks β-oxidation, can block the increased respiration by palmitate-BSA treatment in UCP1 KO cultured primary brown adipocytes. We found comparable levels of oxygen consumption driven by palmitate-BSA treatment between control and etomoxir treated group (see below), demonstrating that the increased oxygen consumption by palmitate-BSA treatment was not mediated by beta-oxidation but by uncoupling. Of note, under this condition, the FCCP has no further uncoupling effect on the cells, further indicating the cells were under uncoupled state. Furthermore, basal respiration was decreased by etomoxir.

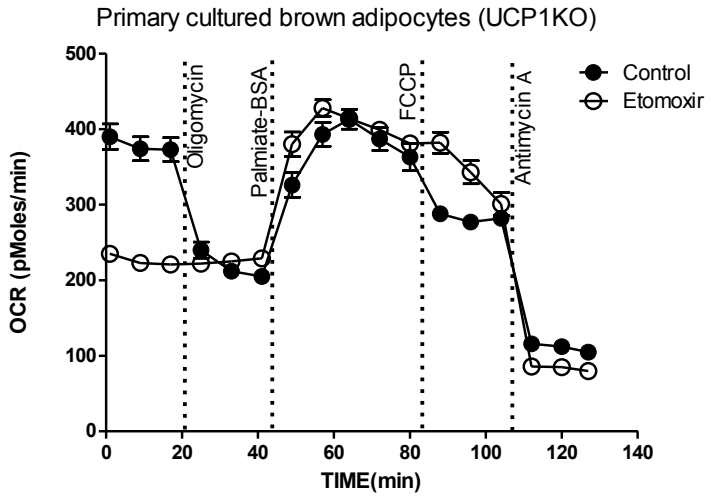


Figure legend: Time course of oxygen consumption rate (OCR) of primary brown adipocytes from UCP1 knockout (KO) mice. Adipocytes were pretreated with Etomoxir (100μM, 1h) or vehicle prior to bioenergetic profiling. Comparable levels of oxygen consumption driven by palmitate-BSA treatment were observed between control and Etomoxir treated group, demonstrating that the increased oxygen consumption by palmitate-BSA treatment was not mediated by beta-oxidation but by uncoupling.

 

3- Several points in the paper are massively overstated. For example the term primary brite and primary brown is misleading. The authors use ex vivo differentiated cells from subcutaneous and brown adipose tissue which are not primary brown or brite cells. To make such a statement the authors would need to redo the experiments with primary mature adipocytes isolated from different depots. Alternatively the title and the manuscript have to be changed accordingly.

We thank the reviewer for this comment. We have modified our terminology from primary brown/brite adipocytes to cultured primary brown/brite adipocytes.

Also the statement in the abstract: "Our study provides the essential guidelines to assess the bioenergetic and thermogenic capabilities of brown and brite/beige adipocytes in cell cultures." and the statement in the discussion: "demonstrate the pitfalls that have repeatedly led to misinterpretation of data in a number of past studies [26-34]". One has to be careful with these kind of statements because of the two different aspects that are studied in brown adipose tissue biology. On the one hand people study adipose tissue browning and the concomitant increase in mitochondria and FA flux. For such studies it does not matter where the uncoupling effect comes from and the conclusions drawn from such studies is perfectly valid since whole BA uncoupling and not only the Ucp1 effect is considered. The paper presented here is relevant for the people studying the specific effect of Ucp1 to BA uncoupling which according to this paper are negligible. In my opinion this is actually the most important finding of the paper as it suggests that BAs do not really need Ucp1 to uncouple but that they can do it by increasing FA flux. The problem I see is whether this holds true for BAs in vivo as well and whether we just see this unspecific effect due to the strong induction of lipolysis by Isop. in cell culture. These aspects need to be taken into consideration in the discussion.

We are grateful for this advice. As far as we understand, UCP1 expression and function analysis are the hallmarks of adipose tissue browning. Only showing increment of mitochondria genesis and maximal uncouple respiration (FCCP induced maximal respiration) does not really mean 'browning' occur.

Regarding the issue whether brown adipocytes really need UCP1 to do uncoupling, our study suggests that in the situation of controlling intracellular level of FFA with 2% BSA, which mimic the in vivo situation (4%BSA is the level of albumin approximately found in the blood), UCP1 is essential. This finding also questions the speculation that brown adipocytes do not really need UCP1 to perform uncoupling in vivo. Actually, we have noticed that some fatty acid binding proteins such as FABP3 are not expressed in cultured brown and brite adipocytes, though highly expressed in vivo. Whether lack of some fatty acid binding proteins makes the cultured adipocytes less efficient in controlling the intracellular level of FFA is under investigation and beyond the scope of the current MS.

Minor comments:

1- In the material and methods section there is no reference to the palmitate-BSA experiments. Also, it is not clear to me how the authors determined the BSA concentration. Do they reflect circulating albumin levels.

We have described the palmitate-BSA experiments in the method section of our revised MS.

Based on the literature 4%BSA is the level of albumin approximately found in the blood.

2- In figure 8 the authors claim that UCP1 activation is largely dependent on ATGL rather than on HSL. The authors do not discuss this unexpected finding, as HSL is the lipase reported from the literature to be activated upon beta-adrenergic stimulation. One possibility would be that free fatty acids release by ATGL are better activators of UCP1 than those released by HSL due to transport associated mechanisms. The pertinent literature for that effect should be included.

We thank the reviewer for pointing out this unexpected finding. Actually our data are consistent with the *in vivo* data showing that HSL knockout mice exhibit normal thermogenesis and are not cold sensitive; while ATGL knockout mice are extremely cold sensitive and die after cold exposure of more than 6 h (See review Zechner et al., 2009.JLR. 50, 3-21). However the underling

 

mechanism is not clear. Our speculation is that the activity of HSL may depend on ATGL based on the facts that ATGL catalyzes the first step of lipolysis, while HSL catalyzes the second step.

**Referee #3:**

According to the introductory review of the literature, apparently little progress been made to understand the role of fatty acids in the regulation of brown and brite versus white adipocytes thermogenesis for many years The present work sets out to resolve some of the issues.

By comparing wt brown (BAT) and brite adipocytes with UCP1 Ko cells, the authors perform a systematic study of the influence of the source of fatty acids on the respiratory activity of the adipocytes , whether they are externally added or originate from general lipolysis or from hormone induced lipolysis. Contrary to conventional wisdom and evidence , here it is shown , that under "normal" conditions FA induced uncoupling is independent of UCP1 in BAT cells. Further, most of the FA effective in uncoupling originate from general and not from hormone induced lipolysis, as shown by different lipase inhibitors. By employing cyclosporine A it is claimed that Fa induce transition pore opening rather than activate UCP1 in the adipocyte uncoupling. Only when FA are scavenged by BSA, the uncoupling becomes UCP1 dependent.

The difference to the previous work on the uncoupling in adipocytes resides in the more systematic controls, using ko mice and and inhibitors against lipases.

The low FA concentrations obtained by BSA addition are assumed to mimic the in vivo conditions. Also under these conditions the major source of uncoupling FA comes from general and not from hormone induced lipolysis. This again is against the textbook concept of hormonal control of BAT thermogenesis.

We thank the reviewer for pointing out that our data are contradictory to the current concept that BAT activation is largely dependent on hormone sensitive lipase (HSL), which actually highlights the importance of our work. By using specific lipase inhibitors we demonstrate UCP1 activation is largely dependent on adipose triglyceride lipase (ATGL) rather than HSL. In reference to our response to reviewer #2, we consider our data to be consistent with the *in vivo* data but the underlying mechanisms are unclear. However, at no time we wanted to create the impression that 'ATGL mediated UCP1 activation' is against the concept of 'hormonal control of BAT thermogenesis'. ATGL is also a target downstream of β-adrenergic hormones (See *Lass A., Zimmermann R., Oberer M., Zechner R. (2011) Prog. Lipid Res. 50, 14–27*).

It seems paradox that only at low FA concentration UCP1 can be activated in BAT cells. This would contradict the view that FA activate UCP1 by competitively removing nucleotides. The authors do not discuss this crucial question. At any rate this point is unresolved similar as the contradictory evidence reported for over 30 years, using isolated BAT mitochondria , on how UCP1 is regulated by FA and nucleotides.

UCP1 activity is extremely sensitive to fatty acids. It has been demonstrated by David G. NICHOLLS and colleagues that as little as 0.2 nM unbound free fatty acid in equilibrium with BAT mitochondria causes a substantial conductance increase (Locke et al, 1982).

*Locke RM, Rial E, Scott ID & Nicholls DG (1982) Eur. J. Biochem.129, 373-380.*

*Locke RM, Rial E & Nicholls DG (1982) Eur. J. Biochem. 1299 381-387.*

Regarding how UCP1 is regulated by FA and nucleotides is still under debate. Actually, Rial and Nicholls (1989) suggests that UCP1 is transformed by free fatty acids and by GDP into two different states and this could result in the apparent competition observed.

*Rial E and Nicholls DG. On the mechanism of transport by the uncoupling protein from brown adipose tissue mitochondria. In: Anion Carriers of Mitochondrial Membranes, edited by A Azzi, KA Nalecz, MJ Nalecz, and L Wojtczak. Berlin: Springer-Verlag, 1989, p. 261–268.*

Based on those two points, our results are actually consistent with these early studies.

 

The present work is sound and well performed but experimentally quite simple, relying only on the measurements of respiration. Therefore the work stays on a more overall metabolic level . All the figures contain recordings of respiration abundantly illustrating the results. The column in the figures repeat the recordings and should be omitted. In some cases only the columns but not the recordings might be presented.

We thank the reviewer for pointing out that our study is sound and well performed. We show raw data traces from a representative experiment and the bar chart summarize the normalized data from 3 independent experiments.

The introduction might be condensed and more focused on the issue of FA mediated thermogenesis.

Due to space limitations the revised manuscript has been strongly condensed to communicate and discuss the main findings.

2nd Editorial Decision 23 July 2014

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.

 