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Transcriptional expression changes of glucose metabolism genes after exercise in thoroughbred horses

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ABSTRACT

Physical exercise induces gene expression changes that trigger glucose metabolism pathways in organisms. In the present study, we monitored the expression levels of *LDHA* (lactate dehydrogenase) and *GYS1* (glycogen synthase 1) in the blood, to confirm the roles of these genes in exercise physiology. *LDHA* and *GYS1* are related to glucose metabolism and fatigue recovery, and these processes could elicit economically important traits in racehorses. We collected blood samples from three retired thoroughbred racehorses, pre-exercise and immediately after 30 min of exercise. We extracted total RNA and small RNA (≤200 nucleotide-long) from the blood, and assessed the expression levels of *LDHA*, *GYS1*, and microRNAs (miRNAs), by using qRT-PCR. We showed that *LDHA* and *GYS1* were down-regulated, whereas eca-miR-33a and miR-17 were up-regulated, after exercise. We used sequences from the 3′ UTR of *LDHA* and *GYS1*, containing eca-miR-33a and miR-17 binding sites, to observe the down-regulation activity of each gene expression. We observed that the two miRNAs, namely, eca-miR-33a and miR-17, inhibited *LDHA* and *GYS1* expression via binding to the 3′ UTR sequences of each gene. Our results indicate that eca-miR-33a and miR-17 play important roles in the glucose metabolism pathway. In addition, our findings provide a basis for further investigation of the exercise metabolism of racehorses.

1. Introduction

Thoroughbred horses possess many genetic features related to racing ability, robustness, recovery, and stamina. These traits can be distinguished by using SNP (single-nucleotide polymorphism) or gene expression (Doan et al., 2012; Hill et al., 2010a,b). Despite the importance of glucose metabolism genes in the selection of superior racehorses (Schroder et al., 2011), few published studies have focused on the characterization of these genes in thoroughbred horses. Physical exercise induces changes in glucose metabolism; therefore, the maintenance of blood glucose metabolism in galloping horses is very important for homeostasis and recovery after exercise. In addition, physical exercise changes the physiological state of the body in various species, including the horse. Hence, the genes related to physiological traits have been analyzed in horses (Gu et al., 2009; Petersen et al., 2013; Suontama et al., 2013). Gene expression profiles have frequently been shown to change after exercise in horses (Eivers et al., 2010; Park et al., 2012), humans (Barres et al., 2012; Jemiolo and Trappe, 2004), mice (Handschin et al., 2007), and rats (Jin et al., 2000). Further, exercise-dependent differential expression of genes related to exercise physiology – including glucose transport, glucose metabolism, angiogenesis, myogenesis, mitochondrial biogenesis (Eivers et al., 2010; McGivney et al., 2010), actin and myosin (Jin et al., 2000; McGivney et al., 2010), and the extracellular matrix (Jin et al., 2000; Mienaltowski et al., 2009) – has previously been demonstrated.

Physical exercise is classified into aerobic exercise and anaerobic exercise, according to whether or not oxygen is required to exploit energy via metabolic pathways (Hughson et al., 2001; Laursen, 2010). When organisms undergo an intensive activity, aerobic exercise is concurrent with anaerobic exercise, because the energy demands exceed the output energy of the aerobic exercise pathway. In anaerobic exercise, lactate is generated through pyruvate fermentation; the lactate is accumulated in muscles, leading to fatigue (Duffield et al., 2005; Laursen,

Abbreviations: LDHA, lactate dehydrogenase; GYS1, glycogen synthase 1; miRNA, microRNA; M-MLV RT, Moloney-Murine Leukemia Virus Reverse Transcriptase; PBMC, peripheral blood mononuclear cell.

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2010). As a consequence of the generation of lactate through pyruvate fermentation in anaerobic exercise, the body performs a recovery mechanism by converting lactate to pyruvate (Scott, 2011). Lactate dehydrogenase A (*LDHA*; EC 1.1.1.27) catalyzes the conversion of L-lactate to pyruvate, and of NAD^+ to NADH. *LDHA* is activated during anaerobic glycolysis as redox cofactors among various organisms (Poso, 2002). After intensive exercise such as racing, lactate is accumulated in the muscles of horses, resulting in fatigue. Fitness is a crucial criterion for racehorses, and therefore it is necessary to clarify *LDHA* expression patterns and the LDHA regulatory pathway after exercise. LDHA is abundant in muscles, but is usually present at low levels in the blood. On the other hand, an increase in blood LDHA levels is an indicator of tissue damage or disease. Many diseases or stress conditions, including liver disease, anemia, muscle trauma, and bone fractures, can cause the release of LDHA into the bloodstream (Wan et al., 2013).

Glucose is derived from glycogen (i.e., the state used for energy storage), as well as from other carbohydrates. The body uses glucose as an energy source. When the blood glucose level decreases during exercise, glycogen is broken down into glucose to meet the energy demand (Rose and Richter, 2005; Sinacore and Gulve, 1993). During the resting state, glucose is converted into glycogen to maintain the optimum glucose concentration. In this situation, glycogen synthase 1 (GYS1) catalyzes the conversion of glucose into glycogen. *GYS1* (EC 2.4.1.11) regulates the blood glucose level and accumulates the excess glucose as an energy reserve, by converting glucose into glycogen (Pederson et al., 2005). *GYS1* is an important gene in fatigue recovery after exercise; however, few studies have investigated the expression and physiological role of *GYS1* in horses.

MicroRNAs (miRNAs) are small endogenous, 21–23 nucleotide- (nt) long RNAs that play various roles in the regulation of gene expression by binding to their target genes. Primary miRNAs are transcribed from miRNA genes, and are then processed into precursor miRNAs by the Drosha enzyme, and into mature miRNAs by the Dicer enzyme (Bartel, 2004; Kim, 2005; Lee et al., 2002). A mature miRNA has a seed region of approximately 2–8 residues long, and recognizes complement seed region sites of the target gene's 3′ UTR (Bartel, 2004). Comparisons of the expression levels of miRNAs and their target genes have demonstrated miRNA-mediated inhibitory effects (Liang et al., 2013). The results of such studies help to clarify the relationships between oncomiRNAs and tumor suppressor genes in cancer biology (Thomas et al., 2012), and also between condition-specific genes and their target miRNAs via assessing expression levels or checking inhibitory effects (Liang et al., 2013). In other words, miRNAs and their target genes present contrasting expression patterns, termed type 2 circuits (Liang et al., 2013; Tsang et al., 2007). In the present study, we identified type 2 circuits related to two genes involved in glucose metabolism.

The aim of our study was to validate the hypothesis that exercise changes the expression profiles of *LDHA* and *GYS1*, and that the expression levels of these genes are regulated by miRNA-mediated mechanisms. Glucose metabolism is an important factor for exercise, energy storage, and recovery from fatigue. Nam et al. (2012) reported that, in the horse, a single gene (*OXCT1*) is down-regulated after exercise, in order to select the optimum energy-exploitation pathways by using ketone bodies (Nam et al., 2012). The authors showed that energy metabolism genes are down-regulated after exercise; however, few studies have investigated miRNA-mediated changes in metabolism genes. Therefore, we compared the expression levels of two genes pre- and post-exercise, and also the expression levels of their target miRNAs in the blood, by using real-time RT-PCR. We subsequently used TargetScan to perform an in silico analysis of searching for conserved miRNA binding sites in the 3′ UTRs of *LDHA* and *GYS1*. In addition, we compared the coding sequences of these genes, to confirm their evolutionary relationship among vertebrates. Our results provide evidence for the miRNA-mediated control of glucose metabolism after exercise in horses. In addition, our data demonstrate the roles of *LDHA* and *GYS1* in glucose metabolism, as well as in exercise-induced gluconeogenesis.

2. Materials and methods

2.1. Horse blood samples and ethics statement

We collected blood samples from three retired thoroughbred horses. The information regarding each horse is provided in Table S1. In order to avoid biased results according to individual horse traits, we considered the gender, weight, and racing achievement records of each horse. Blood samples were drawn from the jugular vein of each horse, before and after exercise (trotting for 30 min). Each blood-collection procedure was performed immediately before and after exercise, to avoid degradation of transcripts by extraneous variables. The pre-exercise samples were collected from each horse at rest. The horses were then trotted for 30 min, and the post-exercise samples were immediately collected. The samples were collected by using a vacutainer, and were stored at −80 °C until analysis. The blood collection followed a previously described procedure, in which the time interval was not considered as an external factor (Nostell et al., 2012). All animal experiments were performed in accordance with and approved by the Pusan National University Institutional Animal Care and Use Committee with approval number PNU-2013-0417. The blood collection was carried out under the supervision of a veterinarian, and appropriate care was taken to minimize suffering of the horses.

2.2. Selection of target miRNAs

We identified the *LDHA*- and *GYS1*-inhibiting target miRNAs by prediction through TargetScanHuman ver. 6.2 (http://www.targetscan.org), and selected the conserved target regions in the horse genome. We predicted the interactions by using the RNAhybrid ver. 2.1 program (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/). The alignments of target genome assembly were equCab2 in the horse and hg19 in humans.

2.3. Total and small RNA extraction and cDNA synthesis

We used hybrid-R miRNA for total and small RNA isolation procedures (GeneAll, South Korea). We used Turbo DNA-free™ (Ambion) to prevent contamination of genomic DNA. Extracted total and small RNA were quantified by using a NanoDrop® ND-1000 UV–Vis Spectrophotometer. Moloney-Murine Leukemia Virus Reverse Transcriptase (M-MLV RT; Promega) was used to synthesize cDNA from 500 ng of total RNA at an annealing temperature of 42 °C. Small RNAs were polyadenylated by using a poly (A) tailing kit (Ambion). The poly (A) tailing reactions were performed in E-PAP buffer at an incubation temperature of 37 °C for 1 h. Reverse transcription reactions were subsequently performed by using M-MLV RT with RNase inhibitor (Promega) at an annealing temperature of 42 °C. To select target miRNAs in the reverse transcription reactions, we used 1 μL of 10 nmol/μL oligo-dT adaptor (5′-CTGTGAATGCTGCGACTACGA-18dTs-3′).

2.4. Quantitative real-time RT-PCR amplification

The amplification reaction mixture (15 μL) contained 7 μL of H_2O, 5 μL of QuantiTech SYBR Green PCR master Mix (Qiagen, Hilden, NW, Germany), 1 μL each of forward and reverse primers, and 1 μL of cDNA template. In addition, to confirm non-specific background amplification, we amplified a template control without cDNA. Real-time RT-PCR amplifications for target genes and housekeeping genes were conducted as follows: 50 cycles each of 94 °C for 10 s, 58 °C for 15 s, and 72 °C for 15 s. Melting curve analysis was performed for 30 s at 55–99 °C. To guarantee reproducibility, we amplified all samples in triplicate. We determined the level of significance (paired *t*-test) of expression for all samples. As a standard control, we used *GAPDH* in gene expression,