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Comparative metabolomic analysis in horses and functional analysis of branched chain (alpha) keto acid dehydrogenase complex in equine myoblasts under exercise stress

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Abstract

The integration of metabolomics and transcriptomics may elucidate the correlation between the genotypic and phenotypic patterns in organisms. In equine physiology, various metabolite levels vary during exercise, which may be correlated with a modified gene expression pattern of related genes. Integrated metabolomic and transcriptomic studies in horses have not been conducted to date. The objective of this study was to detect the effect of moderate exercise on the metabolomic and transcriptomic levels in horses. In this study, using nuclear magnetic resonance (NMR) spectroscopy, we analyzed the concentrations of metabolites in muscle and plasma; we also determined the gene expression patterns of branched chain (alpha) keto acid dehydrogenase kinase complex (*BCKDK*), which encodes the key regulatory enzymes in branched-chain amino acid (BCAA) catabolism, in two breeds of horses, Thoroughbred and Jeju, at different time intervals. The concentrations of metabolites in muscle and plasma were measured by ^{1}H NMR (nuclear magnetic resonance) spectroscopy, and the relative metabolite levels before and after exercise in the two samples were compared. Subsequently, multivariate data analysis based on the metabolic profiles was performed using orthogonal partial least square discriminant analysis (OPLS-DA), and variable important plots and *t*-test were used for basic statistical analysis. The stress-induced expression patterns of *BCKDK* genes in horse muscle-derived cells were examined using quantitative reverse transcription polymerase chain reaction (qPCR) to gain insight into the role of transcript in response to exercise stress. In this study, we found higher concentrations of aspartate, leucine, isoleucine, and lysine in the skeletal muscle of Jeju horses than in Thoroughbred horses. In plasma, compared with Jeju horses, Thoroughbred horses had higher levels of alanine and methionine before exercise; whereas post-exercise, lysine levels were increased. Gene ex-

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Upon reasonable request, the datasets of this study can be available from the corresponding author.

Authors' contributions
Conceptualization: Park JW, Cho BW, Song KD.
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Ethics approval and consent to participate
All animal procedures used in the study were conducted in compliance with international standards and were approved by the Institutional Animal Care and Use Committee of Pusan National University (Approval Number: PNU-2013-0417, PNU-2013-0411, PNU-2015-0864).

pression analysis revealed a decreased expression level of *BCKDK* in the post-exercise period in Thoroughbred horses.

Keywords: Metabolite, mRNA expression, Nuclear magnetic resonance (NMR) spectroscopy, Branched chain (alpha) keto acid dehydrogenase kinase complex (*BCKDK*) gene, Equine myoblast

INTRODUCTION

The performance of racing horses is primarily related to their energy metabolism, and numerous enzymes and metabolites are involved in this process [1]. The total muscle blood flow, oxygen consumption, and cardiac output also play key roles in race performance [2]. During racing, horses experience a metabolic stress intricately linked with electrolytic loss and energy metabolism [3]. The energy consumption is mainly dependent on the production of adenosine triphosphate (ATP) in muscle, which generates energy through three mechanisms. The phosphocreatine-ATP system provides instant energy when performing short and high-intensity exercises; the muscle glycolytic system involves anaerobic production of ATP, and is limited when lactate concentration reaches its threshold range; and the oxidative system provides more energy through oxidation of glucose, fatty acids and, proteins [4]. To reveal the role of metabolites in race performance, high-throughput techniques such as nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry are being used [5]. In recent years, NMR-based studies have been used to quantify many metabolites in serum, plasma, urine, and tissues [6,7].

Branched-chain amino acids (BCAAs), e.g., isoleucine, leucine, and valine, play important roles in the skeletal muscle metabolism. These amino acids are essential amino acids which activate protein synthesis after exercise. Supplementation with BCAAs in combination with resistance exercise led to an increase in the phosphorylation of p70 (S6k) in human skeletal muscle [8]. Leucine regulates signaling pathways involved in translational control of protein synthesis in skeletal muscle [9]. Inhibition of AMP-activated protein by leucine stimulates mammalian target of rapamycin (mTOR) signaling in C2C12 myoblasts [10]. The catabolism of BCAAs in skeletal muscle is well studied in human and rats [11,12]. Two enzymes, namely the branched chain (alpha) keto acid dehydrogenase complex (BCKDH) and branched chain (alpha) keto acid dehydrogenase kinase complex (BCKDK), tightly regulate this pathway [12,13]. These enzymes are abundant in the inner mitochondrial membrane in various tissues. The BCKDH multienzyme complex consists of three enzyme units namely E1 (α-ketoacid dehydrogenase), E2 (dihydrolipoyltransacylase), and E3 (dihydrolipoamide dehydrogenase). The E1 subunit consists of E1-α and E1-β chains encoded by *BCKDHA* and *BCKDHB* [14,15]. The catalytic activity of this enzyme is further regulated by the BCKDK complex. This complex is encoded by *BCKDK*. Various mutations and defects in *BCKDHB* and *BCKDK* are associated with maple syrup urine disease and neurological defects in human [16,17].

Various studies focused on BCCA concentrations in plasma or serum in the post-exercise period have reported an increased concentration of BCCAs [18]. Most of the studies quantified metabolite levels in endurance racehorses [19,20]. The effects of metabolites in the skeletal muscle have not been studied well, which restricts the analysis of metabolites for muscle physiology and energy metabolism. Moreover, studies evaluating differences between breeds of horses at the metabolomic and gene expression levels have not been reported to date, despite its importance in the analysis of racing performance in horses. The purpose of this study was to compare the metabolite and gene expression levels involved in energy production during the pre- and post-exercise period in two breeds of horses.

MATERIALS AND METHODS

Sample collection

Two stallions and one mare Throughbred horses aged 5, 9, and 10, weighing from 500 to 700 kg which were maintained at Ham-an Racing Horse Resort and Training Center were used to obtain the blood and skeletal muscle samples before and after exercise. Exercise was performed by trotting at the speed of 13 km/h for 30 min and cantering through lunging and long-reining (circular bridge lunging).

Three Jeju horses (3 mares), which were maintained at The National Institute of Subtropical Agriculture, Rural Development Administration were used to obtain tissue samples skeletal muscle, kidney, thyroid, lung, appendix, colon, spinal cord and heart. Venous blood samples were collected using a 20 mL syringe and transferred to ethylenediaminetetraacetic acid (EDTA)-containing tubes. For the skeletal muscle biopsy, local anesthesia was administered to the *gluteus medius* in muscle, and a biopsy collection syringe was then used to obtain the muscle samples. All samples were stored at −80℃ before RNA extraction. All procedures were conducted by following the protocol that had been reviewed and approved by the Institutional Animal Care and Use Committee at Pusan National University (protocol numbers: PNU-2013-0417, PNU-2013-0411, PNU-2015-0864).

Equine muscle cell culture and in vitro stress-induced systems

The horse muscle-derived cells were established in our previous study [20]. The horse muscle cells were routinely cultured in medium 199 (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Invitrogen, Waltham, MA, USA) and 1% antibiotic–antimycotic (Invitrogen), and kept at 37℃ and 5% CO_2 environment. Routine medium changes were performed three times a week. Cells at 70% to 80% confluency were gently washed twice with phosphate-buffered saline (PBS) and harvested using 0.05% trypsin-EDTA (Welgene, Gyeongsan, Korea) for expansion.

To induce various stresses, horse muscle cells at 70% to 80% confluency were incubated with 20 μg/mL cortisol [21].

RNA extraction and complementary DNA synthesis

Horse muscle-derived cells from the initial culture were plated in a 6-well plate and incubated for 24 h. They were then treated with 20 μg/mL cortisol and incubated for 8 h then harvested. A mixture made of lysis buffer and 2-mercaptoethanol (1 mL:10 μL) was added to the harvested cells, followed by an equivalent volume of 70% ethanol, and the mixture was vortexed thoroughly to ensure complete cell lysis. The mixture was then transferred to the spin cartridge with a collection tube and centrifuged at 12,000×g for 15 s at room temperature. After centrifugation, the flow-through was discarded and the spin cartridge was reinserted into the same collection tube. Then, 700 μL of wash buffer I was added, and the mixture was centrifuged at 12,000×g for 1 min at room temperature. The flow-through was discarded and the spin cartridge was inserted into a new collection tube. After, 500 μL of wash buffer II was added and the mixture was centrifuged at 12,000×g for 1 min. The flow-through was discarded and the spin cartridge reinserted into the same collection tube. This process was repeated and additionally centrifuged at 13,000×g for 1 min to dry the membrane with bound RNA. After, the flow-through was discarded and the spin cartridge inserted into a recovery tube of 1.5 mL. Thirty μL of RNase-free water was added to the center of the spin cartridge and incubated for 1 to 5 min and then centrifuged at 12,000×g for 1 min to elute the RNA from the membrane into the recovery tube. RNA quantity was determined using a spectrophotometer. RNA measurements obtained were then used to calculate the volume of

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RNA, H_2O, 5xBF, dNTP, RNAse inhibitor, OligodT, and RTase needed for cDNA synthesis, and the mixture was subject to reverse transcription.

Quantitative reverse transcription polymerase chain reaction

To quantitate the gene expression levels of *BCKDK* in muscle tissues and blood cells before and after exercise, a quantitative reverse transcription polymerase chain reaction (qRT-PCR) was conducted using the BioRad CFX-96 apparatus (BioRad, Hercules, CA, USA). Each reaction was conducted in a 25 μL mixture containing 14 μL of SYBR Green Master Mix, 2 μL of forward primer (5 pmol), 2 μL of reverse primer (5 pmol), 5 μL of distilled water, and 2 μL (50 ng/μL) of cDNA. PCR conditions were as follows: a predenaturation step at 94℃ for 5 min; 39 cycles at 94℃ for 20 s, 56℃ for 20 s, and 72℃ for 30 s; and a final step at 72℃ for 10 min. All measurements were performed in triplicate for all specimens, and the $2^{-\Delta\Delta Ct}$ method was to compare the data [22]. The relative expression level of each target gene was calculated by normalizing the expression level against that of glyceraldehyde-3-phosphate dehydrogenase.

Statistical analysis

Student's *t*-test and analysis of variance were conducted to determine significance levels. Data were shown as the mean ± SE.

RESULTS

Comparison of metabolites between Thoroughbred and Jeju horses

In our previous study, differentially present metabolites were identified in Thoroughbreds [23]. In this study, we identified differentially present metabolites in Jeju horses, and conducted a comparison analysis between Thoroughbred and Jeju horses. We obtained massive metabolomic data from equine plasma (Table 1) and muscle (Table 2). Among the massive metabolites, we obtained each of the four metabolites, which were present at different levels in both the plasma (Table 3) and muscle (Table 3). Alanine, methionine, and taurine were significantly expressed in the plasma sample before exercise, while lysine was significantly expressed after exercise. In muscle samples, aspartate, isoleucine, leucine, and lysine were significantly expressed before exercise, whereas none were significantly expressed after exercise. In addition, we analyzed the levels of metabolites in Thoroughbred and Jeju horses. Jeju horses had a significantly lower level of alanine, lysine, and methionine; and a higher level of taurine in plasma ($p < 0.05$) than in Thoroughbred horses (Fig. 1). On the other hand, no other metabolites were found to be either significantly low or high in plasma. No significant differences were found between the amino acids and other metabolites after exercise, except for lysine. When compared to Jeju horses, Thoroughbred horses had a significantly higher level of lysine ($p < 0.05$), (Table 1). The metabolite profile of skeletal muscles in both breeds indicate very few differences at the region of BCCAs and lysine (Table 2). In muscles during the pre-exercise period, Jeju horses had a significantly higher level of aspartate, isoleucine, leucine, and lysine than in Thoroughbred horses ($p < 0.05$) (Fig. 2). Other metabolites related to exercise did not have a significant difference in skeletal muscle. Thoroughbred horses had a significantly higher level of phospholipid derivative o-phosphocholine than in Jeju horses (p value: <0.05), and no other significant differences were seen for metabolites in muscle (Table 2).

Functional analysis and evolutionary analysis of BCKDK as branched-chain amino acid related genes (DEGs)

Based on metabolomic data, we found isoleucine and leucine, which are BCAAs significantly

Table 1. Comparison of metabolites composition between Thoroughbred and Jeju horses in plasma

Metabolites	Before ppm			After ppm		
	TH	JH	*p*-value	TH	JH	*p*-value
Acetate	5.36 ± 6.88	0.48 ± 0.18	0.29	4.96 ± 7.57	1.29 ± 1.07	0.45
Alanine	7.34 ± 0.42	5.40 ± 0.94	0.03*	5.40 ± 2.22	5.53 ± 1.60	0.94
Choline	1.77 ± 2.06	0.45 ± 0.30	0.33	0.38 ± 0.31	0.52 ± 0.18	0.55
Creatine	1.21 ± 0.55	1.32 ± 0.65	0.83	1.19 ± 0.49	1.14 ± 0.62	0.92
Glutamate	0.92 ± 0.13	2.01 ± 0.93	0.12	1.81 ± 1.06	1.99 ±0.54	0.81
Glutamine	0.57 ± 0.09	0.72 ± 0.34	0.50	0.69 ± 0.25	0.92 ± 0.69	0.62
Glycine	7.73 ± 0.84	5.88 ± 1.83	0.19	8.33 ± 3.68	6.32 ± 1.77	0.44
Histidine	1.22 ± 0.49	1.17 ± 0.19	0.87	1.58 ± 0.54	1.04 ± 0.14	0.17
Isoleucine	1.09 ± 0.20	0.76 ± 0.44	0.31	0.83 ± 0.33	0.60 ± 0.41	0.50
Lactate	47.10 ± 9.83	46.96 ±15.33	0.99	37.38 ± 11.59	44.95 ± 11.49	0.47
Leucine	1.81 ± 0.22	1.11 ± 0.38	0.05	2.07 ± 0.40	1.48 ± 0.49	0.18
Lysine	1.30 ± 0.46	0.78 ± 0.26	0.16	1.54 ± 0.26	0.70 ± 0.13	0.008*
Methionine	0.38 ± 0.04	0.21 ± 0.08	0.03*	0.23 ± 0.09	0.26 ± 0.14	0.81
Myo-Inositol	0.25 ± 0.16	0.34 ± 0.09	0.45	0.36 ± 0.08	0.52 ± 0.37	0.49
Phenylalanine	0.29 ± 0.16	0.25 ± 0.09	0.69	0.22 ± 0.09	0.37 ± 0.09	0.11
Proline	1.29 ± 0.36	1.33 ± 0.81	0.95	1.40 ± 0.40	1.10 ± 0.87	0.61
Pyruvate	12.38 ± 14.39	21.24 ± 6.50	0.39	18.25 ± 17.79	22.16 ± 6.41	0.74
Taurine	0.66 ± 0.41	2.01 ± 0.13	0.01*	0.99 ± 0.55	1.67 ± 0.49	0.18
Threonine	0.65 ± 0.28	1.36 ± 0.50	0.10	0.78 ± 0.17	1.16 ± 0.38	0.19
Tyrosine	0.49 ± 0.13	0.34 ± 0.08	0.17	0.30 ± 0.13	0.47 ± 0.20	0.26
Valine	3.24 ± 0.26	2.87 ± 1.26	0.65	2.74 ± 0.74	2.80 ± 1.13	0.94

All values expressed in ppm as mean ± SD.

*$p < 0.05$.

TH, Thoroughbred horse; JH, Jeju horse.

expressed in Jeju and Thoroughbred horses. As mentioned, the BCKDH and BCKDK are tightly involved in the BCAA signaling pathway. Therefore, we evaluated *BCKDK* expression in the muscle tissue of Jeju and Thoroughbred horses.

Equine *BCKDK* is located in chromosome 13, and the genomic structure is shown in Fig. 3. The BCKDK gene consists of 11 exons, and the full lengths is 1,239 bp. Equine *BCKDK* encodes 412 amino acids. To investigate the evolutionary relationships of BCKDK in horses, we extracted and compared amino acid sequences from eight species (frog, mouse, rat, cow, horse, wild horse, dog, human) from Ensembl 62, and conducted a phylogenetic analysis (Fig. 3B). Multiple alignment using the 'histidine kinase-like ATPases' domain showed higher identity (Fig. 3C, solid box). Therefore, we suggest that BCKDK is highly conserved between various species and these domains would have an important role in the exercise stress response.

Validation of BCKDK expression in equine muscle tissue and horse muscle-derived cells under stress

To validate BCKDK gene expression, which we deduced from metabolomic data as a differentially expressed metabolite related gene, we conducted qRT-PCR with Thoroughbred and Jeju horse muscle tissue (Fig. 4). The expression level of *BCKDK* significantly decreased after exercise in both horse breeds, even though the expression level in Thoroughbred horses decreased more than that of Jeju horses. Additionally, we investigated the expression patterns of *BCKDK* in horse muscle-

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Table 2. Comparison of metabolites composition between Thoroughbred and Jeju horses in skeletal muscle

Metabolites	Before ppm			After ppm		
	TH	JH	*p*-value	TH	JH	*p*-value
Acetate	0.18 ± 0.04	0.47± 0.24	0.11	0.30 ± 0.11	0.18 ± 0.03	0.13
Alanine	1.02 ± 0.43	1.01 ± 0.31	0.97	1.41 ± 0.48	0.93 ± 0.20	0.18
Anserine	0.30 ± 0.02	0.29 ± 0.14	0.96	0.50 ± 0.14	0.34 ± 0.13	0.22
Arginine	0.57 ± 0.09	1.07 ± 0.29	0.05	0.52 ± 0.18	0.42 ± 0.25	0.60
Aspartate	0.71 ± 0.28	1.62 ± 0.43	0.04*	0.84 ± 0.84	1.03 ± 0.42	0.75
Betaine	0.44 ± 0.15	0.28 ± 0.18	0.32	0.27 ± 0.12	0.28 ± 0.21	0.95
Carnitine	1.79 ± 1.38	1.30 ± 0.45	0.59	1.55 ± 0.43	1.32 ± 0.26	0.48
Choline	0.54 ± 0.22	0.25 ± 0.08	0.10	0.44 ± 0.29	0.28 ± 0.17	0.47
Creatine	29.22 ± 5.79	28.24 ± 3.02	0.81	29.28 ± 8.23	34.47 ± 4.47	0.39
Cysteine	1.72 ± 0.27	2.20 ± 0.47	0.20	1.21 ± 0.40	0.57 ± 0.51	0.17
Fumarate	0.11 ± 0.04	0.10 ± 0.02	0.65	0.11 ± 0.06	0.09 ± 0.01	0.39
Glucose	2.78 ± 0.52	2.95 ± 0.77	0.77	4.31 ± 1.60	3.70 ± 0.24	0.55
Glutamate	0.49 ± 0.17	0.81 ± 0.18	0.09	0.75 ± 0.52	0.56 ± 0.24	0.60
Glutamine	1.35 ± 0.64	1.47 ± 0.48	0.80	1.52 ± 0.27	1.43 ± 0.32	0.72
Glycine	0.82 ± 0.21	1.20 ± 0.60	0.36	1.18 ± 0.38	1.26 ± 0.36	0.81
Isoleucine	0.16 ± 0.03	0.41 ± 0.12	0.02*	0.29 ± 0.18	0.18 ± 0.07	0.39
Lactate	45.19 ±10.63	43.07 ± 6.67	0.78	42.12 ±6.85	42.45 ± 2.70	0.94
Leucine	0.35 ± 0.07	1.19 ± 0.23	0.01*	0.62 ± 0.37	0.43 ± 0.14	0.45
Lysine	0.19 ± 0.05	0.62 ± 0.22	0.03*	0.24 ± 0.10	0.22 ± 0.11	0.84
Methionine	0.75 ± 0.09	1.23 ± 0.61	0.25	0.70 ± 0.07	1.03 ± 0.47	0.30
Myo-inositol	0.57 ± 0.21	0.58 ± 0.16	0.97	0.65 ± 0.14	0.52 ± 0.10	0.25
O- Phosphocholine	0.47 ± 0.14	0.35 ± 0.07	0.27	0.61 ± 0.15	0.28 ± 0.08	0.03*
O- Phosphoethanolamine	1.32 ± 0.68	0.94 ± 0.62	0.52	1.36 ± 0.48	0.40 ± 0.34	0.05
Phenylalanine	0.19 ± 0.16	0.17 ± 0.03	0.80	0.18 ± 0.06	0.11 ± 0.06	0.24
Proline	1.30 ± 0.74	1.15 ± 0.33	0.77	0.92 ± 0.28	0.92 ±0.32	0.98
Pyruvate	0.22 ± 0.30	0.50 ± 0.38	0.38	0.43 ± 0.33	0.34 ± 0.09	0.67
Serine	1.19 ± 0.29	1.26 ± 0.62	0.87	1.35 ± 0.18	0.70 ± 0.65	0.17
sn-Glycerol-3-phosphate	0.62 ± 0.11	0.49 ± 0.10	0.22	0.67 ± 0.30	0.46 ± 0.21	0.21
Succinate	0.06 ± 0.02	0.05 ± 0.01	0.34	0.63 ± 1.03	0.06 ± 0.01	0.39
Taurine	3.56 ± 1.21	2.97 ± 1.16	0.57	3.12 ± 0.65	3.48 ± 1.79	0.76
Threonine	0.69 ± 0.12	0.43 ± 0.16	0.08	0.50 ± 0.34	0.41 ± 0.12	0.67
Tyrosine	0.15 ± 0.04	0.19 ± 0.04	0.30	0.21 ± 0.03	0.16 ± 0.02	0.07
Valine	0.28 ± 0.06	0.39 ± 0.12	0.22	0.52 ± 0.18	0.25 ± 0.05	0.07

All values expressed in ppm as mean ± SD.

*$p < 0.05$.

TH, Thoroughbred Horse; JH, Jeju Horse.

derived cells under stress. To validate *BCKDK* expression under stress, we conducted qRT-PCR on cortisol treated horse muscle-derived cells. In a previous study, we established a cortisol treatment system [21]. In this study, quantitative expression analysis was performed on *BCKDK* by cortisol reactivity (Fig. 5). To verify the effect of cortisol on stress induction, expression patterns of stress marker genes were investigated (Fig. 5B). We found that the expression levels of the marker genes of stress increased substantially. Next, we examined the effects of cortisol on the horse muscle-derived cells. *BCKDK* expression level increased after cortisol treatment ($p < 0.01$, Fig. 5C). In addition, we investigated the effect of methyl sulfonyl methane (MSM) on stress reduction ($p <$

Table 3. Comparison of metabolites in plasma and muscle in Thoroughbred horses (TH) and Jeju horses (JH)

S.No.	Metabolites	Before (Mean ±SD)			After (Mean ±SD)		
		TH	JH	*p*-value	TH	JH	*p*-value
Plasma							
1.	Alanine	7.34 ± 0.42	5.40 ± 0.94	0.03*	5.40 ± 2.22	5.53 ± 1.60	0.94
2.	Lysine	1.30 ± 0.46	0.78 ± 0.26	0.16	1.54 ± 0.26	0.70 ± 0.13	0.008*
3.	Methionine	0.38 ± 0.04	0.21 ± 0.08	0.03*	0.23 ± 0.09	0.26 ± 0.14	0.81
4.	Taurine	0.66 ± 0.41	2.01 ± 0.13	0.01*	0.99 ± 0.55	1.67 ± 0.49	0.18
Muscle							
1.	Aspartate	0.71 ± 0.28	1.62 ± 0.43	0.04*	0.84 ± 0.84	1.03 ± 0.42	0.75
2.	Isoleucine	0.16 ± 0.03	0.41 ± 0.12	0.02*	0.29 ± 0.18	0.18 ± 0.07	0.39
3.	Leucine	0.35 ± 0.07	1.19 ± 0.23	0.01*	0.62 ± 0.37	0.43 ± 0.14	0.45
4.	Lysine	0.19 ± 0.05	0.62 ± 0.22	0.03*	0.24 ± 0.10	0.22 ± 0.11	0.84
5.	O-Phosphocholine	0.47 ± 0.14	0.35 ± 0.07	0.27	0.61 ± 0.15	0.28 ± 0.08	0.03*

*$p < 0.05$.

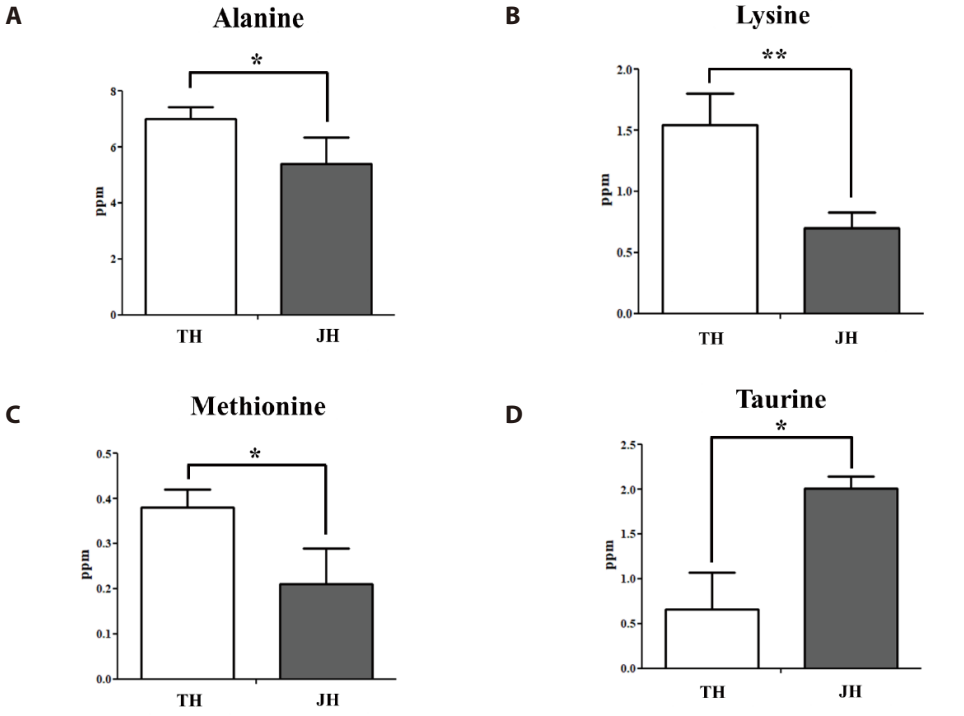

Fig. 1. Significant differences in plasma metabolite levels between Thoroughbred and Jeju horses (A) alanine (before exercise), (B) lysine (after exercise), (C) methionine (before exercise) and (D) taurine (before exercise). *$p < 0.05$. All values are expressed in ppm as the mean ± SD. TH, Thoroughbred horse; JH, Jeju horse.

0.01, Fig. 5D). We found that MSM did not reduce stress by regulating *BCKDK*. It is assumed that MSM may reduce exercise stress through another signaling pathway.

DISCUSSION

Comparison studies in horses showed that there are variations between amino acid concentrations

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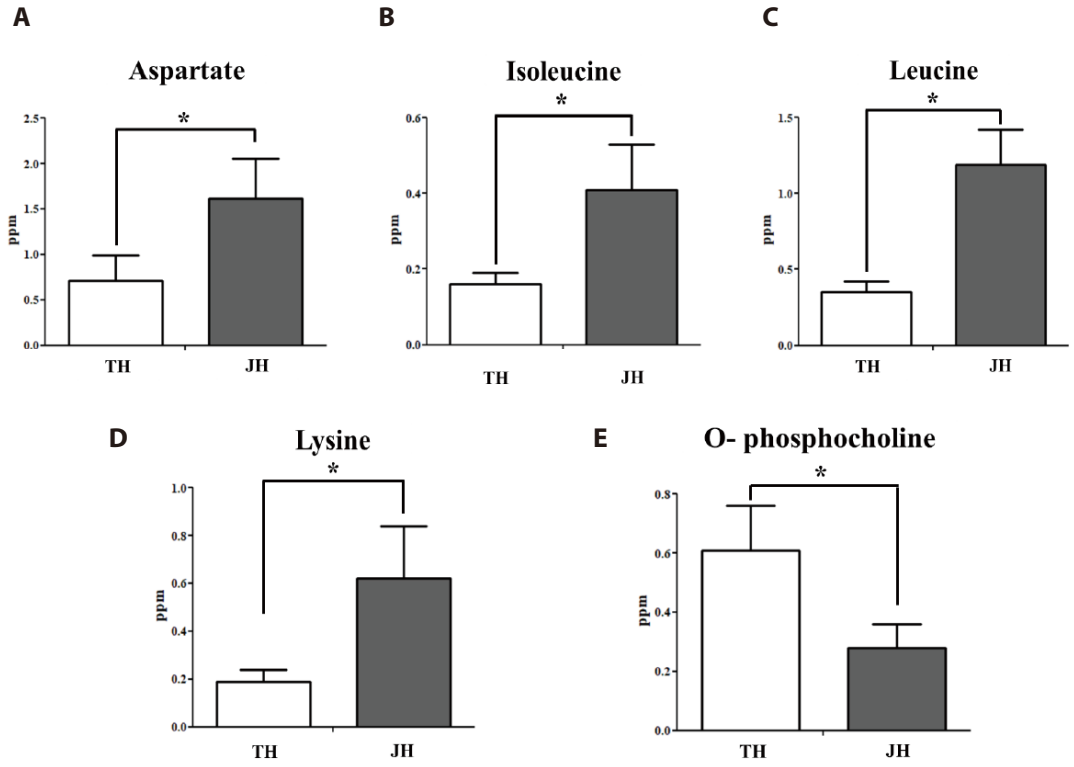

Fig. 2. Significant differences in skeletal muscle metabolite levels between Thoroughbred and Jeju horses (A) leucine (before exercise), (B) isoleucine (before exercise), (C) lysine (before exercise), (D) aspartate (before exercise) and o-phosphocholine (after exercise). *$p < 0.05$. All values are expressed in ppm as the mean ± SD. TH, Thoroughbred horse; JH, Jeju horse.

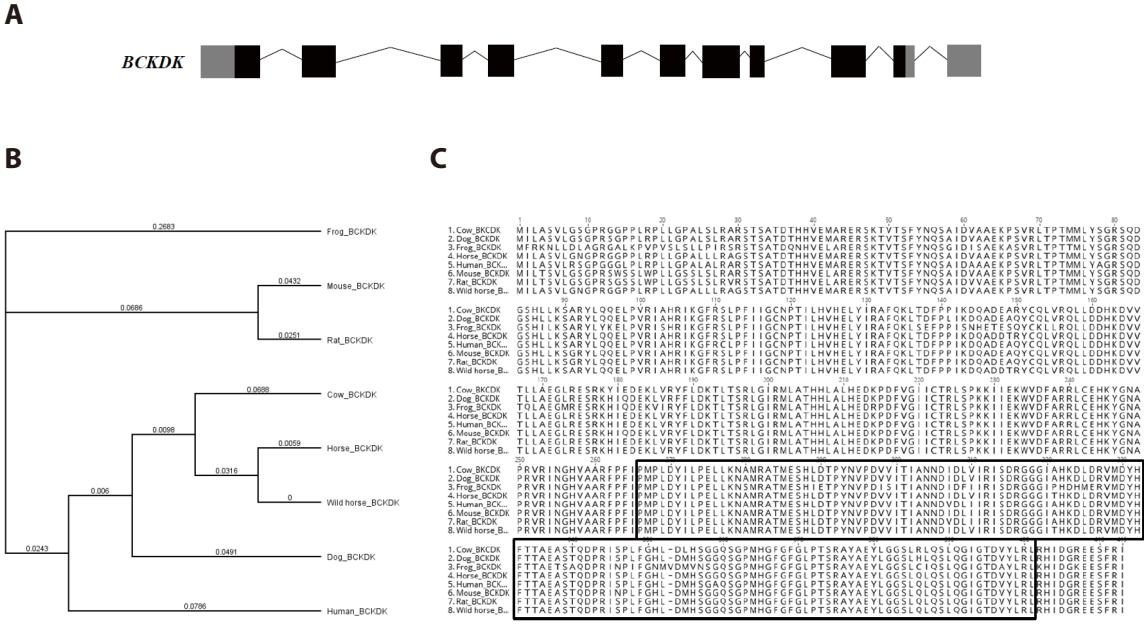

Fig. 3. Analysis of amino acid sequences and phylogenetic tree of branched chain (alpha) keto acid dehydrogenase kinase complex (BCKDK) gene among various species. (A) Gene structure of the branched chain (alpha) keto acid dehydrogenase kinase complex (BCKDK) gene in horses. Black boxes indicate exons, grey boxes indicate untranslated regions (UTR), and black lines indicate introns. (B) Phylogenetic tree of BCKDK. The phylogenetic tree was made with the full amino acid sequences of each species by Neighbor-Joining method after alignment by the MUSCLE method using GENEIOUS. Horse AXL was more similar to cow and dog than to frog and mouse. (C) 'Alignments of histidine kinase-like ATPases' domain of BCKDK from various species. The sequences were aligned by the MUSCLE method in GENEIOUS program.

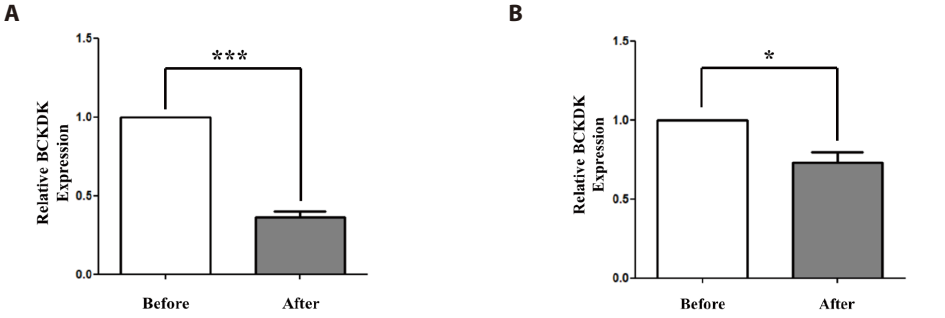

Fig. 4. BCKDK gene expression in skeletal muscle of Thoroughbred and Jeju horses. (A) BCKDK in Thoroughbred horses. (B) BCKDK in Jeju horses. BCKDK gene expression significantly decreased after the exercise in Thoroughbred horses (n = 3, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Error bars indicate standard error). The relative expression for each gene was normalized to that of GAPDH and calculated with the 2−rr CT method (mean ± SD of triplicate experiments; two-tailed Student *t*-test). BCKDK, branched chain (alpha) keto acid dehydrogenase kinase complex.

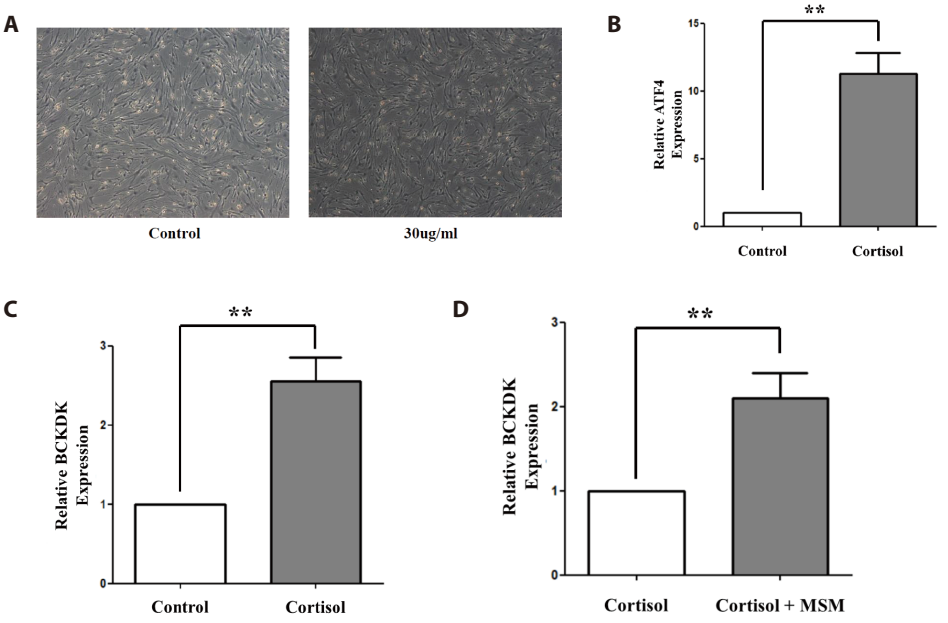

Fig. 5. Effects of exercise stress on BCKDK gene expression in horse muscle-derived cells and effects of methylsulfonylmethane (MSM) on stress reduction. (A) Morphology of horse muscle-derived cells. (B) ATF4 gene, as exercise stress marker gene expression using qRT-PCR. white and grey bars represent gene expression in the presence and absence of cortisol treatment (30 ug/mL). (C) Expression of horse BCKDK analyzed using RT-PCR in horse muscle-derived cells after treatment with 30 μg/mL cortisol. Data are presented as one of three independent experiments. (D) Analysis of relative BCKDK gene expression using qRT-PCR under cortisol and MSM treatment. white and grey bars represent gene expression in the presence and absence of MSM treatment (100 mM), under exercise stress (30 μg/mL cortisol). The relative expression for each gene was normalized to that of GAPDH and calculated with the $2^{-\triangle\triangle}$ CT method (mean ± SD of triplicate experiments; two-tailed Student *t*-test). BCKDK, branched chain (alpha) keto acid dehydrogenase kinase complex; qRT-PCR, quantitative real-time polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

in skeletal muscle [24]. Among these amino acids, isoleucine and leucine, which are BCAAs, play pivotal roles in exercise physiology [25]. Additionally, the basic amino acid lysine also plays important roles in skeletal muscle metabolism [26]. The comparison analysis shows the physiological status of Thoroughbreds and Jeju horses. Elevated amounts of alanine, methionine, and taurine in plasma pre-exercise in Thoroughbreds, suggest their capacity to perform in

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races. In skeletal muscle, high amounts of aspartate, isoleucine, leucine, and lysine in Jeju horses indicate their slow ability to respond to exercise. This finding is supported by the low amount of phosphocholine in Jeju horses. In general, alterations in the concentrations of essential amino acids such as methionine, isoleucine, leucine, and lysine in plasma and /or in skeletal muscle reflects the important functions of essential amino acids in moderate exercise. Moreover, various studies on horses showed decreased BCCAs in plasma and changes in skeletal muscle [27]. Generally, exercise induces protein degradation in skeletal muscle, but several studies on humans and horses showed that supplementation of amino acids reduces this process [28]. Furthermore, Thoroughbred horses participate in daily racing practice; this may contribute to the lesser amounts of these amino acids in their skeletal muscle. Thoroughbred horses have been specially bred for sports; and the racing ability of this breed is higher than in Jeju horses [29]. Physiological factors such as body weight and height contribute to racing ability. In contrast to Thoroughbreds, Jeju horses have low weight and height which has been used for mechanical work. A decreased expression level of *BCKDK* after exercise in Thoroughbred horses indicates their catabolic ability to BCAAs. As a result, low levels of BCKDK enzymes available in skeletal muscle could activate the BCKDH enzyme complex while performing exercise. Despite these results, we propose that low levels of BCKDK in Thoroughbred horses leads to the activation of the BCKDH enzyme complex, and as a result the catabolism of BCAAs is increased in skeletal muscle. These consequent reactions may lead to BCCAs acting as fuels, as well as anabolic signals for protein synthesis in Thoroughbred horses. For Jeju horses, the lack of change in BCKDK gene expression level may lead to continued suppression of the BCKDH complex, which would result in high levels of BCAAs in skeletal muscle. Moreover, binding of the BCKDH complex with BCKDK also plays an important role in this catabolic process. The process has been well studied in rats, and the results have shown that BCKDK capacity to bind to the BCKDH complex crucially affects BCKDH catalytic activity [30]. In this study, we focused on metabolomes and transcriptomes, but not proteomes. Collectively, the results presented indicate that BCKDK genes play important roles in the exercise response.

REFERENCES

1. Muñoz A, Riber C, Trigo P, Castejón-Riber C, Castejón FM. Dehydration, electrolyte imbalances and renin-angiotensin-aldosterone-vasopressin axis in successful and unsuccessful endurance horses. Equine Vet J. 2010;42:83-90. https://doi.org/10.1111/j.2042-3306.2010.00211.x
2. Treiber KH, Hess TM, Kronfeld DS, Boston RC, Geor RJ, Friere M, et al. Glucose dynamics during exercise: dietary energy sources affect minimal model parameters in trained Arabian geldings during endurance exercise. Equine Vet J. 2006;38:631-6. https://doi.org/10.1111/j.2042-3306.2006.tb05617.x
3. Coenen M. Exercise and stress: impact on adaptive processes involving water and electrolytes. Livest Prod Sci. 2005;92:131-45. https://doi.org/10.1016/j.livprodsci.2004.11.018
4. Hargreaves M. Skeletal muscle metabolism during exercise in humans. Clin Exp Pharmacol Physiol. 2000;27:225-8. https://doi.org/10.1046/j.1440-1681.2000.03225.x
5. Beckonert O, Keun HC, Ebbels TMD, Bundy J, Holmes E, Lindon JC, et al. Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts. Nat Protoc. 2007;2:2692-703. https://doi.org/10.1038/nprot.2007.376
6. Keller MD, Pollitt CC, Marx UC. Nuclear magnetic resonance-based metabonomic study of early time point laminitis in an oligofructose-overload model. Equine Vet J. 2011;43:737-43. https://doi.org/10.1111/j.2042-3306.2010.00336.x

7. Ralston SL, Pappalardo L, Pelczer I, Spears PF. NMR-based metabonomic analyses of horse serum: detection of metabolic markers of disease. In: Recent recent advances in Animal Nutrition-Australia; 2011; Armidale, Australia. p. 197-205.
8. Apró W, Blomstrand E. Influence of supplementation with branched-chain amino acids in combination with resistance exercise on p70S6 kinase phosphorylation in resting and exercising human skeletal muscle. Acta Physiol. 2010;200:237-48. https://doi.org/10.1111/j.1748-1708.2010.02151.x
9. Anthony JC, Anthony TG, Kimball SR, Jefferson LS. Signaling pathways involved in translational control of protein synthesis in skeletal muscle by leucine. J Nutr. 2001;131:856S-60S. https://doi.org/10.1093/jn/131.3.856S
10. Du M, Shen QW, Zhu MJ, Ford SP. Leucine stimulates mammalian target of rapamycin signaling in C2C12 myoblasts in part through inhibition of adenosine monophosphate-activated protein kinase. J Anim Sci. 2007;85:919-27. https://doi.org/10.2527/jas.2006-342
11. Shimomura Y, Obayashi M, Murakami T, Harris RA. Regulation of branched-chain amino acid catabolism: nutritional and hormonal regulation of activity and expression of the branched-chain α-keto acid dehydrogenase kinase. Curr Opin Clin Nutr Metab Care. 2001;4:419-23. https://doi.org/10.1097/00075197-200109000-00013
12. Harris RA, Joshi M, Jeoung NH, Obayashi M. Overview of the molecular and biochemical basis of branched-chain amino acid catabolism. J Nutr. 2005;135:1527S-30S. https://doi.org/10.1093/jn/135.6.1527S
13. Suryawan A, Hawes JW, Harris RA, Shimomura Y, Jenkins AE, Hutson SM. A molecular model of human branched-chain amino acid metabolism. Am J Clin Nutr. 1998;68:72-81. https://doi.org/10.1093/ajcn/68.1.72
14. Popov KM, Zhao Y, Shimomura Y, Kuntz MJ, Harris RA. Branched-chainα-ketoacid dehydrogenase kinase. Molecular cloning, expression, and sequence similarity with histidine protein kinases. J Biol Chem. 1992;267:13127-30. https://doi.org/10.1016/S0021-9258(18)42179-5
15. Xu M, Nagasaki M, Obayashi M, Sato Y, Tamura T, Shimomura Y. Mechanism of activation of branched-chain α-keto acid dehydrogenase complex by exercise. Biochem Biophys Res Commun. 2001;287:752-6. https://doi.org/10.1006/bbrc.2001.5647
16. Nobukuni Y, Mitsubuchi H, Akaboshi I, Indo Y, Endo F, Yoshioka A, et al. Maple syrup urine disease. Complete defect of the E1 beta subunit of the branched chain alpha-ketoacid dehydrogenase complex due to a deletion of an 11-bp repeat sequence which encodes a mitochondrial targeting leader peptide in a family with the disease. J Clin Invest. 1991;87:1862-6. https://doi.org/10.1172/JCI115209
17. García-Cazorla A, Oyarzabal A, Fort J, Robles C, Castejón E, Ruiz-Sala P, et al. Two novel mutations in the BCKDK (branched-chain keto-acid dehydrogenase kinase) gene are responsible for a neurobehavioral deficit in two pediatric unrelated patients. Hum Mutat. 2014;35:470-7. https://doi.org/10.1002/humu.22513
18. Trottier NL, Nielsen BD, Lang KJ, Ku PK, Schott HC. Equine endurance exercise alters serum branched-chain amino acid and alanine concentrations. Equine Vet J. 2002;34:168-72. https://doi.org/10.1111/j.2042-3306.2002.tb05412.x
19. Le Moyec L, Robert C, Triba MN, Billat VL, Mata X, Schibler L, et al. Protein catabolism and high lipid metabolism associated with long-distance exercise are revealed by plasma NMR metabolomics in endurance horses. PLOS ONE. 2014;9:e90730. https://doi.org/10.1371/journal.pone.0090730
20. Essén-Gustavsson B, Jensen-Waern M. Effect of an endurance race on muscle amino

https://doi.org/10.5187/jast.2022.e45

acids, pro- and macroglycogen and triglycerides. Equine Vet J. 2002;34:209-13. https://doi.org/10.1111/j.2042-3306.2002.tb05420.x
21. Park JW, Kim KH, Choi JK, Park TS, Song KD, Cho BW. Regulation of toll-like receptors expression in muscle cells by exercise-induced stress. Anim Biosci. 2021;34:1590-9. https://doi.org/10.5713/ab.20.0484
22. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods. 2001;25:402-8. https://doi.org/10.1006/meth.2001.1262
23. Jang HJ, Kim DM, Kim KB, Park JW, Choi JY, Oh JH, et al. Analysis of metabolomic patterns in thoroughbreds before and after exercise. Asian-Australas J Anim Sci. 2017;30:1633-42. https://doi.org/10.5713/ajas.17.0167
24. van den Hoven R, Bauer A, Hackl S, Zickl M, Spona J, Zentek J. Changes in intramuscular amino acid levels in submaximally exercised horses – a pilot study. J Anim Physiol Anim Nutr. 2010;94:455-64. https://doi.org/10.1111/j.1439-0396.2009.00929.x
25. Blomstrand E, Eliasson J, Karlsson HK, Köhnke R. Branched-chain amino acids activate key enzymes in protein synthesis after physical exercise. J Nutr. 2006;136:269S-73S. https://doi.org/10.1093/jn/136.1.269S
26. Sato T, Ito Y, Nagasawa T. Regulation of skeletal muscle protein degradation and synthesis by oral administration of lysine in rats. J Nutr Sci Vitaminol. 2013;59:412-9. https://doi.org/10.3177/jnsv.59.412
27. Assenza A, Bergero D, Tarantola M, Piccione G, Caola G. Blood serum branched chain amino acids and tryptophan modifications in horses competing in long-distance rides of different length. J Anim Physiol Anim Nutr. 2004;88:172-7. https://doi.org/10.1111/j.1439-0396.2004.00493.x
28. van den Hoven R, Bauer A, Hackl S, Zickl M, Spona J, Zentek J. A preliminary study on the changes in some potential markers of muscle-cell degradation in sub-maximally exercised horses supplemented with a protein and amino acid mixture. J Anim Physiol Anim Nutr. 2011;95:664-75. https://doi.org/10.1111/j.1439-0396.2010.01097.x
29. Barrey E, Evans SE, Evans DL, Curtis RA, Quinton R, Rose RJ. Locomotion evaluation for racing in Thoroughbreds. Equine Vet J. 2001;33:99-103. https://doi.org/10.1111/j.2042-3306.2001.tb05369.x
30. Obayashi M, Sato Y, Harris RA, Shimomura Y. Regulation of the activity of branched-chain 2-oxo acid dehydrogenase (BCODH) complex by binding BCODH kinase. FEBS Lett. 2001;491:50-4. https://doi.org/10.1016/S0014-5793(01)02149-4