Metabolic response in skeletal muscle of horses fed a forage-only diet and a forage-concentrate diet

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Abstract

The aim of this project was to analyse metabolic changes in muscle induced by diets using proteomics. The hypotheses were that feeding high fermentable fiber diets will trigger aerobic energy metabolism more than a high starch diet and will cause metabolic effects resembling the effects of training. Six Standardbred geldings in race training were used, aged 6.5 ± 0.4 years (mean ± SD). The horses were offered a forage-only diet (F) consisting of early-cut haylage (timothy, meadow fescue mixture) and a mixed diet (FC) consisting of late-cut haylage (timothy, meadow fescue mixture) supplemented with concentrate (50:50 dry matter basis) in a change-over design experiment with 29-day experimental periods. Muscle biopsies were taken from *m. gluteus medius* before warm-up and after an exercise test. The muscle biopsies taken before warm-up from four horses were selected for this study and were prepared to isolate mitochondrial proteins. The isolated protein samples were stored at -80^{o}C until analysis. The peptides were separated in reversed-phase on a C18-column and electrosprayed online into a Q Exactive mass spectrometer. Tandem mass spectrometry was performed applying HCD. The initial qualitative analyses were performed in Sequest towards proteins in a FASTA database containing proteins from *Equus caballus* (UniProtKB/TrEMBL) using Proteome Discoverer 1.4. The search criteria for protein identification were set to at least two matching peptides of 95% confidence level per protein.

The total number of identified proteins was 703. The equine characterized proteins were 138 in total which corresponded to 19.6% of the total number of identified proteins. Expression of proteins involved in the aerobic energy metabolism in the mitochondria was up-regulated in horses fed a forage-only diet. This support our hypothesis that feeding a forage-only diet cause metabolic changes resembling those achieved by exercise training. Thus, proteins of the multi-enzyme pyruvate dehydrogenase complex were up-regulated. In addition, the membrane respiratory chain NADP dehydrogenase, cytochrome c oxidase, ubiquinol-cytochrome c oxidoreductase complex, mono-oxygenase (required for the synthesis of coenzyme Q10) and ATP synthase were all up-regulated. In horses fed the forage-only diet, the expression of the mitochondrial trifunctional protein (HADA), which catalyzes the last three steps of mitochondrial beta-oxidation of long chain fatty acids, was up-regulated. The expression of apo-lipoprotein O was up-regulated on the forage-only diet. On the forage-only diet, the expression of a moonlighting protein (DLD) and prohibitin were up-regulated, both involved in regulation of energy metabolism.

Proteomics appears to be a very useful tool to gain information on the dynamics of cellular performance at a molecular level and to get an insight into important biological and biochemical reactions related to cellular metabolism.

Introduction

Feeding management of horses is quite well documented and has shown that Standardbred and Thoroughbred horses from all over the world are fed high concentrate diets (Jansson & Harris, 2013) but also that leisure horses seem to be fed large amounts of concentrates (Harris, 1999; Henricson, 2007). Concentrate-rich diets are associated with a number of disorders, such as gastric ulcers (Coenen, 1990), disturbances in the intestinal microbiota (Bailey et al., 2004; Willing et al., 2009) and colic (Tinker et al., 1997). Colic has recently been identified as one of the most frequent health issues in the insured Swedish horse population (Penell, 2005). Concentrate-rich diets have also been associated with rhabdomyolysis (McLeay et al., 1999) and stereotypic behaviour (high concentrate/low forage diets, Redbo et al., 1998).

We have recently conducted a number of studies, with the aim to clarify possible benefits and limitations with high energy fibre or forage diets to athletic horses compared to traditional high concentrate/starch diets (Connysson et al., 2006; Jansson and Lindberg, 2008; Muhonen et al. 2009; Connysson et al. 2010). These studies have focused on the possibilities to exchange the traditional high concentrate/starch diets with high energy fibre/forage diets. The general hypothesis has been that both performance and health of Standardbred horses would benefit from a diet adapted to the biology of the horse, i.e. a high energy forage/fibre diet and not a high starch diet. The results from these experiments are promising but more detailed evidence is needed to confirm and better understand the mechanisms involved in adaptations to diets.

The aim of this project was to analyse metabolic changes in muscle induced by diets using proteomics. Proteomics has the potential to provide this in depth analysis. The first publication using proteomics in human training studies was published in 2009 (Holloway et al.) and so far only a few publications appears to be available on exercising horses (Ichibangase et al. 2009; Bouwman et al. 2010).

The hypotheses were that feeding high fermentable fiber diets (which promote health and increases the production of acetate) will trigger aerobic energy metabolism more than a high starch diet and will cause metabolic effects resembling the effects of training.

Material and methods

Horses

Six Standardbred geldings in race training were used, aged 6.5 ± 0.4 years (mean ± SD). The average number of races in which the horses had competed was 27 ± 8 and the average racing record was 77.3 ± 0.8 s/1000 m. They had an initial body weight (BW) of 515 ± 21 kg. The horses were kept at a training camp for harness racing 20 km south of Uppsala, Sweden. They were housed in individual stalls on wood shavings during the night and were kept together in a sand/clay paddock between 08:00-15:00 h on days without training. All horses had passed a flexure test prior to the study and were regarded as healthy. The experiment was approved by the Uppsala ethical committee.

Diets and feeding

The horses were offered a forage-only diet (F) consisting of early-cut haylage (timothy, meadow fescue mixture) (Table 1) and a mixed diet (FC) consisting of late-cut haylage (timothy, meadow fescue mixture) supplemented with concentrate (50:50 dry matter basis) in a change-over design experiment with 29-day experimental periods. Feed allowance was based on individual BW and was 13-17.4 kg haylage and 180-240 g sugar (only to ensure complete intake of the salt, mineral and vitamin supplements) for diet F and 6.3-8.4 kg haylage, 5.3-7.1 kg oats, 0.9-1.2 kg soy bean meal, 0.18-0.24 kg wheat bran and 90-120 g sugar for diet FC. The diets were estimated to be iso-caloric and iso-nitrogenous, and provided energy and

nutrients according to requirements specified by NRC (1989). Horses on both diets were offered a mineral and vitamin supplement (51 ± 2 g/day, Miner Röd, Krafft, Falkenberg, Sweden), NaCl (36 ± 1 g/day) and those on diet FC ground chalk (calcium carbonate, 34 ± 1 g/day) to meet mineral and vitamin requirements specified by NRC (1989). Water was provided *ad libitum* from graded buckets. The forage allowance was fed in the afternoon and the concentrate and mineral and vitamin supplement (diet FC) at 15.00, 23.00 and 06.00 h. With diet F, the mineral and vitamin supplement was fed at 23.00 and 06.00. Diet FC was introduced gradually during the experimental period (on days 1 and 2, horses were fed 50% of the F diet and 50% of the FC diet, and then the FC diet was increased by 10% per day until the full ration was reached on day 7). Diet F was introduced abruptly on day 1.

Muscle samples

Muscle biopsies were taken from *m. gluteus medius* at a depth of approximately 6 cm according to the method described by Lindholm and Piehl (1974). A local anaesthetic (Carbocain 20 mg/ml, Astra Zeneca AB, Sweden) was applied to the area and a nose twitch was used. Biopsies were taken before warm-up and immediately after an exercise test, frozen in liquid nitrogen and stored at -80ºC until analysis. The present study reports data from the muscle biopsies collected before warm-up.

Sample preparation

The muscle biopsies were transferred to a glass homogenizer and 200 µL ice-cold sucrose buffer (sucrose 0.25M, tris 5 mM, EDTA 0.5mM; pH 7.2) was added. The sample was homogenized for 3 x 30 seconds, with 30 seconds of rest on ice between each round. The homogenate was transferred to an Eppendorf tube and centrifuged at 600 x g for 10 minutes. The supernatant was decanted and fresh ice-cold sucrose buffer was added (10% w/v) and centrifuged at 13200 x g for 10 minutes. The supernatant was decanted and the pellet was suspended in 200 µL ice-cold sucrose buffer and centrifuged at 13200 x g for 10 minutes. The supernatant was decanted and the pellet was suspended in 10 µL sucrose buffer and transferred to cryo tubes and stored at -80^{o}C until analysis.

Proteins in the samples were extracted in a urea buffer using sonication according to a standard operating procedure. The total protein concentration in the samples was measured using the Bradford Protein Assay with bovine serum albumin (BSA) as standard. Aliquots corresponding to 15 µg protein were taken out from all samples except for two samples for which 6 and 4 µg protein, respectively, were taken out. The proteins were reduced, alkylated and in-solution digested by trypsin according to a standard operating procedure. Thereafter the samples were purified by ZipTips®, dried and resolved in 0.1% FA to a concentration of 0.5 µg/µL.

LC-MS/MS

The peptides were separated in reversed-phase on a C18-column and electrosprayed online into a Q Exactive mass spectrometer (Thermo Finnigan). Tandem mass spectrometry was performed applying HCD. Each sample was run once. One sample was chosen as technical replicate and was therefore run three times; in the beginning, in the middle and in the end of the running series.

Qualitative and quantitative analysis

The initial qualitative analyses were performed in Sequest towards proteins in a FASTA database containing proteins from *Equus caballus* (UniProtKB/TrEMBL) using Proteome Discoverer 1.4 (Thermo Scientific™). The search criteria for protein identification were set to at least two matching peptides of 95% confidence level per protein. Biological annotation of

the identified proteins was performed in ProteinCenter Software (Thermo Scientific™) and was included as the final step in the database search.

The second analysis part, including the quantitative analysis, was performed using the MaxQuant software (Version 1.4.1.2) against the same protein database as for the initial analysis. Signal processing was performed in all 23 MS raw files. Peptides were grouped into proteins and a Label Free quantitation was performed.

A quality analysis was performed before the quantitative analysis and only proteins that could be identified in all samples were included in the following quantitative analysis. Comparison of changes in protein expression was calculated before warm-up on the forage-concentrate diet (diet FC) relative to before warm-up on the forage-only diet (diet F). Statistical significance of the results was calculated using Student's t-test (n=4 or 5 or 6, paired). A p-value of <0.05 was considered significant.

Results

The total number of identified proteins in the MaxQuant software was 703. The equine characterized proteins were 138 in total which corresponded to 19.6% of the total number of identified proteins. Annotations for the equine uncharacterized proteins were obtained from the human gene compendium (www.genecards.org).

The quantitative analysis of protein expression in muscle biopsies collected before warm-up on diet FC and diet F was performed on samples from four horses (Bertil, Tubbe, Spöket and Victor). A total of 35 proteins showed a significant difference in expression level. The identities, log ratios and p-values of the proteins are presented in Table 2.

All 35 proteins were up-regulated in horses fed the forage-only diet. No proteins were exclusively identified when the horses were fed one of the diets.

Two proteins of the multi-enzyme pyruvate dehydrogenase complex (PDC) were identified, one belonging to the E1 beta unit (PDHB) and one belonging to the E2 unit (DLAT).

Twelve of the proteins (34.3 %) belonged to the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I), which functions in the transfer of electrons from NADH to the respiratory chain. The genes coding for these proteins were NDUFA6, 9, 10, 12 & 13; NDUFB8 & 10; NDUFS1, 2 & 8; and NDUFV1 & 2.

Six proteins (17.1 %) belonged to mitochondrial ATP synthase, which is composed of two linked multi-subunit complexes: the soluble catalytic core F1 and the membrane-spanning Fo, which comprise the proton channel. Three of these proteins were subunits beta (ATP5B), delta (ATP5D) and gamma (ATP5C1) of the catalytic core F1. Two of the proteins were subunits f (ATP5J2) and g (ATP5L) of the Fo complex.

Three proteins (MT-CO2, COX5A & COX4I1) belonged to cytochrome c oxidase, which is a multi-subunit enzyme complex that couples the transfer of electrons from cytochrome c to molecular oxygen.

Two proteins (UQCRCB & UQCRC1) were components of the ubiquinol-cytochrome c oxidoreductase complex (complex III). One of the proteins (UQCRC1) is thought to mediate formation of the complex between cytochrome c and c1, and the other protein (UQCRB) binds ubiquinone and participates in the transfer of electrons when ubiquinone is bound.

One protein (COQ6) was an evolutionarily conserved monooxygenase required for the biosynthesis of ubiquinone (coenzyme Q10).

Two proteins were characterized as prohibitin (PHB & PHB2). Prohibitin act as a mediator of transcriptional repression by nuclear hormone receptors and are probably involved in regulating mitochondrial respiration activity.

One protein (HADHA) belonged to the alpha subunit of the mitochondrial trifunctional protein, which catalyzes the last three steps of mitochondrial beta-oxidation of long chain fatty acids.

One protein (DLD) was identified as a moonlighting protein based, which have the ability to perform mechanistically distinct functions.

One protein (APOOL) belonged to the apo-lipoprotein O superfamily domain. This domain is found on proteins in circulating lipoprotein complexes.

Discussion

The present study has shown that the expression of proteins involved in the aerobic energy metabolism in the mitochondria in *m. gluteus medius* were up-regulated in horses fed a forage-only diet. These observations therefore support our hypothesis that feeding a forage-only diet cause metabolic changes resembling those achieved by exercise training. The present study also show that proteomics is a very useful tool to gain information on the dynamics of cellular performance at a molecular level and to get an insight into important biological and biochemical reactions related to cellular metabolism.

Proteins of the multi-enzyme pyruvate dehydrogenase complex (PDC) were up-regulated. The PDC catalyzes the conversion of pyruvate to acetyl coenzyme A and is a key enzyme for the aerobic energy metabolism through the TCA cycle. In addition, the membrane respiratory chain NADP dehydrogenase, cytochrome c oxidase, ubiquinol-cytochrome c oxidoreductase complex, mono-oxygenase (required for the synthesis of coenzyme Q10) and ATP synthase were all up-regulated. The transfer of electrons through the electron transfer chain in the mitochondria results in the pumping of H+ across the membrane creating a proton gradient across the membrane, which is used by ATP synthase (located on the membrane) to generate ATP. Coenzyme Q10 functions as an electron carrier from enzyme complex I and enzyme complex II to complex III in this process. This is crucial in the process, since no other molecule can perform this function.

Feeding high-fiber or forage diets will increase hindgut fermentation and volatile fatty acid (VFA) production (mainly acetate), compared to a high starch diet, and this will enhance body lipid and aerobic glucose metabolism in accordance with observations on a high fat diet (Pagan et al. 2002). We have also shown that plasma acetate concentration is increased on high-fiber or forage diets (Connysson et al. 2010; Jansson and Lindberg 2012), that the lactate threshold might be higher (Jansson and Lindberg 2012) on a forage-only diet and that glycogen utilisation can decrease during exercise (Palmgren-Karlsson et al. 2002) on a high-fiber diet. The horses used for the analyses in the present study showed increased plasma acetate concentrations, reduced plasma lactate concentrations post exercise and a tendency for lower lactate threshold in response to the forage-only diet. The lactate threshold is known to be correlated to true exercise performance (race) in Standardbred horses (the higher the better).

The higher lactate threshold and glycogen saving effect could possibly be explained by the provision of an alternative substrate for the aerobic energy metabolism in the muscle. VFA will, after transport into the cell, be transformed to acetyl-CoA via specific fatty acyl-CoA ligases, thus resulting in the same key metabolite that is being produced from the ß-oxidation of fat. In horses fed the forage-only diet, the expression of the mitochondrial trifunctional protein (HADA) was up-regulated. This protein catalyzes the last three steps of mitochondrial beta-oxidation of long chain fatty acids. The mitochondrial membrane-bound hetero-complex is composed of four alpha and four beta subunits, with the alpha subunit catalyzing the 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase activities. In addition, the expression of apo-lipoprotein O was up-regulated on the forage-only diet. Apo-lipoprotein is part of the circulating lipoprotein complex in the body.

Moreover, on the forage-only diet, the expression of a moonlighting protein (DLD) and prohibitin were up-regulated. Prohibitin act as a mediator of transcriptional repression by nuclear hormone receptors and is probably involved in regulating mitochondrial respiration activity. The moonlighting protein functions as a dehydrogenase in homo-dimeric form and is found in several multi-enzyme complexes that regulate energy metabolism. Interestingly, as a monomer, the moonlighting protein can function as a protease. This ability may be of importance for the gluconeogenesis in situations where the glucose supply is limiting, which could be the case when forage-only diets low in water-soluble sugars are fed. We have previously observed very low insulin levels on forage diets (Connysson et al., 2010; Jansson and Lindberg 2012) and also a significant reduction of the muscle glycogen content (-13%). The importance of such a reduction for performance is unclear. Insulin is a strong activator of muscle glycogen synthesis due to its stimulating effects on glycogen synthase, protein kinase B and atypical protein kinas C. However, Waller et al. (2009) showed that oral acetate supplementation of horses might increase glycogen synthesis some hours post exercise but our results indicate that elevated plasma acetate concentrations has no positive effect on glycogen content, at least not when plasma insulin levels are low. Knowledge on the importance of muscle tissue adaptation to diets inducing low and high insulin levels is of general interest and may contribute to the understanding of problems related to over-nutrition.

In conclusion, the present study show that proteomics is a useful tool to assess metabolic changes caused by diet in the horse and that increased quantities of proteins involved in the TCA cycle, ß-oxidation and the respiratory chain could serves as markers for relevant alterations in the metabolism during exercise.

References

Bailey SR, Marr CM, Elliott J. (2004) Current research and theories on the pathogenesis of acute laminitis in the horse. *Vet J* 167, 129-142.

Bouwman F, van Ginneken M, Noben J et al. (2010) Differential expression of equine muscle biopsy proteins during normal training and intensified training in young standardbred horses using proteomics technology. *Comp. Biochem. Phys., Part D,* 55-64.

Coenen M. (1990) The occurrence of feed-induced stomach ulcers in horses. *Schweizer Archiv fur Tierheilkunde* 132, 3, 121-126.

Connysson M, Essén-Gustavsson B, Lindberg JE, Jansson A (2010) Effects of feed deprivation on Standardbred horses in training fed a forage-only diet and a 50:50 forage-oats diet. *Equine Vet J. Suppl.* 38(42), 335-340.

Connysson M, Muhonen S, Lindberg J E, Essén-Gustavsson B, Nyman G, Nostell K, Jansson A (2006) Effects on exercise response, fluid and acid-base balance of protein intake from forage-only diets in Standardbred horses. Equine vet. J. Suppl. 36, 648-653.

Harris P (1999) Review of feeding and stable management practices in the UK concentrating on the last decade of the 20th century. *Equine Vet J. Suppl.* 28, 46-54.

Henricson A (2007) Utfodring och hälsa hos privatägda hästar. Examensarbete 248. Institutionen för HUV, SLU.

Holloway, K, Gorman M, Woods P et al. (2009) Proteomic investigation of changes in human vastus lateralis muscle in response to interval-exercise training. *Proteomics* 9, 5155-5174.

Ichibangese T, Imai K (2009) Application of fluorogenic derivatization-liquid chromatography-tandem mass spectrometric proteome method to skeletal muscle proteins in fast Thoroughbred horses. *J Proteome Res* 8, 2129-2134.

Jansson A, Harris PA (2013) A bibliometric review on nutrition of the exercising horse from 1970 to 2010. *Comparative Exercise Physiology,* 9**,** 169-180.

Jansson A, Lindberg JE (2008) Effects of a forage-only diet on body weight and response to interval training on a track. In: Nutrition of the exercising horses, edited by Saastamoinen M T, Martin-Rosset W. *EAAP Publication* 125, 345-350.

Jansson A, Lindberg JE (2012) A forage-only diet alters the metabolic response of horses in training. *Animal* 6(12): 1939-1946.

Lindholm A, Piehl K (1974) Fibre composition, enzyme activity and concentrations of metabolites and electrolytes in muscles of standardbred horses. *Acta Veterinaria Scandinavica* 15, 287-309.

MacLeay JM, Sorum SA, Marsh WE, Sorum MD (1999) Epidemiologic analysis of factors influencing exertional rhabdomyolysis in Thoroughbreds. *Am J Vet Res.* 60 (12), 1562-1566.

Muhonen S, Lindberg JE, Bertilsson J, Jansson A (2009) Effects on fluid balance and exercise response in Standardbred horses feed silage, haylage and hay. *Comparative Exercise Physiology* 5(3-4), 133-142.

NRC (1989) National Research Council, Nutrient Requirements of Horses. 5:th edition. National Academic Press, Washington, DC, USA.

Pagan J, Geor RJ, Harris PA et al. (2002) Effects of fat adaptation on glucose kinetics and substrate oxidation during low-intensity exercise. *Equine Vet J* Suppl 34, 33-38.

Palmgren Karlsson C, Jansson A, Essen-Gustavsson B, Lindberg JE (2002) Effect of molassed sugar beet pulp on nutrient utilisation and metabolic parameters during exercise. *Equine Vet. J. Suppl.* 34, 44-49.

Penell J (2009) Secondary databases in Equine Research. *Acta Universitatis Agriculturae Sueciae* 2009:59.

Redbo I, Redbo-Torstensson P, Ödberg FO, Hedendahl A, Holm J. (1998) Factors affecting behavioural disturbances in race-horses. *Anim. Sci*. 66, 475-481.

Tinker MK, White NA, Lessard P et al. (1997) Prospective study of equine colic risk factors. *Equine Vet J* 29**(**6**),** 454-458.

Waller AP, Geor RJ, Spriet LL et al. (2009) Oral acetate supplementation after prolonged moderate intensity exercise enhances early muscle glycogen resynthesis in horses. *Exp Physiol.* 94, 888-898.

Willing B, Vörös A, Roos S, Jones C, Jansson A, Lindberg JE (2009) Changes in faecal bacteria associated with concentrate and forage-only diets fed to horses in training. *Equine Vet J.* 41, 908-914.