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Acute Exercise Decreases Insulin-Stimulated Extracellular Vesicles in Conjunction with Augmentation Index in Adults with Obesity

Emily M Heiston, Anna Ballantyne, Sabrina La Salvia, Luca Musante, Uta Erdbrügger, and Steven Kenneth Malin
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The following individual(s) involved in review of this submission have agreed to reveal their identity: Daniel Stephen Lark (Referee #1); Ivan Vechetti (Referee #2)

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Dear Dr Malin,

Re: JP-RP-2021-282274 "Acute Exercise Decreases Insulin-Stimulated Extracellular Vesicles in Conjunction with Augmentation Index in Adults with Obesity" by Emily M Heiston, Anna Ballantyne, Sabrina La Salvia, Luca Musante, Uta Erdbrügger, and Steven Kenneth Malin

Following your successful appeal, we would now like to give you the opportunity of responding to the original reviewer comments (copied again below) and submitting a revised version for further consideration.

I hope you will have no difficulty returning your revisions within 4 weeks.

Your revised manuscript should be submitted online using the links in Author Tasks Link Not Available.

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- Article file, including any tables and figure legends, must be in an editable format (eg Word)

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- Upload each figure as a separate high quality file

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I look forward to receiving your revised submission.

If you have any queries please reply to this email and staff will be happy to assist.

Yours sincerely,

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EDITOR COMMENTS

Reviewing Editor:

The present manuscript has been reviewed by two experts in the field. Both recognise that the manuscript does provide an important advancement for the field, but that the manuscript in current form is somewhat limited in scope. For example, with a lack of some mechanistic underpinnings and extracellular vesicle dynamics.

REFEREE COMMENTS

Referee #1:

Basic Overview: The authors performed hyperinsulinemic-euglycemic clamps on obese individuals. Authors measured insulin action, augmentation index and EV subpopulations using spectral flow cytometry.

Strengths:

1) Hyperinsulinemic-euglycemic clamp to measure insulin action.

2) EV analysis using antibodies and spectral flow cytometry.

3) Characterizing multiple circulating EV populations.

Weaknesses:

1) The authors mention NTA and TEM as methods used to characterize EVs, but data is not reported in manuscript. While it is widely recognized that lipoprotein contamination is prevalent in plasma EV preparations from a variety of methods, these data are still useful to compare to previous reports and to build upon using the immunolabeling approach described here. Furthermore, NTA and TEM data would address the EV size question that the authors note as a limitation of their study in the discussion.

2) There are some details missing from the flow experiments. While some of this information is provided in the "MI Flow-Cyt" checklist, this might be best reported in the Methods section of the paper. For example...

3) How was EV abundance calculated from flow data?

4) FITC, PE-Dazzle, PE, AF 647 and Pacific Blue were conjugated to antibodies. Given the limited abundance of target proteins in EVs, Could a difference in brightness between probes affect the ability to detect EVs with these markers?

5) Catalog numbers are provided (in the supplement) but not a vendor.

6) Are there other EV-sized particles that express CD31, CD45, etc.?

7) Also, building off of those questions, could the authors report the co-expression of tetraspanins and CD proteins?

8) How do you know your flow events are EVs? Size range?

9) The authors mention serial dilutions were performed to address swarming, but do not report the data from these dilutions. Was a specific dilution chosen and why? If the dilution used as different between samples, then this needs to be reported and explained.

10) In the discussion, it is not clear to this reviewer how "no change in fasting glucose" suggests "a potential EV-muscle "cross-talk" mechanism" in response to exercise. By and large, it did not appear that acute exercise improved insulin sensitivity in this study. Furthermore, why argue SkM-liver crosstalk when SkM EVs were not measured? Based on the results described, platelets appear to be the only EV population associated with insulin action.

Referee #2:

The study conducted by Heiston and colleagues aimed to evaluate the effects of a single bout of moderate aerobic exercise on EV responses to insulin in relation to vascular function. The main findings in the study were a single bout of exercise lowers fasting and insulin-stimulated EVs in adults with obesity, and that exercise decreases insulin-stimulated AIx75 and increases metabolic insulin sensitivity. The study is well designed and written, bringing an exciting new hypothesis to the exercise-EV field. This reviewer also commands the authors of this study for a clear and comprehensive limitation section in the manuscript. The authors have also followed the guidance of EV societies, which makes the study stronger. Despite all these positive aspects of the manuscript, this reviewer has the same concerns raised by the authors in the limitation section. Specifically about EV dynamics. The decrease in EV concentration could be due to an increase in EV uptake rather than a deficit in EV biogenesis. A time-course would be beneficial to better understand this process. Additionally, the lack of a mechanism reduces the excitement of the manuscript.

Minor: The authors mentioned in the methods, the use of cryo-EM, NTA, and western blot for EV characterization, however, no figure has been provided.

END OF COMMENTS

Confidential Review **16-Aug-2021**

Responses to Reviewers: JP-RP-2021-282274

EDITOR COMMENTS

Reviewing Editor:

The present manuscript has been reviewed by two experts in the field. Both recognise that the manuscript does provide an important advancement for the field, but that the manuscript in current form is somewhat limited in scope. For example, with a lack of some mechanistic underpinnings and extracellular vesicle dynamics. It is in part, this reason that has led to the recommendation regarding this manuscript.

We thank the editor as well as both reviewers for their comments as it has strengthened the current manuscript.

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REFEREE COMMENTS

Referee #1:

Basic Overview: The authors performed hyperinsulinemic-euglycemic clamps on obese individuals. Authors measured insulin action, augmentation index and EV subpopulations using spectral flow cytometry.

Strengths:

1) Hyperinsulinemic-euglycemic clamp to measure insulin action.

2) EV analysis using antibodies and spectral flow cytometry.

3) Characterizing multiple circulating EV populations.

Weaknesses:

1) The authors mention NTA and TEM as methods used to characterize EVs, but data is not reported in manuscript. While it is widely recognized that lipoprotein contamination is prevalent in plasma EV preparations from a variety of methods, these data are still useful to compare to previous reports and to build upon using the immunolabeling approach described here. Furthermore, NTA and TEM data would address the EV size question that the authors note as a limitation of their study in the discussion.

We appreciate these comments. We have added a new figure (*Figure 1*) that summarizes the basic characterization of EVs from our EV prep/enrichment protocol, including a picture of EVs with cryo-EM imaging, an example for particle detection with NTA, and Western blot analysis of EV markers.

We did not perform NTA analysis for each single sample, as it only provide particle counting (including lipids and co-sedimented proteins mixed with EVs). The flow cytometry analysis, employed by us for this project, performs single EV analysis and offers characterization of the surface proteins on the detected EVs but also enumeration of EVs through a volumetric method provided by the flow cytometer. We are using flow cytometry for enumeration of specific EVs as

this is more reflecting impact of insulin on EVs compared to impact of insulin on particles (measured by NTA). Offering more specific EV analysis opens up new mechanistic insight into pathophysiology as the markers used derived from cells/organs releasing the EVs during stress and stimulus (here insulin and exercise). The reviewer is correct that other investigators have studied smaller EVs, however those have mostly used NTA (detecting particle counts) or performed bulk analysis with Western blotting (describing EV protein cargo). Importantly, those studies have not focused on single EVs. In this manuscript we analyzed medium-sized and larger EVs and performed targeted phenotyping of each single EV detecting surface markers of interest coming from the circulation (here CD105, CD41, CD31, CD45, Tetraspanins). Of note, most of the conventional flow cytometers which are used by EV researchers do not detect EVs <600nm (JThromb Haemost2018 Jun;16(6):1236-1245. doi: 10.1111/jth.14009. Epub 2018 Apr 26). We are using a flow cytometer which can detect EVs down to 150-200nm (J Extracell Vesicles. 2021 May; 10(Suppl 1): e12083.Published online 2021 Ma15. doi: 10.1002/jev2.12083). Therefore, we believe our data offers insight into single EV phenotype analysis of medium size to large EVs which has not been done in this area of research. We have added a sentence in the manuscript to stress this fact and point out in the conclusion that additional analysis of smaller EVs is part of future investigations. We have also added comments within the discussion and explained why we used high resolution flow cytometry as our EV detection tool. Throughout the text we also highlight the different methods utilized by EV researchers.

2) There are some details missing from the flow experiments. While some of this information is provided in the "MI Flow-Cyt" checklist, this might be best reported in the Methods section of the paper. For example...

The missing information has been added in the manuscript as recommended.

3) How was EV abundance calculated from flow data?

EVs numbers are calculated with a volumetric method provided through the instruments. This information has been added in the method section.

4) FITC, PE-Dazzle, PE, AF 647 and Pacific Blue were conjugated to antibodies. Given the limited abundance of target proteins in EVs, Could a difference in brightness between probes affect the ability to detect EVs with these markers?

Flow cytometry provides targeted EV phenotyping. Yes, some fluorescent dyes are "stronger and brighter", however for each antibody tested we performed a titration study to assess the concentration needed for best sensitivity for each marker. In addition, we have tested the fluorescent dyes with single staining and in the "cocktail" (mixing them all) to assess their detection capacity and interference. We also used several controls as recommended by the EV Flowcytometry working group of ISEV to assure that we are detecting single EVs. Those include: EV sample testing without labeling, antibodies tested in buffer only (to test background noise generated artificially with antibody complexes), Isotype control, detergent use (to lyse EVs and demonstrate reduced fluorescence uptake), dilution experiments (to rule out coincidences ("swarm phenomenon")). According the MyCytFlow guidelines by the Flowcytometry working group of

ISEV we have uploaded our flow raw data on a publicly available server so that the data is available to reviewers and readers.

In addition, we have added an example of the dilution process as a supplemental figure (*Figure S1*) and added this information about our dilution experiments in the method section.

5) Catalog numbers are provided (in the supplement) but not a vendor.

We apologize that we did not provide this information. We used fluorescent labeled antibodies from BD Biosciences. This is added in the method section.

6) Are there other EV-sized particles that express CD31, CD45, etc.?

Our method is designed to provide single EV high throughput analysis. We use several controls as described above. In particular, the fact the EV counts decrease after use of a detergent, demonstrate that mostly EVs are measured. However, we cannot rule out that there are other non-EV particles such as small debris, protein complexes and other EVs (exomers) which are detected as well, however we suspect that that proportion is minimal. As additional EV markers we have added a "cocktail" of 3 Tetraspanin markers, see answer below to point 7.

7) Also, building off of those questions, could the authors report the co-expression of tetraspanins and CD proteins?

The Reviewer is correct that this study is using staining with antibodies directed against Tetraspanins. This was intended to implement another control/ tool for detecting EVs. However, in this manuscript we wanted to focus on the other markers and not create further subgroups depending on tetraspanin positivity. In addition, we used a "cocktail" of tetraspanins (CD9/CD63 and CD81). The co-expression would be for all 3 tetraspanins and not specific for individual ones.

8) How do you know your flow events are EVs? Size range?

Spectral flow cytometry provides "high throughput single EV analysis". As previously mentioned, we are using several controls to assure that we are detecting EVs (also outlined above: EVs unstained, Buffer with antigens (as these can also produce artificial EVs), lysed EV samples (reduction in numbers shows, that lysis is disrupting EVs), isotype controls, dilution experiments to r/o swarm phenomenon and provide a list recommended by the Flow cytometry working group of ISEV ("MIFlowCyt-EV", Extracell Vesicles, 2020 Feb 3;9, (1):1713526. doi:10.1080/20013078.2020.1713526.eCollection 2020). All these controls help to assure that single EVs are measured. However, we cannot rule out that some non-EV particles are also measured. However, this is true for other methods, e.g. NTA with fluorescence.

In addition, the NTA data used for our basic EV characterization demonstrates the overall particle distribution is around (mean 175nm +/- 61nm) (including debris, protein complexes, etc). The detection with flow cytometry focuses on medium to larger EVs. We agree that this is a limitation of the study, however we are analyzing more specifically EVs. Flow cytometry is one of the few single

EV detection tools which can also detect surface proteins on EVs. We are planning to also characterize smaller EVs, e.g. with chip technology (Exoview (based on interferometry) by Nanoviewbiosciences). Some novel NTA tools have also 1 or 2 lasers, however we do not have this capability and only limited markers can be tested, therefore high resolution flow cytometery with spectral flow cytometry as used here for our preferred method. We added a comment about our method choice in the method section and also commented in the abstract which method we are using and that we focus our analysis on medium to large sized EVs.

Currently available flow data does not allow comparison between groups. We are following the MiFlowCyt guidelines also using MESF values which are helping different groups to compare data (see also flow checklist). We have also added comments within the abstract, introduction, and methods, to be more clear about how we measured size and assured that mostly EVs were detected by using several recommended controls.

9) The authors mention serial dilutions were performed to address swarming, but do not report the data from these dilutions. Was a specific dilution chosen and why? If the dilution used as different between samples, then this needs to be reported and explained.

We did perform serial dilutions: Each sample was diluted 3 times e.g. 1:500,1000,2000. In this way we were able to pick the right dilution without swarming. We have provided a supplemental figure (*Figure S1*) and have also added a sentence in the method section.

10) In the discussion, it is not clear to this reviewer how "no change in fasting glucose" suggests "a potential EV-muscle "cross-talk" mechanism" in response to exercise. By and large, it did not appear that acute exercise improved insulin sensitivity in this study. Furthermore, why argue SkM-liver crosstalk when SkM EVs were not measured? Based on the results described, platelets appear to be the only EV population associated with insulin action.

We appreciate this critique regarding a potential EV-muscle "cross-talk" mechanism. Although we believe that based upon our data (fasting glucose and insulin, M-Value) and EV work by others that cross-talk likely occurred between the liver and skeletal muscle during insulin infusion, without quantification of these organ-specific derived EVs and isotope tracing for tissue glucose uptake, it is a purely speculative statement. Therefore, we have removed this from our discussion and instead highlighted that additional work is warranted regarding EVs and their subsequent functions.

Referee #2:

The study conducted by Heiston and colleagues aimed to evaluate the effects of a single bout of moderate aerobic exercise on EV responses to insulin in relation to vascular function. The main findings in the study were a single bout of exercise lowers fasting and insulin-stimulated EVs in adults with obesity, and that exercise decreases insulin-stimulated AIx75 and increases metabolic insulin sensitivity. The study is well designed and written, bringing an exciting new hypothesis to the exercise-EV field. This reviewer also commands the authors of this study for a clear and comprehensive limitation section in the manuscript. The authors have also followed the guidance of EV societies, which makes the study stronger. Despite all these positive aspects of the manuscript, this reviewer has the same concerns raised by the authors in the limitation section. Specifically about EV dynamics. The decrease in EV

concentration could be due to an increase in EV uptake rather than a deficit in EV biogenesis. A time-course would be beneficial to better understand this process. Additionally, the lack of a mechanism reduces the excitement of the manuscript.

We appreciate the positive comments by the Reviewer.

We agree that this work is not analyzing the full range of EV sizes which is a known limitation of flow cytometry. However, flow cytometry is one of the few high throughput single EV analysis tools which can detect specific phenotypes of EVs. Our approach can also be called targeted phenotyping. We tested surface markers from circulating cells and the endothelium which likely play a role in the pathophysiology of the patients tested. In addition, our results show significant and novel findings. This study directly tested the effects of insulin on EVs and how a single bout of exercise impacts these responses. And taken together, these data highlight the positive effects of a single bout of exercise on fasting and insulin-stimulated EVs, with the latter relating to increased insulin sensitivity and decreased augmentation index. It is not known yet, if medium to larger EVs or smaller EVs are better clinical markers. Our study addresses medium to larger EVs. There is not one tool/technique which allows to test the whole size range of EVs with focus on single EV detection. One would need to combine our flow cytometry study with e.g. chip technology like e.g. Exoview from Biosciences (using interferometry) or utilizing NTA with a laser, however that techniques is also limited on testing only a few surface markers including particles (not as many as we did) and not very well established. Nevertheless, interferometry technique only captures the very small EVs between 40 and 100nm with first generation instruments and it is not known if results of both techniques can be compared directly. It is also important to highlight that the flow cytometer used in this study belongs to the higher resolution tools to detect EVs. As outlined above in the answers to reviewer #1, most researchers who use flow cytometry do detect larger EVs 400-600nm, our technique goes down to 150-200nm. It is true that some EV tools analyze the whole range of EVs, however this is only possible with omic analysis (proteomic/RNA) of EVs, which is only providing bulk analysis and not single EV analysis. Therefore, this study is novel in that regard that it provides high throughput single EV analysis of specific phenotypes from medium to large sized EVs. Our future work will include the study also of smaller EVs with capture tools.

We also agree that mechanistic studies are needed, however this was not the purpose for this study. The clinical cohort is well characterized and we think that the current results show enough novelty. Mechanistic studies would be beyond the scope of this study. Likewise, we agree that time course studies are needed to understand EV dynamics. Unfortunately, these data were not collected in the current study and believe that a new study should be designed to assess time-course data.

We have added now more information about high resolution flow cytometry, our technique used here. We hope that this information will make our method choice clearer.

Minor: The authors mentioned in the methods, the use of cryo-EM, NTA, and western blot for EV characterization, however, no figure has been provided.

We have now added a figure for basic EV characterization including cryo EM, NTA, WB. See *Figure 1.*

Dear Dr Malin,

Re: JP-RP-2021-282274R1 "Acute Exercise Decreases Insulin-Stimulated Extracellular Vesicles in Conjunction with Augmentation Index in Adults with Obesity" by Emily M Heiston, Anna Ballantyne, Sabrina La Salvia, Luca Musante, Uta Erdbrügger, and Steven Kenneth Malin

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All queries at proof stage should be sent to TJP@wiley.com

Yours sincerely,

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EDITOR COMMENTS

Reviewing Editor:

Thank you for addressing the comments raised in review and congratulations on an insightful piece of work.

REFEREE COMMENTS

Referee #1:

I would like to thank the authors for their thorough response to my questions and concerns. My only other suggestion is that if the authors believe tracking SkM EVs is important for the future directions of this area, they might consider noting that Estrada et al. 2021 (AJP-Cell) have just described an approach in mice to do this. I have no further questions or concerns about this manuscript.

Referee #2:

The authors have addressed all my concerns and I have no further questions.

1st Confidential Review **22-Dec-2021**