Author's response to reviews

Title: Exercise training with dietary counselling increases mitochondrial chaperone expression in middle-aged subjects with impaired glucose tolerance

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Version: 2 Date: 18 December 2007

Author's response to reviews: see over
Author’s response to reviews:

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Version: 1 Date: 14.12.2007

Author’s response to review: see over
Dear Editor and Reviewers,

We are pleased to hear that our manuscript interests you. We would like to thank you for your supportive and elucidative comments and proposals which helped us to improve our paper. We have made changes in the manuscript according to your comments. Below you will find our answers to all of the questions raised by you. In the revised manuscript, the changes are marked with red.

Reviewer's report 1

Title: Exercise training with dietary counselling increases mitochondrial chaperone expression in middle-aged subjects with impaired glucose tolerance
Version: 1 Date: 13 August 2007 Reviewer: Peter Csermely

Reviewer's report:

General

Authors provide evidence that 2 yrs of exercise training together with dietary counseling induces mitochondrial Hsp expression and reduces oxidative stress in middle-aged subjects with impaired glucose tolerance.

Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)

N.A.

Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

1 The statement on page 15 “Most studies showing that exercise induced HSP72, have used low force exercises with continuous activity.” Has to be substantiated by appropriate references.
Reply: References are now included on page 15.

2 The number of total experiments have to be included to figure legends. Error bars are missing from Fig.1.

Reply: Error bars are now added in Figure 1 and the number of total experiments are now included on Figure legends.

Discretionary Revisions (which the author can choose to ignore)

N.A. **What next?:** Accept without revision  **Level of interest:** An article of outstanding merit and interest in its field **Quality of written English:** Acceptable  **Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**
I declare that I have no competing interests.
Reviewer's report 2

Title: Exercise training with dietary counselling increases mitochondrial chaperone expression in middle-aged subjects with impaired glucose tolerance

Version: 1 Date: 12 September 2007

Reviewer: Allan Vaag

Reviewer's report:

In this manuscript, the authors have measured the expression of several key mitochondrial chaperones and level of oxidative stress in skeletal muscle in 22 subjects with impaired glucose tolerance before and after a 2-year intervention programme with exercise and dietary counselling. Briefly, they report an upregulation of mitochondrial Heat Shock Proteins and decreased oxidative stress after the intervention. Somewhat surprisingly, the beneficial changes in mitochondrial Heat Shock Proteins and oxidative stress were not related to the changes in HOMA insulin resistance and degree of oxidative stress.

The questions posed by the authors in the manuscript are indeed new and well defined, and the methods are well described and in general appropriate. One drawback, however, is that insulin resistance was not measured using the gold standard hyperinsulinaemic clamp technique, which in fact may explain the lack of any association of the changes in heat shock proteins with degree of HOMA insulin resistance. Thus, HOMA insulin resistance is more likely to represent hepatic -and not muscle -insulin resistance, given that HOMA insulin resistance is based only on fasting plasma glucose and insulin measurements, and that the main "target" of insulin in the basal state is the liver, and not skeletal muscle. The authors should explain and discuss this important issue in a revised version of the paper. Apart from this somewhat minor issue, the data appears sound and well controlled, the paper is well prepared and clearly written, and the discussion and conclusions are well balanced and supported by the data. All together, this is indeed an interesting and potentially very important paper.
Reply: We thank the referee for the supportive comments; unfortunately, we have not used standard hyperinsulinaemic clamp technique to determine insulin resistance. Fasting values of glucose and insulin (HOMA-IR) depend on the hepatic glucose control, but not peripheral glucose metabolism. Petersen et al. (2005) have shown that improvements in basal and insulin-stimulated hepatic glucose metabolism were associated with the marked reduction in intrahepatic lipid (IHL) without any significant changes in insulin-stimulated peripheral glucose uptake after a moderate weight loss (8% of their body weight).


Reviewer's report 3

Title: Exercise training with dietary counselling increases mitochondrial chaperone expression in middle-aged subjects with impaired glucose tolerance

Version: 1 Date: 11 October 2007

Reviewer: Kathryn H Myburgh

Reviewer's report:

General

The background is logically written and, in general, supports the study. Some specific comments are provided.

This study was part of a larger study and 22 subjects volunteered. This is likely a
good number for this complexity of work, however, it must be discussed how this
could have influenced your results.

The methods are sound and the data presented are for the most part relevant
and good. A few specific comments are provided.

However, the discussion does not reflect the standard of the data.

Major Compulsory Revisions (that the author must respond to before a decision
on publication can be reached)

1 Introduction: Although it is commendable that it is not too long, it should be
expanded (briefly) in some aspects. See specific comments.

2 Where relevant do power analyses to determine if this may have affected
your conclusions).

Reply: We did not perform power analyses in this study, because the number
of subjects volunteered to give muscle samples both before and after 2-year
intervention was 22 and our permission to take biopsies was limited to the
intervention group. We did not have possibility to recruit more study
subjects.

3 There is little or no discussion of the comparison between the two groups,
which was an objective of the study.

Reply: Skeletal muscle is a highly heterogeneous tissue and metabolic
responses of the different muscle fibre types vary depending on their
phenotype. However, most of the studies have been done with animals and
very little is known on the effects of exercise and dietary interventions in
human skeletal muscle. Glucose homeostasis depends on several factors,
including glucose transport into the muscles, which is influenced by the
muscle fibre type composition and the regulation of glycogen synthesis.
Previous studies have shown that obese or type 2 diabetic subjects have a higher proportion of type IIb fibres (cf. MHC IIx) in the skeletal muscle tissue [Mårin et al. 1994, Hickey et al. 1995, Tanner et al. 2002], and for insulin sensitivity, the muscle fibres follow the order type I > type IIa > type IIb [James et al. 1985, Henriksen et al. 1990, He et al. 2001]. Also, a number of studies have shown the expression of HSPs to vary depending on the muscle fibre type [Liu Y and Steinacker 2001, Locke et al. 1991, Ornatsky et al. 1995]. Therefore we feel that even, our limited results may make a contribution to the literature.


4 P4 Sentence 3: This sentence is too general: in which tissue/system was oxidative stress measured in the rats? If the HSP responses were impaired, what was the challenge? Or do you mean in a baseline condition in response to the diabetes itself?

Reply: The change has been done in the revised manuscript.

5 PKB/Akt – what is the relevance of this part of the sentence? Explain.

Reply: Akt/PKB has been linked to the activation of the glucose transport, based on the findings that over-expression of the constitutively active Akt construct leads to enhanced glucose transport in 3T3-L1 adipocytes and L6 muscle cells (Wojtaszewski et al. 2000). HSP90 has several physiological roles, including mediating protein kinase B (PKB/AKT) stability (Basso et al. 2002, Csermely et al. 1998). We added a brief explanation to the revised manuscript.

- Csermely P, Schnaider T, Soti C, Prohaszka Z, Nardai G: The 90-kDa molecular
haperone family: structure, function, and clinical applications. A comprehensive

6 P5 Sentences 2 and 3 are too generalized. Expand each sentence with one
more sentence explaining more specifically e.g. can you give relevant examples of
some cellular functions and adaptations that are regulated by ROS in their capacity
as 2nd messengers? Similarly, for the next sentence, please explain e.g. why you
state that ROS is involved in i) pathophysiology of insulin resistance ii)
pathophysiology of diabetes and iii) the complications of diabetes. Or, is the only
evidence that it is involved related to the indirect evidence in the following
sentence? This is not clear.

*Reply: These changes have been done in the revised manuscript.*

7 P5 Par 2 Sentence 2: Very generalized statement with no references. If very
little is know, it should be easy to actually mention exactly what has been done in
humans and which animals models have provided relevant information or
investigated parameters relevant to your study.

*Reply: Mattson et al. (2000) have reported that both HSP60 and GRP75 are up-
regulated in rat skeletal muscle after 8 weeks of endurance training and in
type 2 diabetic subjects. Insulin resistance correlates with decreased
expression of HSP72 in skeletal muscle at mRNA level (Kurucz et al. 2002,
Bruce et al. 2003). Induced HSP60 and HSP72 expressions in skeletal muscle
have also been reported in a short-term exercise intervention in healthy
humans (Khassaf et al. 2001, Morton et al. 2006). However, to our knowledge
there have been no papers in the literature studying simultaneously the
effects of exercise and dietary interventions on the antioxidant and HSPs
defences in humans with impaired glucose metabolism. We mentioned the limited number of references in the appropriate sections, as advised.


8. You have used various markers for oxidative stress in your study, yet none are mentioned in the Background in terms of prior use in a similar population or any papers indicating their acceptance as relevant and repeatable markers (in serum and muscle). What was you purpose in measuring serum urate? I was under the impression that urate is in fact an anti-oxidant. Please discuss the various possible interpretations of a plasma urate concentration.

**Reply:** Niskanen et al. (Niskanen et al. 2006) have recently showed that high concentration of serum uric acid at baseline predicted a poorer metabolic outcome independent of baseline insulin and glucose levels in the IGT
subjects of DPS main study. Uric acid acts in certain situation as pro-oxidant and is dependent on several factors like the surrounding oxidant milieu, depletion of other local antioxidants, the supply and duration of oxidant substrate and its oxidant enzyme (Hayden et al. 2004).


Methods

9. P6 There is too little information on the subject selection “based on 2 oral glucose tolerance tests”. What exactly was your definition of IGT? What cut-offs were used?

Reply: The change has been done in the revised manuscript.

10. P7 There is insufficient information regarding the training intervention, particularly since it was individualized, it is also possible that some individuals did a lot less exercise. What was adherence of the subjects in this sub-study? Please provide data on the range of training volume actually performed.

Reply: The change has been done in the revised manuscript.

11. P 7: Was the biopsy taken under resting conditions? When was the last exercise
session prior to the biopsy? It would be helpful to the reader if you could specify the typical size of the muscle sample required to perform these analyses. Was one homogenate used for all further analyses? Or were separate small pieces used for the various analyses? Were any homogenates or extract aliquots refrozen between assays?

Reply: The change has been done in the revised manuscript.

12. P8: MHC analysis: Too little information on the gel electrophoresis method. This is an incorrect reference. Reference the method you have used.

Reply: The change has been done in the revised manuscript.

Results
13 It is unclear why the comprehensive dietary analyses are provided. The effect of dietary intervention was not a priority in the introduction or the discussion. Limit to essentials. I suggest you remove everything except the total energy intake and the vitamin C and E intakes.

Reply: The change has been done in the revised manuscript. The comprehensive dietary analyses were provided because we want to make sure that there are no differences in the dietary intake between the sub groups.

14. P11: Is there any relevance to the differences in dietary fat and fibre intake between the slow and fast twitch muscle subject groups? If this is important to explain fat soluble vitamin absorption, then you need to make this one of the issues of investigation. Otherwise, remove data that isn't relevant.
Reply: The change has been done in the revised manuscript.

15. P12. Correlations should be presented graphically for proper evaluation of the spread of data. I suggest that you provide a graph with 2 panels: indicating the positive relationship in the one group and the lack of relationship in the other group.

Reply: The change has been done in the revised manuscript.

16. A second correlation graph that would be relevant to see is the relationship between VO2max (do you mean the final Vo2max, or the change in VO2max?) and the increased GRP75 (again, do you mean the change in GRP75? Or the new, elevated value post-intervention?). Again, it would be relevant to have two panels: one for the significant result and one for the lack of correlation in the other group.

Reply: The change has been done in the revised manuscript.

Discussion

17 Par 1 is lightly confusing because in the introduction you have emphasized the roles of HSP60 and GRP75 as chaperones involved in trafficking and processing, whereas here you are emphasizing a cytoprotective role, which you assigned to the 70kDa HSP family in the introduction, whereas here you are assigning the HSP72 and 90 the roles of chaperones.

Reply: Chaperones protect other proteins against aggregation, solubilize initial, loose protein aggregates, assist in folding of nascent proteins or in refolding of damaged proteins, target severely damaged proteins to degradation and in case of excessive damage, sequester damaged proteins to larger aggregates (Sõti et al. 2005). Therefore, in our opinion, chaperone
function of HSPs is tightly connected to their role in the protein homeostasis and cytoprotective functions. Moreover, many members of HSP family work in concert with each other and have overlapping chaperon and cytoprotective functions, despite their different induction patterns and subcellular locations.


“Although the IGTslow group represents a type of ordinary people”. Rephrase appropriately. E.g. refer to this fibre type distribution as more common (or if you meant less prone to type II diabetes?) and provide references of a larger population study.

Of course the fibre type of the 2 groups differed since you selected them to differ.

Explain properly why you are discussing results presented in reference number 19 – does this reference report on the exact same subject cohort? Then say so, since in this paragraph you start out specifically stating “In this study”… If a change in MHC proportion post-intervention for these subjects was not statistically significant, then you cannot say that one type of MHC increased and another decreased. How many subjects would have been required to make this conclusion? If these data are presented elsewhere, do they warrant a paragraph of discussion on their own?

Reply: The study subjects are exactly the same in the paper of the reference number 19 (at now 24) and the present study. We have used expression of slightly increased and decreased. We have now added (n.s) after these word to avoid confusion of the statistically significance. It is difficult to know how many subjects would have been required more to reach statistically significant change in MCH because skeletal muscle highly is heterogeneous
tissue, and the standard deviation affect highly in this study with limited number of study subjects.

19 14: It is confusing that you are discussing results presented elsewhere. You can’t try to correlate some data presented in one paper and other data presented in the current paper. It is more important that you attempt to explain the last sentence, since you objective here was to compare the two groups.

Reply: We present in two papers, the results of two years of dietary and exercise intervention study. The first published paper focused on the glucose homeostasis and the second paper submitted to BMC focuses mainly on the skeletal muscle antioxidant and HSP defences. It is practically impossible to bring all the results into one paper. On the other hand, we observed some important correlations between the results of the first and second reports of the same study. We aimed to bring this information to the attention of the readers.

20. P14 last Par and P15 first Par: This is a confusing piece of writing: It is a very long paragraph – what is the actual point you wish to make? That the mitochondria are dysfunctional? Or that mitochondria are not generated? Or that dysfunctional mitochondria are generated? Is there sufficient evidence to distinguish? Which DNA damage is reduced? Mitochondrial DNA? If the oxidative enzymes did not increase and only mRNA was elevated (which genes?) it is very possible that mitochondrial biogenesis did not increase. It is unclear why you have a sentence here about the anti-oxidative processes. Which genes are you talking about? Anti-oxidant enzymes, or? Are they cytosolic or mitochondrial in location? Do you mean matrix or membrane? Mitochondrial function and biogenesis are too loosely linked, when you are actually discussing biogenesis. Suddenly adding some discussion on HSP72 doesn't fit in this paragraph. The relation of HSP72 and anti-oxidant defence is not explained.
Increased concentrations of reactive oxygen species (ROS) during oxidative stress may cause dysfunction of mitochondria either directly by damaging or through the deregulation of mitochondrial functions. Direct damage is the oxidative insult to the mitochondrial components, i.e. mitochondrial proteins, lipids, phospholipids, mitochondrial membranes and mitochondrial DNA (which is limited in number and in amount). It has been shown that ROS, especially H2O2 can easily penetrate membranes including mitochondrial membranes and regulates several transcription factors, kinases phosphatases and as well activities of enzymes. Although in physiological concentrations ROS have regulatory role especially in the signal transduction, during oxidative stress, redox regulation of cellular functions are also impaired. A wide range of proteins, enzymes and transcription factors are susceptible to H2O2, including small ubiquitin related modifiers, cytosolic and mitochondrial antioxidant enzymes, peroxiredoxins, superoxide dismutase, heat shock proteins, especially heat shock factor1-α. Furthermore, in mitochondria. mitochondrial enzyme aconitase, α-ketoglutarate dehydrogenase and various subunits of the respiratory complex I are some of the targets of H2O2 (Giorgio et al. 2007). Because of the critical role of HSPs in protein homeostasis and for minimizing the protein damage to mitochondria, we would still like to keep this part of the discussion as it was.


If your study used a similar type of exercise to ref 46, and reference 46 found a decrease in HSP72 expression, then how does this explain why you found no change? Discuss other possibilities: your study duration was 18 months – are
changes possibly transient?

Reply: In the study of Gjøvaag et al., the authors investigated the effect of exercise training on heat shock protein responses in well trained athletes. Unlike our subjects in Gjøvaag et al.’s study the subjects were heavily trained. An acute bout of exercise induces HSP response in dose and time dependent manner and this response is usually more significant in the individuals who have lower starting levels of HSP or who are less fit. Therefore, the comparison of less fit, sedentary subjects with very well trained athletes may not be appropriate and we admitted this problem in the revised manuscript. However, we referred the study of Gjøvaag et al. for the comparison of HSP responses because their training protocol was the closest in the literature to ours.

22 P16: How do you explain this? HOMA-IR may be more closely related to GLUT4 than to mitochondria or chaperones.

Fasting values of glucose and insulin (HOMA-IR) depend on the hepatic glucose control, but not peripheral glucose metabolism. All OGTT-derived indexes rely upon the measurement of plasma glucose and insulin concentrations, either fasting or post-load values. These indexes mostly correlated with whole-body insulin resistance. Abdul-Ghani et al. (2007) have developed a new OGTT-derived index for skeletal muscle metabolism. Unfortunately, we could not use it in our study because we do not have same time points for glucose and insulin calculation. Also we did not have the measures for protein concentration of GLUT4, and we cannot correlate it for HOMA-IR. Decreased glucose uptake can not been explained by a decreased expression of the GLUT-4 but a decreased function and distribution of GLUT-4 has been suggested to account for the decreased glucose transport in skeletal muscle from subject with type 2 diabetes (Garvey et al. 1992 and Vogt et al. 1992). Also our samples are taken at rest not in insulin stimulated
situation which would be needed to study translocation of GLUT-4 to plasma membrane.


P16: ORAC: Explain why sentence 2, which explains completely different experimental design is “in agreement” with your results? What do you mean? That it is difficult to ‘get a significant change in ORAC’? Is it really? What other methods were used in ref 48?

We agree with the referee that our statement may mislead the readers and may give a false impression that ORAC assay does not have enough sensitivity compared with the other antioxidant capacity methods. Indeed, ORAC assay is more specific for the endogenous sources of reactive oxygen species and therefore ORAC method gives less exaggerated results and is more relevant to the measurement of antioxidant capacity from the biological samples (Prior and Cao 1999). On the other hand, changes in the antioxidant levels in the serum levels may not always reflect the adaptations gained in skeletal muscle, especially if the samples were taken at rest. Therefore we modified this paragraph accordingly.

Regarding the reference of Fatourus et al., they have used a commercially available total antioxidant capacity assay, originally called as “Trolox equivalent antioxidant capacity (TEAC)”. The TEAC assay is based on the inhibition by antioxidants of the absorbance of the radical cation of
2,29-azinobis(3-ethylbenzothiazoline 6-sulfonate) (ABTS). The original TEAC assay measures the ability of a compound in reducing ABTS radical. Because ABTS radical is not necessarily a pro-oxidant and it is quite stable, the relevance of TEAC method in biological samples is questionable and because of the same reason, we did not prefer this method in our studies. In order to avoid any confusion this reference is removed from the revised manuscript.


24 P16 Last sentence of this paragraph: There are other possible mechanisms for the cardiac protection, not necessarily anti-oxidant capacity (see Valko et al, 2007).

Reply: We agree with the referee, that antioxidant and HSP defences are only some of the factors that contribute to tissue defences and cardiac protection. We modified this sentence accordingly. As, it has been accepted for a long while among free radical researchers that in physiological levels, reactive oxygen species have major role in the modulation of cellular functions especially by mediating intracellular signalling. Moreover, the term oxidative stress was recently redefined as “the state of disruption of redox signalling and redox control” (Jones 2006). Endogenous antioxidants have an important role in the regulation of the redox signalling since many of the endogenous antioxidants, their selves are signalling molecules (Jones 2006). Therefore, adaptation of endogenous antioxidant defences during exercise training improves the balance between oxidants and antioxidants and is a part of the health beneficial effects of physical exercise.

25 Conclusions

The changes in oxidative stress markers are in no way remarkable, did not change in both groups and did not change in muscle.

The evidence does not support the last sentence.

Reply: The change has been done in abstract and conclusion

Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

Reply: The all of these changes have been done in the revised manuscript.

1. Grammar:

P5: Furthermore, we aimed to investigate....

2. P9: Give precise information on the anti-bodies (catalogue number or reference number).

23. P9: Where was your oxidized BSA standard purchased?

24. Check all units: if you use a forward slash (/), then do not also use -1

25. P11: The mean and S.E. for change in body weight has great range. Please supply the actual ranges.

26. Figure 1: Fix spelling error

27. P12: Give the % changes in the chaperone responses in the text.

28. P12: Par 2: Insert words: muscle before HSP60 and serum before ORAC.

29. P13 last line: CS activity is normally assayed in a muscle homogenate, not a mitochondrial fraction. It is not necessary to state that the CS is in mitochondria, since it is a bit confusing in the context of this sentence.

210. P14, Par 2: In relation to the sentence with ref 41, also check the 2007 literature.
P14, Par 2: in relation to the sentence with ref 42: this is important to mention in the introduction as well and should satisfy some of the comments above about the need to explain the dual roles of chaperones/cytoprotection.
P14 Par 2: Grammar: “due to”; “reduces” DNA damage; “increases”
P14 par 2 last line: insert “and lack of exercise training”

14. P15 Par 2: A sentence starting with “Most studies” should have multiple references, not none at all.

14 P16 Grammar: delete “have been used”

P17: Oxidative stress: remove “In other words”, because production of ROS is different from synthesis of anti-oxidant enzymes.
P17 Sentence 2: Should be explained in the Introduction.

P17 Grammar: rephrase “in this weightlifters study”

P17 rephrase “determining protein oxidation” – this is the term usually used for the oxidation of protein as a fuel, not for oxidation-induced protein damage.

References
24, 37 incomplete. If ePub, still need to provide the information.

Table 1: Remove the last column – the data is in the next table anyway. No units for BMI. Footnote: mention the statistical test used.
Table 2: The abbreviation VO2maxind is not commonly used. Take out the ‘ind’ Use 3 decimal places for the P. carbonyl results – means nothing as stated.
Table 3: Are these the correct units to use for ORAC?

Reply: Trolox (Aldrich, Milwaukee, WI) was used as a control standard. Final results were calculated using the differences of areas under the FL decay curves between the blank and a sample and quantified according to Trolox standards and expressed as µmol.

Table 4: Remove. Place data only in text. All the data is not required.
Discretionary Revisions (which the author can choose to ignore)

What next?: Unable to decide on acceptance or rejection until the authors have responded to the major compulsory revisions

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Needs some language corrections before being published

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:

I declare that I have no competing interests at all