THE EMBO JOURNAL

Exercise-induced α-ketoglutaric acid stimulates muscle hypertrophy and fat loss through OXGR1-dependent adrenal activation

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Editor: Daniel Klimmeck

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 4th Nov 2019

Thank you for the submission of your manuscript (EMBOJ-2019-103304) to The EMBO Journal. Your manuscript has been sent to three reviewers, however, please note that one referee was not able to send us his/her comments as to unexpected additional obligations. We did receive reports from the other two reviewers, which I enclose below.

As you will see, the reviewers acknowledge the interest and novelty of your results, although they also express a number of issues that will have to be conclusively addressed before they can be supportive of publication of your manuscript in The EMBO Journal. Given the referees' recommendations, I would like to invite you to submit a revised version of the manuscript, addressing their criticism.

REFEREE REPORTS:

Referee #1:

The paper describes the role of AKG as a secreted myometabolite on systemic metabolism. The authors show that AKG via the adrenal leads to increased circulating E levels and induces brown fat function and lipolysis. In addition, they identify Oxgr1 as the receptor which mediates this effect. The paper contains a fast amount of in-depth data which is presented in a very systematic manner. I have listed a few minor points which should be addressed in a revised version.
1. Fig. 1 Please present lean and fat mass independently.
2. The human data looks very interesting, please use a multivariate analysis to assess the contribution of AKG to the different risk factors

3. Please show 3E ang G using a scale starting from 0
4. 3N-O and all other quantifications of BAT function, please include a Wblot of Ucp1
5. Change figure 3 to fit with the text, in its current form it is cited out of order
6. Increased circulating E levels should lead to enhanced britening in the sc AT. This should be addressed and discussed especially since it can be expected that brite cells are formed by interconversion.

Referee #3:

The manuscript by the Shu group proposes that α-ketoglutarate (AKG) is a metabolite whose levels increase in the bloodstream after resistance exercise and exert a beneficial effect on lean mass after high fat diet (HFD). They show that the effects of AKG are due to the stimulation of OXGR1 receptors and epinephrine release from the adrenal gland. The study contains an impressive list of experimental approaches spanning from the physiology of muscle exercise, the metabolomics analysis, the whole body energy homeostasis and the inter-organ cross-talk of metabolites and hormones as validated by novel OXGR1 mouse mutants. The study is therefore complete as it is, with 8 multi-panel main figures and 8 extended views.
Minor points:
1) The authors report that AKG derivatives also change after resistance training (Fig. 1F). It would be interesting to see how these metabolites vary after AKG administration.
2) It would be clearer to have a direct comparison of AKG levels in WT and KO mice (Fig. 7H)
3) In the discussion, the authors could mention that the data on the NF-kB pathway (Fig. 8) awaits in vivo validation in mice.

1st Revision - authors' response 23rd Dec 2019

The authors greatly appreciate the critiques and constructive suggestions that helped to improve this manuscript submitted to EMBO J. In the initial review, this manuscript was praised for “a fast amount of in-depth data”, “presented in a very systematic manner”, and “an impressive list of experimental approaches”. All concerns have been addressed as detailed below point-to-point. Major revisions have been labelled with red lines by the left side margin in the revised manuscript.

Referee #1:

The paper describes the role of AKG as a secreted myometabolite on systemic metabolism. The authors show that AKG via the adrenal leads to increased circulating E levels and induces brown fat function and lipolysis. In addition, they identify Oxgr1 as the receptor which mediates this effect. The paper contains a fast amount of in-depth data which is presented in a very systematic manner. I have listed a few minor points which should be addressed in a revised version.

1. Fig. 1 Please present lean and fat mass independently.

This point is well taken. Fig. 1B has been re-organized. Lean and fat mass has been independently presented.

2. The human data looks very interesting, please use a multivariate analysis to assess the contribution of AKG to the different risk factors

We highly appreciate this point. The contribution of AKG to the different metabolic risk factors, including BMI, HCF, WCF, Fat, Weight, NCF, VF, Systolic, Diastolic, Height and Age has been assessed by a multivariate analysis. The results have been summarized in Fig. EV1H.

3. Please show 3E and G using a scale starting from 0

We appreciate the point. The scale bar has revised to start from 0 in Fig. 3C and E (re-organized from Fig. 3E and G).

4. 3N-O and all other quantifications of BAT function, please include a Wblot of Ucp1

This is an excellent point. Western quantification of UCP1 protein levels have been added in Fig. 3G. Other mouse models involving quantifications of BAT function have been listed as follows:

1. **Acute AKG effects on BAT functions in WT mice: Fig. 4B-C (BAT temperature); Fig. EV4D (Wblot of UCP1).**
2. **Long-term AKG water supplementation effects on BAT functions in adrenalectomized WT mice: Fig. 4O-P (BAT temperature); Fig. 4Q and 4T-U (RT-PCR and Wblot of UCP1).**
3. **Long-term AKG water supplementation effects on BAT functions in OXGR1KO mice: Fig. EV7B (Wblot of UCP1).**
4. **Long-term AKG water supplementation effects on BAT functions in OXGR1RE$_{AG}$ mice: Fig. EV7I (Wblot of UCP1).**
5. **Long-term AKG water supplementation effects on BAT functions in OXGR1OE$_{AG}$ mice: Fig. EV8J (Wblot of UCP1).**
6. **Resistance exercise effects on BAT functions in OXGR1KO mice: Fig. 7J-K (RT-PCR and Wblot of UCP1).**

5. Change figure 3 to fit with the text, in its current form it is cited out of order

We highly appreciate this point. We have revised Figure 3 to fit with the described text.

6. Increased circulating E levels should lead to enhanced britening in the sc AT. This should be addressed and discussed especially since it can be expected that brite cells are formed by interconversion.

This is an excellent point. The effects of AKG water supplementation on cold-induced britening in the sc AT has been tested. The results have been summarized in Fig. EV3E-I. We found that AKG increased brite adipocyte formation at room temperature and enhanced cold-formed brite adipocytes that expressed UCP1 in the iWAT. The following discussion has been added.

"While activation of the BAT thermogenesis has clinically significant effects, BAT is found in negligible volumes in adult humans (Yoneshiro, Aita et al., 2013). Indeed, cold exposure does not induce measurable metabolic response of small BAT depots in humans (Chondronikola, Volpi et al., 2014). Instead, data from rodents and humans (Betz & Enerback, 2015, Wu, Bostrom et al., 2012) suggest BAT-like white adipose tissue (beige or brite) as an intriguing and potentially more physiologically significant target for human metabolic syndrome. Indeed, human pre-adipocytes can be differentiated into brite adipocytes, which resemble classical brown adipocytes, and respond to cold adaptation or other stimuli (Betz & Enerback, 2015, Giralt & Villarroya, 2013, Rosenwald & Wolfrum, 2014). Interestingly, increased circulating E levels have been shown to enhance britening in the subcutaneous white adipose tissue in both mice and humans (Cannon & Nedergaard, 2004, Kajimura, Spiegelman et al., 2015, Sidossis, Porter et al., 2015). Consistently, we found that water supplementation of AKG

significantly increased brite adipocytes that expressed UCP1 and other brite and thermogenic genes in the iWAT. These data suggest that britening, in rodents, may have therapeutic relevance, which can be enhanced by AKG supplementation. ”

Referee #3:

The manuscript by the Shu group proposes that α-ketoglutarate (AKG) is a metabolite whose levels increase in the bloodstream after resistance exercise and exert a beneficial effect on lean mass after high fat diet (HFD). They show that the effects of AKG are due to the stimulation of OXGR1 receptors and epinephrine release from the adrenal gland. The study contains an impressive list of experimental approaches spanning from the physiology of muscle exercise, the metabolomics analysis, the whole body energy homeostasis and the inter-organ cross-talk of metabolites and hormones as validated by novel OXGR1 mouse mutants. The study is therefore complete as it is, with 8 multi-panel main figures and 8 extended views.

Minor points:
1) The authors report that AKG derivatives also change after resistance training (Fig. 1F). It would be interesting to see how these metabolites vary after AKG administration.

We appreciate this constructive suggestion. We examined the levels of AKG derivatives (SUA: Succinic acid; FUMA: Fumaric acid; OAA: oxaloacetic acid; α-keval: Alpha-ketoisovaleric acid; 2H3MA: 2-Hydroxy-3-methylbutyric acid; α-kehex: α-ketoleucine; Pyr: Pyruvic acid) in the circulating blood 3 hrs after acute i.p. injection of AKG (10 mg/kg). We found that AKG increased the level of α-kehex and decreased the level of FUMA. The results have been summarized in Fig EV4M-S.

2) It would be clearer to have a direct comparison of AKG levels in WT and KO mice (Fig. 7H)

This is an excellent point. We tested serum AKG levels of male WT and OXGR1KO mice before or after 40 min resistance exercise. The results have been summarized in Fig. 7H. We found that the stimulatory effect of resistance exercise on serum AKG was attenuated by OXGR1KO. We speculated that this attenuation is attributed to the reduction of total muscle mass induced by OXGR1KO when mice were fed on HFD.

3) In the discussion, the authors could mention that the data on the NF-kB pathway (Fig. 8) awaits in vivo validation in mice.

Revise：We highly appreciate this point. The following discussion has been added.

“As the first step to explore the intracellular mechanism of AKG-induced E release, we performed transcriptome analysis on adrenal chromaffin cells before or after AKG treatment. Interestingly, inflammatory signaling pathways were identified as the transcriptome signature induced by AKG treatment. We further provided in vitro evidence to support a model in which AKG acts through OXGR1 to induce NF-κB signal transduction cascade, and by doing so, it stimulates E release from chromaffin cells. Consistent with this model, pro-inflammatory cytokines, tumor necrosis factor α, and interleukin 1β/α have been shown to modulate catecholamine secretion and long-term gene regulation in chromaffin cells of adrenal medulla (Jenkins, Sreenivasan et al., 2016, Rosmaninho-Salgado, Araujo et al., 2009, Tamura, Nemoto et al., 2014). The activation of a cytokine-specific intracellular signaling pathway and following E2 release in adrenal medulla may be one part of a systemic stress response induced by exercise. Chromaffin cells may act as an integrative center responding to both immune and endocrine stimuli induced by exercise. Our lab is currently validating this model and exploring if p65/NF-κB inflammatory pathway is required for the stimulatory effects of AKG on E release of adrenal chromaffin cell in vivo.”

2nd Editorial Decision 22nd Jan 2020

Thank you for submitting your revised manuscript for consideration by The EMBO Journal. Your revised study was sent back to the two referees for re-evaluation, and we have received comments from both of them, which I enclose below. As you will see the referees find that their concerns have been sufficiently addressed and they are now broadly in favour of publication.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal, pending some minor issues related to formatting and data representation points listed below, which need to be adjusted at re-submission.

REFEREE REPORTS:

Referee #1:

The authors adressed all my comments very convincingly, the paper in my opinion should be accepted for publication.

Referee #3:

The authors addressed the previously raised issues

2nd Revision - authors' response 25th Jan 2020

The authors performed all requested editorial changes.

3rd Editorial Decision 28th Jan 2020

Thank you for submitting the revised version of your manuscript. I have now evaluated your amended manuscript and concluded that the remaining minor concerns have been sufficiently addressed.

Thus, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Gang Shu
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2019-103304R

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- ➔ the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ➔ figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- ➔ graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- ➔ if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be iustified
- ➔ Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- ➔ a specification of the experimental system investigated (eg cell line, species name).
- ➔ the assay(s) and method(s) used to carry out the reported observations and measurements
- ➔ an explicit mention of the biological and chemical entity(ies) that are being measured.
- ➔ an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- ➔ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- ➔ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- ➔ a statement of how many times the experiment shown was independently replicated in the laboratory.
- ➔ definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	All the experiments were repeated three times independently and standard deviations were calculated from those data.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	We had remarked all the sample size for animal studies.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data were excluded intentionally.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	We had described that animals were assigned randomly in methods.
For animal studies, include a statement about randomization even if no randomization was used.	We had described that animals were assigned randomly in methods.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Blinding of investigator was required for our experiment in group allocation.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Blinding was required for animal studies.
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	The range of variability and the standard error were comparabale among group.
Is there an estimate of variation within each group of data?	Standard error.

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http://www.ebi.ac.uk/ega

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http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Company and catalog numbers commercially available antibodies had been provided in material and method.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes, we had identified in method.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Genotypes of all animals and the details of housing conditions were described in methods.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N/A
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We had confirmed the compliance of these recommentation.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Yes
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Yes.We had updated 16S-seq, RNA-seq and metabolomics data, which were described in data availability.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	N/A
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A