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cc. Lene Knuppel (Head of Section)

Danish Committees on Scientific Dishonesty (DCSD/UVVU)

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Re: The Penkowa fraud case

The University of Copenhagen has encouraged the scientific community to contribute to the exposure of possible dishonesty associated with the scientific work of Milena Penkowa. An interesting aspect is whether she alone is to blame for such fraud, or whether the fraud reflects lenient scientific ethics in the milieu around her. Primarily, Penkowa has been accused of cheating with data from her main areas of interest, i.e. neuro- and cancer research. However, in order to elucidate her interaction with collaborators, I have chosen to review the 12 scientific papers, she has co-authored about muscle physiology and pathophysiology. Her histochemistry contributions to these papers have been instrumental for their publication; but muscle has not been her own primary field of interest, neither has she conducted the underlying experiments or carried out the biochemical analyses. So, one may assume that the interest in her muscle findings was at least as high among her collaborators as her own. My background for writing this report is that I have been in the scientific frontline within the area in question, i.e. endocrinology as it relates to muscle, for about 35 years.

Below you will find my conclusion followed by the grounds for this given in comments, in chronological order, to each of the 12 papers mentioned above. I have understood from discussions with Lene Knuppel that Rector, and not DCSD/UVVU, will be dealing with material of relevance for the Penkowa case. Accordingly, I hope that my report will be made available to the international committee, which the University has employed to evaluate Milena Penkowa´s research.

Conclusion:

All 12 papers about muscle physiology and patophysiology, to which Milena Penkowa has contributed, have been reviewed. Throughout one gets the impression of a lenient scientific milieu, in which various aspects of scientific misconduct are allowed or indeed encouraged to flourish. Thus, the material indicates extensive reuse of subject populations and tissue material as well as reuse of data, both without notifying the reader. Rather, the reuse was disguised intentionally or unintentionally (e.g. by imprecise and differing informations on population sizes, use of different ways of expressing material dispersion, different number of decimals, figures instead of numbers in text or tables).

Key analyses were immunohistochemical determination of various peptides and biochemical determination of corresponding mRNAs in muscle. The histochemical data clearly indicate fraud, e.g. the same stained section being used to illustrate presence of different peptides or of the same peptide in different situations. mRNA data were generally presented in such a way that the actual level of expression and its asserted correspondence with histochemical and other data cannot be properly evaluated by the reader, who accordingly potentially may be mislead.

Throughout, the attitude in the dealing with data and literature apparently was to promote own hypotheses, rather than attempting falsification of hypotheses. In this context one can mention conclusions not supported by data or exaggerated beyond what data justified. It is also remarkable that in several instances conflicting data obtained and reported by the group in different publications were simply ignored and not discussed. In addition, there is ground for suspicion that undesired results may have been excluded. Characterizing the milieu, lack of sufficient carefulness with data and interpretation also applied to other variables than those mentioned above.

Milena Penkowa did, of course, have a major responsibility regarding the histochemical work, she was in charge of. However, the weaknesses inherent in this work were so obvious as regards the description of methodology and evaluation as well as regarding the quality and manipulations of resulting illustrations (e.g. use of the same muscle section to illustrate two time points within one figure) that all coauthors should have reacted, even if they were not directly involved in the actual work. They all have a clear responsibility for publication of the fraudulent material emanating from Penkowa. This is the more so, as histochemical

findings changed between papers, and often did not have a convincing and consistent relationship with other findings. Finally, several of Penkowa´s coworkers were at an early stage warned of possible defects in her work.

For the other aspects of the studies Penkowa essentially had only a peripheral responsibility. Regarding the other main variable, the group was also warned against overinterpretation of weak mRNA expression in muscle. Besides Penkowa, the only other author involved in all the studies reviewed here, BK Pedersen, was also senior author of all but one of the resulting papers. She had the main responsibility for the initiation, organization, implementation and publication of the studies. Importantly, she also had the highest interest in positive results and has, by far, taken most of the credit for the studies. See e.g. the recent paper "BK Pedersen: Muscles and their myokines. J Exp Biol 214: 337-346, 2011" ,in which you may also note that, surprisingly, even some of the above papers that have now been retracted continue to be acknowledged.

Even with its limitations (lack of access to laboratory data (e.g. on mRNA), notes and files (e.g. those giving a complete account of subject groups and analyses performed) as well as to interviews with persons involved in the work), the present investigation, primarily based on review of published papers, leaves little room for doubt that BK Pedersen is at least guilty of gross negligence of her scientific duties both as a group leader and supervisor, as a senior author and, indeed, as a professor of the University of Copenhagen. Considering not only the glaring obviousness of Penkowa´s fraudulent manipulations, but also the numerous inconsistencies in other parts of the work and its interpretation, one has to conclude that it is likely that BK Pedersen chose to close her eyes, i.e. that her negligence was willfull. However, bearing in mind also the obvious motive, deliberate scientific fraud must be suspected. In consequence, a more thorough investigation, with unrestricted access to relevant data and persons, and aiming at disclosing the extent of scientific dishonesty, is warranted.

The report may also give occasion for reflection on how the review process at scientific journals, research foundations and the University of Copenhagen (e.g. in connection with ph.d.-theses) could fail.

Paper 1. Penkowa M, Keller C, Keller P, Jauffred S and Pedersen BK: Immunohistochemical detection of interleukin-6 in human skeletal muscle fibers following exercise. FASEB J. 17: 2166-2168, 2003.

The research group headed by the senior and corresponding author of the present paper (BK Pedersen:"BKP") had previously used femoral arterio-venous concentration and blood flow measurements to demonstrate that in response to prolonged leg exercise interleukin-6 (IL-6) is released from the leg, the output peaking at the end of the exercise (Steensberg et al, J Physiol 537,633, 2001). The aim of the present study was to test whether the released

IL-6 originated in myocytes. Twelve young males had blood and muscle biopsies sampled before and at intervals for up to 21h after 3h of moderate cycling, while 6 other young men served as resting controls for the same period of time.

It was reported that immunohistochemistry showed a significant homogenous accumulation of IL-6 after exercise in all types of myocytes, whereas IL-6 was not found in resting muscle cells or in non-muscle cells. The accumulation peaked 3h after exercise, but was still present 21h after exercise. In accordance with previous findings plasma IL-6 increased during exercise and gradually decreased afterwards. It was concluded that "the study demonstrates that skeletal muscle cells are the dominant cell source of exercise-induced muscle-derived IL-6".

Major concerns:

As judged from the two figures, the IL-6 staining seen after exercise was surprisingly high considering the complete lack of staining at rest. Also, it was not clearly stated how sections were selected for illustration of findings after exercise. The legend to the key Fig. 1 mentioned "exercising subjects", but because only one section was shown for each time point of tissue sampling, it would not be correct to include samples from more than one subject. Although no attempt was made to quantify the IL-6 staining, and although it was not explicitely mentioned, how many sections were evaluated, it was stated in both Results section and legend that IL-6 immunoreactivity/ expression increased "significantly" with exercise. It was not stated, whether more than one scientist examined the sections, nor if this was done in a blindfolded manner.

An 11-fold increase was found in IL-6 mRNA from pre-exercise to end of exercise. Unfortunately, however, the pre-exercise values were not given. Furthermore, the absence of IL-6 protein in muscle fibers in the presence of the corresponding mRNA at rest requires an explanation. The need for an explanation becomes more urgent, when a previous paper from the group is taken into account. In the latter they reported that IL-6 mRNA could not be detected in muscle before a marathon race, and only in 5 of 8 subjects after the race (Ostrowski et al, J Physiol 508, 949, 1998). These findings were not mentioned. It was stated that the finding of a peak in mRNA at the end of exercise fitted nicely with a peak in intramyocellular IL-6 protein 3h later. Again, this statement could not be evaluated from the sparse immunohistochemistry data presented. The possibility exists that the evaluation of IL-6 stainings was influenced by knowledge about mRNA behavior in the present or previous studies.

The above deficiencies raise serious doubts above the article as a whole, and as described below these doubts are confirmed by the fact that one of the sections shown in this article (Fig.1, panel H) was also used to illustrate an oxidative stress marker (NITT) in paper 4 (Fig. 3, Panels D and F). Furthermore, the group has subsequently published conflicting findings

as regards IL-6 protein expression in resting myocytes and distribution among various fiber types (see comments on Paper 6 below).

However, even if the data had been correct they would not justify the conclusion. An accumulation of IL-6 in myocytes was found, whereas a depletion would be necessary to account for the release of IL-6 from an exercising leg and the accompanying increase in plasma IL-6 concentrations. Furthermore, from endocrinology It should be common knowledge that generally an endocrine gland has hormone stores which are depleted early, when hormone secretion is stimulated. This knowledge should have made the authors suspicious as regards the data.

And if not earlier they knew these arguments from a CMRC (Copenhagen Muscle Research Centre) Scientific Meeting the 19th of June, 2003, before acceptance of the paper for publication. At that meeting the group presented its IL-6 findings, and during the discussion I pointed out the lack of consistency between their results and what would be expected from endocrine physiology. After the meeting I received a letter from a foreign student working in BK Pedersen´s (BKP) laboratory blaming me for criticizing such an extraordinary scientist (BKP). Because I had a special responsibility within the CMRC, I wrote him a lengthy reply stating, among other things, that the progress of science depends on competent criticism, that the criticism basically is not personal, and that, in the discussion, apart from the critique of the data, I had also made constructive proposals.

Other criticism:

A one-way ANOVA was used. Considering the fact that two groups were included in the study, either this was not a correct approach (should be a two-way ANOVA) or the data analyzed statistically (IL-6 mRNA and plasma concentrations) were, in contrast to the impression given to the reader, only obtained in the exercise group.

In the present, as in the other papers, it was not apparent, whether p-values represented one- or two-tailed statistical testing.

Paper 2. Fischer CP, Hiscock NJ, Penkowa M, Basu S, Vessby B, Kallner A, Sjoberg LB and Pedersen BK: Supplementation with vitamins C and E inhibits the release of interleukin-6 from contracting human skeletal muscle. J Physiol. 558: 633-645, 2004.

In this investigation Penkowa was the third and BKP the senior among eight authors indicating a non-dominant responsibility for the final paper. Fourteen young healthy males were randomized to receive either a supplement of vitamins C and E or placebo for 28 days. They then had femoral arterial and venous catheters inserted and carried out moderate knee-extensor exercise for 3 h. Biopsies from the vastus muscle were sampled before exercise as well as 3 and 6 h later. It was concluded that supplementation with vitamins C

and E attenuated the systemic IL-6 response to exercise primarily via inhibition of IL-6 protein release from the contracting skeletal muscle fibers.

Major concerns:

The presented findings disagreed with "common sense" and were mutually disagreeing to an extent that should have made the authors cautious as regards the data and the conclusions; the more so because they had been warned as described above.

Thus:

1.During exercise, in control subjects the histochemical data indicated accumulation of IL-6 in muscle cells, while IL-6 was released from the leg into the venous blood. This is not in obvious accordance with the belief that released IL-6 originated in muscle cells.

2.During exercise, the accumulation of IL-6 in myocytes was similar in the two groups, even though IL-6 was released from the leg in control subjects, but not in vitamin treated subjects.

3.During the 3 h postexercise recovery period a similar marked decrease in intramyocellular IL-6 took place in both groups. This was, in fact, accompanied by release of IL-6 from the legs of both groups. However, the release was much higher in controls than in vitamin treated subjects. The difference between the groups in the relationship between IL-6 depletion and leg release, respectively, was not explained.

To reconcile these various findings within the frames of the overall conclusion is probably not possible, and an attempt was not made.

The objections concerning the histochemical data are the same as described for Paper 1. "Significant increases" in IL-6 expression in muscle fibers in response to exercise were reported, but this was not based on quantification and statistical testing. It was not described how sections were evaluated; it should have been done blindfolded, preferably by more than one investigator. Single sections from three time points obtained from a subject from each group were presented in a figure. However, it was not stated, whether all samples representing controls or vitamin treated subjects were from the same subject.

Based on comparisons between neighbouring sections stained for ATPase and IL-6, respectively, the authors concluded (in contrast to findings in Paper 1) that in response to exercise IL-6 was accumulated predominantly, but not exclusively, in type 1 muscle fibers. It was not reported how many fibers were evaluated, and no attempt was made to give a quantitative estimate of the distribution of IL-6 positive fibers among fiber types.

IL-6 mRNA increased similarly with exercise in the two groups, and responses were comparable to those seen in Paper 1. Again, unfortunately only fold changes were

presented. This makes it impossible to evaluate the actual amounts in the tissue and, accordingly, whether the mRNA most likely originated in myocytes or in less dominant cells. In the present, in contrast to the previous study (Paper 1), IL-6 mRNA and protein levels in muscle declined in parallel after exercise. This discrepancy was not mentioned, even though the time relationship between the two variables was emphasized in paper 1.

Other concerns:

Subject characteristics of the two groups ought not to differ. Correspondingly, no indication of statistical difference was shown in Table 1 regarding BMI (23.5+/- 0.3 (SE), vitamin group, vs 25.9 +/- 0.3, control group, kg/m2 (unit incorrectly stated)) and maximal power (150 +/- 3 vs 140 +/- 3 W). Nevertheless, the values appear to differ. In line with this supposition, during exercise at 50% of maximal power, leg blood flow also appeared lower (20%) in controls compared with vitamin treated subjects (Fig. 1).

Paper 3. Akerstrom T, Steensberg A, Keller P, Keller C, Penkowa M and Pedersen BK: Exercise induces interleukin-8 expression in human skeletal muscle. J Physiol 563: 507-516, 2005.

In this investigation Penkowa was fifth among six authors indicating a non-dominant responsibility for the final paper. BKP was senior and corresponding author. Two protocols were used. In one (study 1), young healthy men were randomized to carry out moderate ergometer cycling for 3 h or to rest, while in the other (study 2), young men performed moderate knee extensor exercise for 3 h. Muscle biopsies were taken in both experiments (but apparently only used for histochemistry in study 1). In study 2 femoral catheterization was used.

Concerns:

Subject population: It is not clear, whether, in study 1, eleven, or twelve, or maybe eighteen subjects participated. Thus, in the Methods section it was stated that the number was 11. However, according to the legends of Figs. 1 and 3, six subjects were included in each of the two groups (exercising or resting). The suspicion arises that one subject may have been excluded without this being mentioned, because it would be natural to have equal numbers of subjects in the two groups. Furthermore, in papers 1 (see above) and 4 (see below) 18 subjects (12 exercising and 6 resting) carried out a protocol identical to the one used in the present study (exercise and repeated biopsies for 24 h). The characteristics of the subjects in the three papers were remarkably similar (age, height, weight and Vo2max), a fact leading to the question, whether the subjects (and, accordingly, tissue samples) used in the present study were a subgroup of the subjects participating in paper 1 and 4, the material studied in the three papers being basically identical.

The suspicion that the three studies represented the same material is further augmented by the fact that in the present paper (on p. 511), it was stated that "IL-8 protein was not expressed in muscle tissue before exercise (n=12)", indicating that the exercise group overall included 12 subjects. Moreover, although in Paper 4, according to information given in the Methods section, twelve subjects participated in exercise, while 6 subjects rested, the legend of Fig. 1 reports 6 exercising and 5 resting subjects – corresponding to the numbers stated in the Methods section of Paper 3.

Histochemistry: Data were illustrated by one section stained for IL-8 protein from each of the six time points at which muscle was sampled (Fig. 2). In addition, two sections at 2-4 times higher magnification, and a section colored for various fiber types were shown. It was not stated, how sections were selected, e.g. if they were from one or more subjects, or evaluated (e.g. one investigator?, blindfolded?). They were all of poor quality and did not confirm the text, which stated that IL-8 was expressed in cytoplasm, membranes (which?), nuclei, and intermittently (meaning?) in endothelium.

Also, in the text (p. 511), it was stated that 21 h after exercise there was a strong immunoreaction for IL-8, whereas in the legend of Fig. 2 it was stated that at this time point, the staining was mildly increased. More devastating, however, the section representing the 21 h time point appears to be the same as the one representing the 0 h (pre-exercise) time point. And this section was also used in Paper 5, where in Fig. 3D, it was said to illustrate the presence of IL-6 receptor protein in muscle fiber membranes.

IL-8 mRNA: It was stated in the Discussion section (p. 513) and in the conclusion (p. 514) that exercise induced a marked increase in IL-8 mRNA within muscle fibers. However, the real time PCR analysis used does not allow localization of the measured mRNA. Furthermore, only fold-changes in mRNA from pre-exercise values were reported. Critical threshold/ number of cycles needed to reach detection (CT values) for the analysis should have been reported in order for the reader to be able to evaluate how big a cell population might contribute to the mRNA measurements. Furthermore, the absence of IL-8 protein (according to histochemistry) in the presence of the corresponding mRNA at rest requires an explanation.

Calculation of IL-8 release from the leg: Femoral veno-arterial plasma concentration differences (in Fig. 5 with the wrong unit: ng/min) were incorrectly multiplied with leg blood flow instead of with plasma flow. It is surprising that in the previous paper with the same senior author (Paper 2) the corresponding calculation for IL-6 release was correctly carried out. In the middle of the exercise period a significant increase in IL-8 release above basal levels was found. However, before as well as after that time point, at rest and during exercise and recovery, IL-8 tended to be taken up in the leg rather than being released into the bloodstream. This pattern is quite unusual, yet the authors did not comment on that. No

change in arterial (systemic) IL-8 concentration occured. The conclusion drawn by the authors that muscle-derived IL-8 may "exert its effect in an endocrine or paracrine fashion" (p. 514), even though tentative (and ambiguous), was therefore not justified by their results.
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Paper 4. Penkowa M, Keller P, Keller C, Hidalgo J, Giralt M and Pedersen BK: Exercise-induced metallo-thionein expression in human skeletal muscle fibres. Exp Physiol 90, 477 – 486, 2005.

In this investigation Penkowa was first and corresponding author, while BKP was senior author. The protocol was identical to that in study 1 of Paper 3.

Concerns:

Histochemistry: Histochemical findings were presented in Fig. 2 (metallothionein expression) and Fig. 3 (expression of the oxidative stress marker nitrotyrosine, NITT). The figures illustrated time courses by showing one section from each time point of muscle biopsy. However, in Fig. 3 the section representing muscle at the end of 3 h of exercise (3D) was the same as the one representing muscle 3 h after exercise (3F), and the very same section was used in Paper 1 to illustrate IL-6 expression in muscle 21 h after exercise (1H).

As was the case with histochemical stainings in the other papers, it is not clear how the presented sections were selected, nor how sections were evaluated. No attempt was made to quantify staining; nevertheless the term "insignificant immunoreactivity" was used. Even though the finding of exercise-induced metallothionein expression was ascribed to oxidative stress, it was not studied on neighbouring sections, whether observed variation in metallothionein content between fibers was accompanied by variation in NITT.

Subject population: As mentioned above (see Paper 3 "Subject population"), discrepancy exists between the number of participants stated in the Methods section (12 exercising and 6 resting=18) and legend to Fig. 1 (on metallothionein mRNA, 6 exercising and 5 resting=11), respectively. Furthermore, albeit not stated, there is reason to suspect that subjects and muscle samples were identical to those of Papers 1 and 3 and also overlapped with those of Paper 5 (see below). The anthropometric data presented in the three papers were compatible with this suspicion.

Metallothionein mRNA: It was stated on p. 480 that mRNA levels were hardly detectable, i.e. essentially not quantifiable, at rest. Nevertheless, fold changes with exercise were calculated. CT-values should have been included. The conclusion in Abstract and beginning and end of Discussion that metallothionein mRNA is expressed in both type 1 and 2 muscle fibers after exercise was not justified by the findings, because in situ hybridization was not performed.

Minor:

In Fig. 1 data were stated to present geometric means +/- SEM. This is not correct as dispersion from means was not symmetrical.

Paper 5. Keller P, Penkowa M, Keller C, Steensberg A, Fischer CP, Giralt M, Hidalgo J and Pedersen BK: Interleukin-6 receptor expression in contracting human skeletal muscle: regulating role of IL-6. FASEB J 19: 1181-1193, 2005.

P. Keller was first and corresponding author, while Penkowa was second and BKP senior author on this paper. Two human and one mouse experiment were carried out.

Major concerns:

Subject population: In one of the human studies, the same ergometer exercise protocol and the same time schedule for blood and vastus muscle biopsy sampling as used in Papers 1, 3 and 4 were applied. Furthermore, compatible with some of the numbers given in papers 3 and 4, in the present protocol six subjects exercised, while only five subjects rested throughout. The anthropometric data presented suggest that study populations and tissue material were identical in the four studies. Correspondingly, in the Discussion it was written that the IL-6 plasma levels obtained were higher in infusion experiments (see below) than in exercise experiments. This is so, although plasma concentration measurements had not been mentioned earlier in the paper. Probably, the measurements referred to are those presented in Paper 1. Finally, the muscle section illustrating IL-6 receptor presence 21 h after exercise in the present study (Fig. 3 D) was in Paper 3 used to illustrate IL-8 presence before exercise.

If the material of the four papers was derived from the same experimental study, this was not mentioned in Paper 5, and papers 3 and 4 were not quoted, while the citation of Paper 1 did not mention an intimate relationship with the present study.

In the other human study recombinant human IL-6 or saline were infused into a femoral artery. This appears to be an unethical approach, if not justified by another purpose than that of the present study. It was not stated from where blood samples were drawn. However, in a preceding study of FFA kinetics from BKP´s laboratory one femoral artery was used for infusions identical to those of the present study, while blood was sampled from the other femoral artery (van Hall et al, J Clin Endocrinol Metab 88: 3005-3010, 2003). The subject characteristics and the plasma IL-6 concentrations appear to be identical in the two studies. So, one may suspect that although it was not stated the two resulting papers were based on the same study.

Histochemistry: Time courses of IL-6 receptor immunohistochemistry in exercise and infusion experiments, respectively, were shown in Figs. 2 and 3. One section was shown for

each time point of muscle biopsy. Most devastating, the section used to illustrate IL-6 receptor presence 21 h after IL-6 infusion (Fig. 3D) was the same as the section used in Paper 3 to illustrate the presence of IL-8 in muscle before (Fig. 2A) and 21 h after (Fig. 2G) exercise. It was not reported how sections were selected for illustration (e.g. representing the same person at all time points?), neither how they were evaluated (e.g. blindfolded?, by more than one investigator?).

In the Discussion it was stated that IL-6 receptor was uniformly expressed in both type 1 and type 2 muscle fibers. However, no evidence for this was presented, and in the Methods section staining for fiber types was not mentioned. While after exercise a very prolonged increase in IL-6 receptor mRNA was paralleled by an increase in staining of the receptor in plasma membrane, in response to IL-6 infusion an increase in receptor staining was, surprisingly, seen in the absence of an increase in mRNA. Here as in the other papers, Western blotting of IL-6 protein would have been appropriate to support histochemical findings.

IL-6 receptor mRNA: In the human experiments the evaluation of data is rendered difficult by the fact that values were reported as fold changes from unreported basal levels. CT-values should have been presented.

Other concerns:

Mouse study: Mice had muscle sampled either before or after swimming. In order for this experiment to be reproducible by other investigators swimming conditions should have been better described (i.e. water depth, number of mice swimming simultaneously per unit water surface). Because skeletal muscles differ, the exact muscle from which samples were obtained should have been stated.

Conclusion in human study: It was stated in the final paragraph that “contractions induce post-exercise expression of IL-6 receptor mRNA and protein levels in human skeletal muscle, possibly to sensitize muscle to the decreasing IL-6 levels elicited by exercise”. However, exercise does not elicit a decrease in IL-6 levels.

Paper 6. Plomgaard P, Penkowa M and Pedersen BK: Fiber type specific expression of TNF-alpha, IL-6 and IL-18 in human skeletal muscles. Exerc Immunol Rev 11: 53-63, 2005.

Penkowa was second, while Plomgaard was first and corresponding, and BKP senior author on the paper. In this small investigation of seven young healthy subjects, the content of three cytokines was determined by immunohistochemistry in the basal state in three human muscles (triceps, vastus lateralis, soleus) known to have different proportions of type 1 and type 2 fibers. Adjacent muscle sections were stained for cytokines and fiber types, respectively.

Major concerns:

In contrast to Paper 1, in the present study IL-6 was found in muscle fibers at rest. This essential discrepancy between findings in the present and previous study by the group was not even mentioned, and, accordingly, no attempt was made to explain it. On the other hand, previous conflicting data regarding IL-6 distribution between fiber types upon contraction were discussed. It was stated that in Paper 1 intense exercise to exhaustion was applied. However, the exercise cannot be considered to be of high intensity, and in the paper it was not described as exhausting either.

In the Title, Abstract ("TNF-alpha and IL-18 being solely expressed by type 2 fibers") and end of the Discussion ("the novel finding of the present study is that normal resting human skeletal muscles express TNF-alpha, IL-8 and IL-6 in a strict fiber-type specific fashion"), specificity of cytokine expression in different muscle fibers types was emphasized. However, in the first paragraph of the Discussion the message was more equivocal: "The expression of TNF-alpha and IL-18 is predominantly found in the type 2 fibers whereas IL-6 is most abundant in type 1 fibers". It is not possible for the reader to evaluate the underlying evidence. Thus, it was not stated how sections were evaluated (how many fibers per section?, by more than one investigator?), and attempts to quantify findings (e.g. simply: per cent of type 1 and 2 fibers, respectively, positive for a given cytokine) were not made.

Cytokine expression detected by immunohistochemistry was not reflected at the mRNA level measured by rtPCR on muscle biopsies. In fact, the variation in IL-6 mRNA expression between the studied muscles tended to be the inverse of what would be expected from histochemistry data, mean IL-6 mRNA content being highest in triceps ("type 2 muscle") and lowest in soleus ("type 1 muscle") muscle (Fig. 3). This discrepancy between contents of cytokine mRNA and protein was suggested to be due to post-transcriptional regulation. However, in order to justify the main conclusion about fiber type specificity of cytokine expression as well as to elucidate the discrepancy between cytokine protein and mRNA contents, in situ hybridization should have been carried out and compared with fiber type composition.

mRNA for myosin heavy chain (MHC 1 and 2a) was also determined. It was claimed that the MHC 1 mRNA level, as would be expected from previous studies, reflected the corresponding protein level (judged from fiber typing). However, this is not fully correct, because the content of this protein was considerably higher in soleus compared with vastus lateralis muscle, whereas mRNA was not.

Reuse of material and data: See comments to Papers 7 and 10.

Minor concerns:

For immunohistochemistry, sections were incubated with goat serum to block non-specific binding. Some control sections were incubated without the blocking serum. It is not immediately apparent, why immunoreactions “were considered only if this control was negative”.

Paper 7. Plomgaard P, Penkowa M, Leick L, Pedersen BK, Saltin B and Pilegaard H: The mRNA expression profile of metabolic genes relative to MHC isoform pattern in human skeletal muscles. J Appl Physiol 101: 817 – 825, 2006.

Plomgaard was first and corresponding, Penkowa second and BKP fourth among six authors of this paper. As in Paper 6 seven young healthy subjects had muscle biopsies (from triceps, vastus lateralis and soleus) taken in the basal state.

Major concerns:

Material: Age, weight and BMI of the subjects in the present paper were identical to corresponding values in Paper 6, and also the experimental protocol was the same. Furthermore, muscle biopsies were analyzed for fiber type distribution and myosin heavy chain (MHC1 and 2a) mRNA content in both papers. Data on these variables, although being presented a little differently in the two papers, appear to be identical, apart from MHC 2a values, which seem to be slightly lower in Paper 6 than in Paper 7. Apparently, subjects and material were the same in Papers 6 and 7 (as well as in Paper 10, see below), respectively, and the same data were included in both (all three) papers. This reuse was reported in none of the papers.

Correlation analysis: The paper was widely based on correlation analysis between mRNA content of various metabolic genes determined on muscle biopsies on the one hand and % type 1 fibers in the biopsies on the other. In each analysis measurements from all subjects and the three muscles were included. This is not a correct statistical approach. Each individual can only contribute to the analysis with one pair of measurements. Furthermore, because % type 1 fibers varied considerably and was well separated between the three muscles, significant correlations were found between mRNA and % type 1 fibers, although it is apparent from the data plots that no such correlation existed within the individual muscles. Accordingly, the conclusion drawn i.e. that some metabolic genes are fiber type specific (should be: dependant) is not justified by the correlation analysis performed. Rather, the differences in mRNA between the three muscles reflected other differences between the muscles than fiber type content. Finally, it is of note that data from the soleus muscle of one of the seven subjects were not included in the correlation analyses. The reason was not stated.

Paper 8. Frydelund-Larsen L, Penkowa M, Akerstrom T, Zankari A, Nielsen S and Pedersen BK: Exercise induces interleukin-8 receptor (CXCR2) expression in human skeletal muscle. Exp Physiol 92: 233 – 240, 2007.

Penkowa was second and BKP senior and corresponding among the six authors of this paper. The hypothesis of the authors was that muscle-derived IL-8 plays a role in stimulation of angiogenesis in response to exercise. The aim of this study, then, was to investigate, whether the IL-8 receptor (CXCR2) is expressed in human skeletal muscle, and whether it co-localizes with the TGF-beta receptor (CD105), which was known to be expressed in activated endothelial cells. Healthy young males were studied either before and after 3 h of moderate cycle ergometer exercise or during an identical period of rest.

It is of note that it was explicitly stated that the design was identical to that used in Paper 3 but that different subjects participated in the two studies. A similar statement was not given in Papers 1, 3, 4 and 5, respectively, and neither in Papers 6 and 7, respectively, a fact supporting the supposition that material from the same subjects were used in those papers (see above).

Major concerns:

Immunohistochemistry: According to the description given in the Methods section, staining for the IL-8 receptor and subsequent examination and recording followed the same procedures used in the former papers (primary antibody against the receptor, detection by secondary biotinylated antibody and streptavidin-biotin-peroxidase complex; examination by light microscopy). Staining for the TGF-beta receptor was not described. Furthermore, in the Results section the stainings mentioned were not reported. On the other hand, results of double immunoflurescense staining for the two receptors were presented (Fig. 2), although neither the fluorescence labeling nor the fluorescence microscopy had been previously described. So, regarding the immunohistochemistry the Results and the Methods sections did not correspond.

In the figure one muscle section from each of six time points before and after exercise was shown. Only sections stained for both receptors, and not sections stained for each alone, were shown. It was also not described, how sections were selected, e.g. whether they were from the same or different subjects, and how they were examined (e.g. by more than one investigator ?, blindfolded ?). It was stated that the IL-8 receptor was “low or absent” in skeletal muscle before and during the first 1.5 h after exercise, but had increased at 3 hours and peaked 6 hours after exercise. However, no attempt was made to quantify the receptor protein content. Western blotting for the receptor protein seems warranted, the more so because of the apparent discrepancy between the absence at rest of immunohistochemically detectable IL-8 receptor protein in the face of expression of IL-8 receptor mRNA.

Essential conclusions not supported by data or imprecise: The authors concluded that the IL-8 receptor "is localized primarily to activated microvascular endothelium". This interpretation fits with the authors´ hypothesis on the role of muscle-derived IL-8. However, according to the illustration (Fig. 2), the receptor was primarily localized to the sarcolemma or adjacent cytoplasm (higher magnification than the one presented is warranted). It was also concluded that 21 h after exercise, the expression of receptor protein content had decreased to pre-exercise levels. Again, this is not in agreement with Fig. 2. The statement that the IL-8 receptor was low or absent in muscle before exercise and during the first 1.5 h after (p. 236) disagrees with the statement (p. 236) that the protein was expressed within the muscle fibers and at the sarcolemma at all time points. Another conclusion was that "exercise induces CXCR2 mRNA and protein expression in the vascular endothelial cells of the muscle fibers" (cf. Abstract). However, because in situ hybridization was not carried out, the cellular origin of the observed increase in receptor mRNA is unknown. Finally, in the Discussion, the present findings of increased IL-8 receptor mRNA and protein in muscle was associated with the finding in Paper 3 of a release in IL-8 from muscle, and a local role of IL-8 in muscle was suggested. However, it was not mentioned that a release in IL-8 was only seen in the middle of a 3h exercise session, whereas an increase in IL-8 receptor expression in muscle was not seen until after exercise.

mRNA measurements: As judged from the description of the PCR analysis, where undiluted cDNA was used for CXCR2 measurement, the expression of the CXCR2 gene was very low in absolute terms. This is also supported by the high variation (100%) between baseline values in exercising and resting subjects, respectively, (Fig. 1), which is indicative of very low copy numbers in the biopsy. The implication for the interpretation of the claimed protein expression was not discussed. Furthermore, the low CXCR2 RNA level was not readily apparent to the reader, because basal CXCR2/GAPDH (house-keeping gene) mRNA ratios shown in Fig. 1 were close to 1. This could not reflect ratios between measurements in absolute terms, but probably reflected use of some normalization procedure, which, however, was not described to the reader.

Minor:

It was stated that data were presented as geometric means +/- S.E.M. However, in Fig. 1 (data on IL-8 receptor mRNA) the dispersion was symmetrical around the mean values.

Paper 9. Petersen AM, Penkowa, Iversen M, Frydelund-Larsen L, Andersen JL, Mortensen J, Lange P and Pedersen BK: Elevated levels of IL-18 in plasma and skeletal muscle in chronic obstructive pulmonary diseases. Lung 185: 161 – 171, 2007.

Penkowa was second and BKP senior and corresponding author on this descriptive study. Plasma and biopsies from the vastus lateralis muscle were sampled from 20 patients with chronic obstructive pulmonary disease and 20 healthy controls.

Critique:

Subjects: It was repeatedly stated (Abstract, Introduction and Discussion) that controls were matched with patients according to age, gender and BMI. However, the statement in the Methods section that “controls were excluded as a result of BMI greater than 30” indicates that no genuine matching procedure was carried out. Still, controls were not randomly picked and, accordingly, the statistical testing for differences in age, gender and BMI carried out between patients and controls was incorrect.

For patients an inclusion criterion was smoking abstinence for at least two months. Nevertheless, in the control group, in addition to ten nonsmokers, ten non-symptomatic smokers were included. The rationale for using smoking controls is not clear, and data were not discussed in the context of impact of ongoing smoking. Four patients were on oral steroid therapy, and it was stated in the Results section that differences between groups in leukocytes and CRP were maintained, if these patients were excluded. However, it was not reported, whether cytokine findings, which certainly might be influenced by steroid therapy, differed between these and the other patients.

Methods: The description of RNA extraction dealt with adipose tissue, which was not studied in the present paper. It is confusing that ATPase staining for fiber type identification was described in two separate sections. Illustrating the confusion, in the interposed section on immunohistochemistry the authors referred to the subsequent section for ATPase staining, but later it becomes clear that the preceding section on ATPase staining was the relevant one.

It was stated that muscle sections were stained for IL-8 (p. 164). Accordingly, it is surprising that IL-8 results, in contrast to IL-18 results, were not reported and compared with previous IL-8 findings by the group. This the more so, since in Paper 8 the angiogenic potential of IL-8 and its interaction with hypoxia were emphasized, while in the present study patients, in fact, suffered from hypoxia and had increased capillary density in skeletal muscle.

Histochemistry: Two figures were presented. Fig. 3 served to illustrate that IL-18 and Caspase-1 levels were very low or absent in muscle fibers from healthy subjects, whereas in COPD patients an increase in expression (surprisingly high and, furthermore, increasing with a 2.5 fold fiber magnification) of these peptides was found. From comparison with neighbouring sections stained for fiber typing it was concluded that the peptide expression mainly occured in type 2 fibers.

Fig. 4 served to illustrate that TNF-alpha and IL-6 contents were also very low in controls, whereas in patients an increase in TNF-alpha was found. It is stated that this was located mainly in type 2 fibers, but, somewhat surprisingly, no section stained for fiber typing is included in this figure. IL-1 beta was “hardly expressed” in any of the groups. This is

somewhat surprising, because IL-1 beta and IL-18 were stated to be closely related and both activated by Caspase-1, which was increased in COPD patients. This discrepancy was not discussed.

IL-18 mRNA levels in muscle – shown in Fig. 2, but without units – were also higher in patients than in controls. In contrast to this parallelism between mRNA and protein, a significant difference of similar magnitude in IL-18 mRNA between smoking and nonsmoking controls (Fig. 2) was not accompanied by a difference in histochemically determined IL-18 protein between these groups (Fig. 3), a finding which was not discussed. Lack of parallelism between mRNA and protein expression was also observed for TNF-alfa, mRNA levels in muscle being at least as high in controls as in patients (Fig. 2). It was suggested that this might reflect a negative feedback by elevated TNF protein on TNF transcription. However, such a mechanism would not be able to fully inhibit an initial rise in mRNA.

Lack of correspondence between mRNA and protein findings also occurred for IL-6, although apparently this went unnoticed by the authors. Thus, IL-6 mRNA was identical between groups (Fig. 2), whereas histochemically determined IL-6 protein was described as “very low” in controls but, nevertheless, “reduced below the levels of controls” in COPD patients. The depicted findings call for a much more exhaustive description of the evaluation of the immunohistochemical data than the one presented (e.g. were sections evaluated blindfolded and by more than one investigator? How many fibers were evaluated and were sections from all subjects examined? Were attempts to quantify staining made?). Also, Western blotting should have been carried out to confirm the histochemical findings.

Other findings: Values presented for plasma levels of IL-8 were 2-4 times as high in the Abstract as in Fig. 1.

Conclusion: Because elevated skeletal muscle expression of IL-18 was found in COPD patients with normal body weight, it was concluded (cf. Abstract) that IL-18 potentially may be involved in COPD-associated muscle wasting. However, the opposite conclusion seems more obvious: Despite increased IL-18 expression no wasting had occured, not even in two patients receiving oxygen therapy and six patients waiting for pulmonary transplantation. In this context, it is surprising that no comparisons were made between the observed changes in various cytokine measurements and muscle histology (e.g. capillarization) on the one hand and the clinical condition of the patients (e.g. degree of hypoxia) on the other.

Minor:

In addition to mRNA (Fig. 2) (see above), units are also missing for BMI and BMD (Table 1), and for fiber size (Table 2). P-values were not given in Table 1.

Paper 10. Nielsen AR, Mounier R, Plomgaard P, Mortensen OH, Penkowa M, Speerschneider T, Pilegaard H and Pedersen BK: Expression of interleukin-15 in human skeletal muscle - effect of exercise and muscle fiber type composition. J Physiol 584: 305 – 312, 2007.

Penkowa was fifth and BKP senior and corresponding among the eight authors. The investigation included two separate studies of healthy young men.

Major concerns:

Muscle biopsies and data from a former study (Paper 6 and 7) seem to have been reused in the present study, but this was not brought to notice. Supporting the suspicion, the protocol used in Study 1 in the present paper was the same as that used in Papers 6 and 7 (biopsies being taken from triceps, vastus lateralis and soleus muscles in the basal state and used for RNA isolation and histochemistry). Furthermore, although it was stated in Methods that 14 subjects took part in this study, it appears from both the legend of Fig. 2 and the section on fiber type composition under Results (p. 310) that histochemistry was only performed in seven subjects, as was the case in Papers 6 and 7. Finally, the range of type 1 fibers in each of the three muscles examined (p. 310) was exactly the same as reported in Paper 7.

This reuse must have been apparent to at least those four, who were authors of both papers. Thus, although Myosin Heavy Chain mRNA 2a was not measured in the present study, in the Results section a negative correlation between this variable and occurrence of type 1 fibers was reported (p. 310). The same finding was reported in Paper 7, and Penkowa did only provide fiber type, not MHC data. Whether the original group of seven subjects from Papers 6 and 7 was supplemented with seven new subjects, or whether 14 new subjects did not contribute to the histochemistry, is not clear.

Illustration of histochemical findings has flaws and deficiencies (Fig. 2). One section stained for IL-15 was shown for each of the three muscles. It was not described how these sections were selected (e.g. from the same subject). It was stated that neighbouring sections were stained for fiber typing, and identical fibers stained for IL-15 and fiber types, respectively, were allegedly depicted by asterisks. However, in conflict with this the two sections from vastus lateralis muscle (c and d) and the fibers depicted in these were apparently not identical. In the figure comparison was also made between a section stained for IL-15 and negative control sections (e.g. incubated without antibody against IL-15). These sections should have been neighbouring, but they obviously were not. In general, the description of the evaluation of the sections was insufficient (e.g. number of fibers evaluated ?, more than one investigator ?).

Lack of parallelism between IL-15 mRNA and protein measurements. In study 1 IL-15 mRNA was significantly higher in triceps than in soleus muscle, and values in vastus were between

these. However, according to both Western blotting and immunohistochemistry IL-15 protein levels did not differ between the three muscles. In study 2 eight subjects carried out knee extensor resistance exercise and had blood and muscle biopsies taken before that and 6, 24 and 48 h later. A significant increase in IL-15 mRNA in muscle was seen at 24 h, whereas no change in IL-15 protein determined by Western blotting was found (histochemistry was not performed). The fact that mRNA increased somewhat late relative to the exercise stimulus was not discussed. The lack of parallelism between mRNA and protein findings in the two experiments was ascribed to existence of transcription without translation, a very unsatisfactory explanation.

Paper 11. Plomgaard P, Nielsen AR, Fischer CP, Mortensen OH, Broholm C, Penkowa M, Krogh-Madsen R, Erikstrup C, Lindegaard B, Petersen AM, Taudorf S and Pedersen BK: Associations between insulin resistance and TNF-alpha in plasma, skeletal muscle and adipose tissue in humans with and without type 2 diabetes. Diabetologia 50: 2562 – 2571, 2007.

Penkowa was the sixth and BKP the senior among 12 authors on this paper. More subjects participated in this than in previous similar studies by other groups. However, some essential variables were only determined in subgroups.

Critique:

Histochemistry: Previous contrasting findings by the group were not mentioned. Thus, a major conclusion in Paper 6 was that in the basal state immunohistochemically determined TNF-alpha is expressed in skeletal muscle, and, furthermore, only in type 2 fibers. Nevertheless, in the present paper, it appears from Fig. 3 that in control subjects studied in the basal state, no TNF-alpha staining could be demonstrated. This discrepancy was not discussed. In fact, Paper 6 was not quoted at all. This is the more surprising because, in Paper 6, the authors quoted a study, in which it was found that TNF-alpha mRNA and protein in humans increased in elderly subjects. In the present study subjects were, in fact, elderly, whereas young subjects were studied in Paper 6.

The conclusion from the histochemistry findings of the present study was that in type 2 diabetics TNF-alpha is increased in skeletal muscle, in particular in an -undefined- subgroup of type 2 fibers. Again, considering the conclusion in Paper 6, it is surprising that the authors emphasized the occurrence of TNF-alpha in type 2 fibers rather than the fact that the presence of TNF-alpha in type 1 fibers also seen in the type 2 diabetic subjects was in contrast to their previous finding that these fibers contained no TNF-alpha in healthy subjects. Finally, the marked difference in TNF-alpha staining of muscle fibers between diabetics and controls shown in Fig. 3 contrasted with the presented Western blot data, according to which TNF-alpha protein content in muscle was on average at most only twice as high in diabetics compared with controls (Fig. 2). This fact was not noted.

As in the other papers the description of the evaluation of the histochemical data was insufficient (e.g. in the present paper: How many fibers were evaluated per section, and what was the percentage of type 2 fibers showing increased TNF-alpha in the diabetics, i.e. the size of the putative subgroup. Were sections evaluated by more than one investigator, were they evaluated blindfolded?). In this as in several previous papers negative control sections were incubated without the goat serum used to block potential nonspecific binding of the primary and secondary antibodies. However, as mentioned in the review of Paper 6, the relevance of this procedure is not clear, and it was not used in the immediately preceding Paper 10. So, it is surprising that it was taken up again here.

Western blotting: A "cleaved" form of TNF-alpha protein in addition to the membrane-bound proTNF-alpha was found in muscle. According to the text and the blots shown (Fig. 2) the amounts of proTNF were higher than those of the cleaved form. However, this is in contrast to Fig. 2, panels b and c, where values were expressed relative to beta-actin protein, and the reverse relationship seems to hold. It was also stated in the Results section that levels of both forms were higher in diabetics than in controls, and in the summary of findings in the beginning of the Discussion that TNF-alpha was elevated in muscle of diabetics compared with controls regardless of the degree of obesity. However, according to Fig. 2b and the Results section, the difference was not significant for proTNF in non-obese subjects. The legend of the figure states that a one-way ANOVA was used, but this contrasts with the outcome described in the legend as well as with the Statistics section.

No differences between patients and controls in TNF-alpha mRNA in muscle (and in adipose tissue) were found. In the present paper the discrepancy between TNF-alpha mRNA and protein levels was tentatively ascribed to posttranscriptional regulation of the protein content, whereas, in Paper 10, a similar discrepancy was ascribed to negative feedback inhibition of the mRNA.

Material and other methods: It was stated in the Introduction as well as in Methods that a case-control design was used. However, this is not correct as controls were dealt with as a group and not matched individually with patients. V02 max as well as fat mass values were reported, but the methods used to analyze these variables were not.

Main conclusion: Based mainly on correlation analysis between plasma concentrations of TNF-alpha and a simple index related to insulin resistance (HOMA2-IR) it was concluded that plasma TNF-alpha is associated with insulin resistance and may play a role in the pathogenesis of chronic insulin resistance. This conclusion seems daring in light of the fact that the correlation was not significant in control subjects. Furthermore, in diabetics the correlation coefficient was only 0.3 and, judged from presented p-values, considerably lower when adjusted for various confounders.

Central unintelligible/ illogical statements; examples: At the end of the Abstract (and similar sentence at the end of Discussion): “However, findings on the TNF-alpha protein level in plasma and skeletal muscle indicate that measurement of TNF mRNA content in adipose or muscle tissue provides no information with regard to the degree of insulin resistance”. In the Discussion, in the context of the finding that TNF-alpha mRNA in adipose tissue did not differ between patients and controls: “Visceral fat in particular has been suggested to have a high level of TNF-alpha (27,35), which is a possible explanation for the lack of difference in subcutaneous adipose tissue between controls and diabetic patients in the present study”. In the Discussion, in the context of the finding of no difference between groups in skeletal muscle TNF-alpha mRNA: “However, patients with type 2 diabetes who performed a resistance exercise demonstrated improved insulin sensitivity despite an increase in TNF mRNA transcripts (37), suggesting no association of insulin sensitivity with TNF mRNA content in skeletal muscle”.

Data dispersion: For several variables this was surprisingly small. E.g. SD can be calculated to be 1-2% for e.g. plasma TNF-alpha, plasma glucose and diastolic blood pressure in healthy subjects. Considering the small dispersions it may also be surprising that it was possible to establish significant correlations, e.g. the 95% confidence interval for sTNFR2 (soluble TNF receptor) was less than 2% in healthy subjects and, nevertheless, this variable correlated significantly with HOMA2-IR.

Paper 12. Matthews VB, Astrøm MB, Chan MH, Bruce CR, Krabbe KS, Prelovsek T, Åkerstrøm T, Yfanti C, Broholm C, Mortensen OH, Penkowa M, Hojman P, Zankari A, Watt MJ, Bruunsgaard H, Pedersen BK and Febbraio MA: Brain-derived neurotrophic factor is produced by skeletal muscle cells in response to contraction and enhances fat oxidation via activation of AMP-activated protein kinase. Diabetologia 52: 1409 – 1418, 2009.

Penkowa was the 11th among 17 authors of this study, which resulted from collaboration between BKP´s group and Australian scientists, and which was co-directed by BKP and Mark Febbraio, who has had a number of papers retracted in recent years. The study consisted in both experiments on humans, electroporation experiments in mice, and in vitro studies on myotubes and rat muscle. It is most likely that the human experiments were conducted in Copenhagen.

Major concerns:

Study population: According to the Methods section eight young men performed two hours bicycle exercise and had blood and vastus muscle biopsies sampled before as well as at multiple time points up to 72 h after exercise. No resting controls were mentioned. In conflict with these informations, in the Results section and legends of Figs. 1 and 2, a resting cohort/ resting controls were mentioned. Analyses from the resting cohort were presented, but the origin and composition of the cohort was not described.

The uncertainty as regards the origin of the study populations is supported by the fact that in Fig. 2 platelet counts were not given in absolute numbers but as percentage of baseline values. Furthermore, while in Fig. 1 the number of subjects was eight, in Fig. 2 the number of subjects was ten, and accordingly higher than the number of subjects included in the study according to the Methods section. It is remarkable that age, weight and height given for the eight subjects included in the present paper were the same as given for the 15 men included in Paper 8.

Brain-derived neurotrophic factor (BDNF) protein: In human vastus muscle, based on Western blotting a significant 50% increase in BDNF expressed relative to beta-actin was found 24 h after exercise compared with pre-exercise values. BDNF mRNA was measured at multiple time points in both exercising subjects and resting controls and peaked 5-8 h after exercise. In light of these facts, it is surprising that BDNF protein in exercising subjects was not measured more frequently before and after the 24 h time point, and that it was not measured in resting controls. Furthermore, it can be doubted, whether the observed 1.5 (-3) -fold increase in BDNF mRNA could account for a 50% increase in BDNF protein, because in previous studies of rat muscle, a 5 fold increase in BDNF mRNA was not accompanied by any increase in protein (ref. 24 in the paper. See also comments on BDNF mRNA below). The immunoblot selected to illustrate the findings does not strengthen the confidence in the measurements as beta-actin contents and image contrast varied considerably between lanes/time points of tissue sampling.

In C2C12 myotubes stimulated electrically to contract in vitro for 2 h, a 70% increase in both BDNF mRNA and protein was found at the end of contractions. The authors did not discuss the observed difference between myotubes and human muscle in the time course of these effects of contractions. In the discussion they concluded that it is likely that muscle-derived BDNF “works in an autocrine and/or paracrine manner”. They did not mention that this view was in conflict with the fact that they did not find any difference in release of BDNF to the medium in contraction-treated versus control C2C12 cells. Because data were not shown, it cannot be evaluated, whether a release occurred at all.

Immunohistochemistry for BDNF protein was performed on muscle samples taken pre-exercise and 24 h post-exercise as well as at identical time points in non-exercising subjects. One muscle section was shown from each of these four categories. It was not reported how sections were selected, how many fibers they represented, and how they were evaluated. An increase in staining, non-uniformly distributed among fibers and appearing gritty, was found 24 h after exercise. The staining looks peculiar and appears to exceed the 50% increase in BDNF found by Western blotting. No comments were made on these features.

BDNF mRNA: The variation between baseline values in exercising compared with resting subjects was high, a fact indicative of low mRNA copy numbers. In agreement with this view

a critical threshold value for BDNF mRNA of above 37, corresponding with mRNA presence in only a small percentage of cells in muscle tissue, has been found in other studies, and also in situ hybridization studies of various skeletal muscles have failed to demonstrate significant BDNF expression within mature myofibers (J Neurosci. 26, 5739, 2006). However, the fact that BDNF mRNA levels in vastus muscle in the present study probably were very low was not readily apparent to the reader, because levels were expressed by some normalization procedure, which was not described. In response to exercise a significant increase in mRNA levels (expressed as fold changes from basal) was not immediately apparent, but could be demonstrated in comparisons of AUCs (area under curve) over time between exercising and resting subjects, respectively. However, the calculation procedure and calculated numbers were not reported, and the procedure would for instance favor a difference, if incremental areas above the differing basal values were used. Anyhow, the, at most small, numerical increase in BDNF mRNA (judged from Fig. 1) above very low basal levels would not be able to account for the widespread increase in intramyocellular BDNF indicated by the immunohistochemical findings.

Other concerns:

A significant increase in palmitate oxidation, albeit less than 10%, was found in L6 myotubes stimulated with BDNF (Fig. 4). However, the illustration of the increase was misleading, because the y-axis did not start from zero, a fact not indicated. Furthermore, dispersions were surprisingly small and differed between similar experiments (SEM being about 1.4% in panel e and up to about 4.3% in panel d).

The authors proposed that BDNF enhanced fat oxidation by stimulating AMPK and, in turn, its downstream target Acetyl coenzyme A carboxylase (ACC). This view was maintained, even though in L6 myotubes ACC was phosphorylated at a lower dose of BDNF than AMPK (Fig. 4), while in isolated rat muscle ACC was phosphorylated earlier than AMPK during treatment with BDNF (Fig. 5).

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