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RESEARCH ARTICLE

Effect of endurance training and branched-chain amino acids on the signaling for muscle protein synthesis in CKD model rats fed a low-protein diet

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Yoshida T, Kakizawa S, Totsuka Y, Sugimoto M, Miura S, Kumagai H. Effect of endurance training and branched-chain amino acids on the signaling for muscle protein synthesis in CKD model rats fed a low-protein diet. *Am J Physiol Renal Physiol* 313: F805–F814, 2017. First published July 12, 2017; doi:10.1152/ajprenal.00592.2015.—A low-protein diet (LPD) protects against the progression of renal injury in patients with chronic kidney disease (CKD). However, LPD may accelerate muscle wasting in these patients. Both exercise and branched-chain amino acids (BCAA) are known to increase muscle protein synthesis by activating the mammalian target of rapamycin (mTOR) pathway. The aim of this study was to investigate whether endurance exercise and BCAA play a role for increasing muscle protein synthesis in LPD-fed CKD (5/6 nephrectomized) rats. Both CKD and sham rats were pair-fed on LPD or LPD fortified with a BCAA diet (BD), and approximately one-half of the animals in each group was subjected to treadmill exercise (15 m/min, 1 h/day, 5 days/wk). After 7 wk, renal function was measured, and soleus muscles were collected to evaluate muscle protein synthesis. Renal function did not differ between LPD- and BD-fed CKD rats, and the treadmill exercise did not accelerate renal damage in either group. The treadmill exercise slightly increased the phosphorylation of p70s6 kinase, a marker of mTOR activity, in the soleus muscle of LPD-fed CKD rats compared with the sham group. Furthermore, BCAA supplementation of the LPD-fed, exercise-trained CKD rats restored the phosphorylation of p70s6 kinase to the same level observed in the sham group; however, the corresponding induced increase in muscle protein synthesis and muscle mass was marginal. These results indicate that the combination of treadmill exercise and BCAA stimulates cell signaling to promote muscle protein synthesis; however, the implications of this effect for muscle growth remain to be clarified.

chronic kidney disease; endurance training; branched-chain amino acids; low-protein diets

MUSCLE WASTING is a frequent complication of chronic kidney disease (CKD) and is associated with high all-cause mortality in the sufferers of the disease (40). In CKD patients, muscle protein synthesis and degradation are altered by such complications as acidosis, inflammation, diabetes, malnutrition, and low physical activity (1, 16), leading to the decline of muscle mass and strength (39). Therefore, maintenance of muscle metabolism is an important therapeutic strategy in CKD patients.

Clinical practice guidelines for the management of CKD published in the United States and Japan (25, 28) recommend a low-protein diet (LPD) with 0.6–0.8 $g \cdot kg^{-1} \cdot day^{-1}$ of dietary protein for CKD patients to protect their renal function. However, LPD may potentially accelerate muscle wasting in patients with CKD. Although a carefully controlled LPD with adequate energy intake is likely to prevent muscle wasting, insufficient energy intake or consumption of proteins with low biological value can increase the risk of negative protein balance. An animal study showed that the activation of the ubiquitin-proteasome system (UPS) and caspase-3-dependent apoptosis contribute to the increase of protein degradation in the muscles of CKD animals fed the LPD (45). Furthermore, the phosphorylation of p70S6 kinase and Akt was suppressed in CKD rats fed the LPD in that study, suggesting that muscle protein synthesis also decreased.

A strategic approach for preventing muscle wasting in CKD includes exercise, diet supplementation with a variety of nutrients, or a combination of both of these. Several studies have shown that exercise activates muscle synthesis (12) and that weight training reduces muscle wasting in CKD (10). Conversely, protein wasting is associated with decreased concentrations of branched-chain amino acids (BCAA) in plasma and tissues of uremic rats (23). Chen et al. (12) examined the effects of BCAA supplementation, primarily leucine, and the work overload of the plantaris muscle on protein metabolism in CKD rats. The combination of leucine supplementation and work overload stimulated the phosphorylation of the mTOR signaling pathway in CKD rats, although the effect was significantly less pronounced than that observed in normal rats. These researchers concluded that the leucine-rich supplements could augment the work-induced activation of the mTOR pathway in CKD rats. However, their experimental setting was far from the reality of human CKD. First, the rats were given standard laboratory food plus 1.35 $g \cdot kg^{-1} \cdot day^{-1}$ of leucine, and thus the total amount of protein and amino acids might be hazardous to the renal function. Second, the compensatory work overload model does not entirely reproduce the intermittent nature of exercise performed by humans. Furthermore, these researchers did not provide anthropometric details of the effects of BCAA and workload on muscle mass.

In the present study, we investigated the effects of treadmill exercise and low-level BCAA supplementation on signaling

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leading to muscle protein synthesis, muscle protein metabolism, and muscle mass in CKD rats fed LPD. The amount of administered BCAA was limited to 1% (0.40 $g \cdot kg^{-1} \cdot day^{-1}$) because this dose was confirmed not to harm renal function in this study.

MATERIALS AND METHODS

Animals. Seventy-one male Wistar rats (9 wk old, weighing 215–245 g) were purchased from Japan SLC (Hamamatsu, Japan). All rats were maintained under standard laboratory conditions (temperature of 23 ± 1°C, humidity of 60% and 12:12-h light/dark cycle) and given access to standard rat chow and tap water ad libitum until the start of the experiments. The rats were familiarized with the animal cages and the treadmill. After an acclimatization period of 1 wk, 45 rats were subjected to two-step 5/6 nephrectomy (5/6Nx) under anesthesia by pentobarbital sodium (50 mg/kg ip). Briefly, two branches of the left renal artery were ligated to infarct two-thirds of the kidney. After 1 wk, the right kidney was removed. The remaining 26 rats underwent a sham operation. All rats were housed in individual cages for 4 wk to recover from the surgery and monitored for renal failure. Unfortunately, four rats died after 5/6Nx, probably because of renal failure. All animal procedures were approved by the Ethics Committee for Animal Experimentation at the University of Shizuoka (approval no. 135026).

Experimental protocols. The 24-h urine sample was collected using metabolic cages, and blood pressure was measured by the tail-cuff method (BP-98; Softron, Tokyo, Japan) 4 wk after the surgery. Blood samples were collected from the tail artery, and the blood urea nitrogen (BUN) concentration was measured. The rats were assigned to eight experimental groups. The sham rats were divided into four groups as follows: in *group 1*, sham rats fed LPD (sham-LPD, $n = 6$); in *group 2*, sham rats fed LPD + BCAA diet (BD) (sham-BD, $n = 6$); in *group 3*, sham rats fed LPD and exercised (sham-LPD + Ex, $n = 6$); and in *group 4*, sham rats fed BD and exercised (sham-BD + Ex, $n = 8$). The Nx rats were also divided into four groups as follows: in *group 5*, 5/6Nx rats fed LPD (5/6Nx-LPD, $n = 10$); in *group 6*, 5/6Nx rats fed BD (5/6Nx-BD, $n = 10$); in *group 7*, 5/6Nx rats fed LPD and exercised (5/6Nx-LPD + Ex, $n = 11$); and in *group 8*, 5/6Nx rats fed BD and exercised (5/6Nx-BD + Ex, $n = 10$). All 5/6Nx groups and all sham groups were matched on the basis of their body weight, systolic blood pressure, and BUN, respectively.

All groups were given their corresponding diets ad libitum on the first day after the start of the test diet administration. On the following days, all rats were offered as much food as the group that consumed the smallest amount on the preceding day. In this study, the smallest consumption group was the sham-LPD group throughout the experiment (~20 g). Although nonexercised groups were pair-fed, exercised groups were provided not only with the same amount of food as the nonexercised groups, but also with additional 2 g of non-protein diet to compensate for the energy loss induced by exercise. Finally, all rats were pair-fed in terms of protein consumption.

The composition of the experimental diets was based on the standard rodent diet, AIN-93M (38). The LPD contained 5% casein and 1% glycine as a nitrogen source, and the BD contained 5% casein and 1% BCAA (leucine, isoleucine, and valine; 3:1:1). The amount of amino acids added to the experimental diets (glycine or BCAA) was determined by a preliminary experiment which revealed that the addition of 3% BCAA to LPD worsened renal function in 5/6Nx rats when compared with the LPD or LPD + 1% BCAA diet.

The rats in the exercise groups ran on a motor-driven treadmill (MK-680; Muromachi Kikai, Tokyo, Japan) at an incline of 0°, 5 times/wk for 7 wk. These rats ran on a treadmill for 10 min at 15 m/min in the first 2 days. The exercise time was then increased by 10 min every day until it reached 60 min. This exercise protocol was characterized as low intensity because the O_2 consumption was less than 55% of the $\dot{V}O_{2max}$, and this level of exercise has been proven to not injure the kidney in 5/6Nx rats (17, 44).

At the end of the 7-wk experimental period, all rats were euthanized immediately after the final exercise session and after the blood samples were collected from the abdominal aorta under anesthesia by pentobarbital sodium (50 mg/kg ip). The gastrocnemius muscle, the soleus muscle, and the epididymal fat were harvested and weighed. The soleus muscle was frozen in liquid nitrogen and stored at −80°C for mRNA and protein analyses.

Analytical procedures. Urinary protein concentration was measured by the pyrogallol red-molybdenum-protein method using a microTP-test kit (Wako, Osaka, Japan). BUN was measured by the urease-indophenol method, and plasma and urine creatinine (Cr) levels were measured by the Jaffe method. Creatinine clearance (Ccr) was calculated using the following equation: Ccr (l/day) = urinary Cr concentration (mg/dl) × 24-h urine volume (dl)/plasma Cr concentration (mg/dl)/10. Plasma interleukin-6 (IL-6) was measured by using a commercial IL-6 ELISA kit (Quantikine ELISA Rat IL-6; R&D Systems, Minneapolis, MN).

Plasma BCAA concentration was analyzed by reversed-phase high-performance liquid chromatography (HPLC) using a previously published procedure (20), with slight modifications. Briefly, the samples were deproteinized by the addition of 5 μl of sulfosalicylic acid (1 g/ml) to 95 μl of the plasma. The mixture was centrifuged at 10,000 rpm for 15 min at 4°C and the supernatant was collected. Sample supernatants and a standard amino acid mixture (Amino Acids Mixture Standard Solution Type H; Wako, Osaka, Japan) were dried under nitrogen gas and neutralized by the addition of 20 μl of 2:2:1 (vol/vol/vol) mixture of ethanol:water:triethyl amine (TEA). Next, the samples were again dried under nitrogen gas. Derivatization was performed by adding 20 μl of 7:1:1:1 (vol/vol/vol/vol) mixture of ethanol:water:TEA:phenylisothiocyanate and mixing well. The reaction was conducted for 20 min at room temperature. Samples were then completely dried under nitrogen gas. The standard and the samples were dissolved in 1 ml of *eluent A* (acetonitrile:60 mM sodium acetate buffer, pH 6.8, 6:94, vol/vol), and injected (10 μl) to a Capcell Pak C_{18} UG120 column (250 × 4.6 mm ID; Shiseido, Tokyo) by an autosampler. Amino acids were separated by a gradient resulting from mixing *eluents A* and *B* (acetonitrile:60 mM sodium acetate buffer, pH 6.8, 60:40, vol/vol). The flow rate was 1 ml/min throughout, and the gradient was set as follows: 100% *A* at the start; 50% *A* and 50% *B* by 20 min; 30% *A* and 70% *B* by 21 min; 100% *B* by 26 min; and 100% *A* by 32 min. The absorbance was measured by a UV detector at 254 nm (Toso, Tokyo).

Histology. Paraffin-embedded soleus muscle sections were stained with hematoxylin and eosin. The cross-sectional areas of soleus muscle fibers were measured using the ImageJ program (http://rsbweb.nih.gov/ij/). A least 100 muscle fibers were randomly chosen for analysis from each sample.

Western blotting. Western blotting for p70s6 kinase and 4E-BP1, downstream signalings of the mTOR pathway, was performed to assess the protein synthesis in the soleus muscle. The soleus muscle was homogenized in the Triton buffer (100 mM Tris, pH 7.4, 1% Triton X-100, 100 mM sodium pyrophosphate, 100 mM NaF, 10 mM EDTA, 10 mM Na_3Vo_4, 2 mM PMSF, and 0.1 mg/ml aprotinin). Protein concentrations in the samples were measured by the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA) and adjusted to the same concentration between samples. The samples boiled for 4 min were then electrophoresed by SDS-PAGE and transferred onto nitrocellulose membranes. First of all, phosphoproteins were immunodetected. The membranes were stained using primary antibodies for phospho-p70S6 kinase (Thr421/Ser424), phospho-4E-BP1 (Thr70), or phospho-Akt (Ser473) and then probed by horseradish peroxidase-conjugated secondary antibodies. The blots were developed using an enhanced chemiluminescence reagent, ECL select (GE Healthcare, Buckinghamshire, UK). Bands were visualized using a luminoanalyzer (Las-3000 mini; Fuji Photo Film, Tokyo), and the band intensity

was determined using an imaging software (Quantity One; Bio-Rad Laboratories). After this procedure, antibodies of phosphoprotein were stripped from the membranes. Finally, the membranes were immunodetected using primary antibodies for the specific total protein of p70s6 kinase, 4E-BP1, and Akt, and with the other reagents described above. All antibodies were purchased from Cell Signaling Technology (Danvers, MA).

Real-time RT-PCR. The expression of target genes was examined by real-time reverse transcription polymerase chain reaction (RT-PCR), as described previously (47). Total RNA was extracted from snap-frozen soleus muscle tissue using Isogen II (Nippon Gene, Toyama, Japan). Next, the first-stranded cDNA was made using the Prime Script RT reagent kit (Takara, Shiga, Japan). Real-time RT-PCR amplification was performed using SYBR Premix Ex Taq II (Takara) with specific primer sets. The primer sets were purchased from Takara and used to assess expression of genes encoding the following proteins: IL-6 (primer set RA060834) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; primer set RA015380). PCR was performed using the Thermal Cycler Dice Real Time System II (Takara). Expression levels of target mRNA were standardized to *GAPDH* mRNA. To quantify the target mRNA abundance, differences in threshold cycles between the gene target and GAPDH were calculated; relative mRNA abundance was then calculated using the $2^{-\Delta\Delta Ct}$ method. The amount of mRNA was expressed as the ratio to the mean value for sham-LPD rats.

Muscle protein synthesis and degradation rate. Another set of all experimental groups (n = 5–7 per group) was prepared as described above to measure muscle protein synthesis and degradation rates. Because the muscle protein synthesis is established to be maximal at 24 h after exercise (21), both the exercise-trained and sedentary rats were euthanized at this point. After euthanasia, the soleus muscle from each animal was immediately harvested and preincubated (37°C, 30 min, in 95% O_2-5% CO_2) in Krebs-Henseleit bicarbonate (KHB) buffer (pH 7.4) supplemented with glucose (10 mmol/l) and phenylalanine (0.5 mmol/l) (14, 33). The muscle was then transferred to 3 ml of fresh medium of the same composition, but with additional [^{14}C]phenylalanine (0.05 μCi/ml), and incubated for a further 2 h. The muscle was then homogenized in 10% cold trichloroacetic acid (TCA), before the acid-precipitated pellet was washed twice with 10% TCA, and once with an equimolar mixture of ether and ethanol. The pellet was next dried under nitrogen gas and dissolved in 1 ml of Tissue Solubilizer (TS-2; Research Products International, Mt. Prospect, IL). Radioactivity was measured using a liquid scintillation spectrophotometer (LSC-7400; Hitachi Aloka Medical, Tokyo).

Release of tyrosine from the incubated muscles was used as a measure of the total protein breakdown rate. Each soleus muscle was individually preincubated and incubated (as described above) in incubation medium modified to contain 0.5 mmol/l cycloheximide, but no phenylalanine. At the end of the 2-h incubation, the tyrosine concentration in the medium was analyzed by HPLC, using the same method as was employed to analyze the concentration of plasma amino acids.

Statistical analyses. The data are shown as means ± SE. Analysis of variance (ANOVA) was used for multiple-group comparisons, followed by the Tukey HSD test. Correlations between two variables were examined by Pearson correlation analysis. A P value < 0.05 was considered to be statistically significant. All statistical tests were performed using SPSS ver.18 for Windows (SPSS, Chicago, IL).

RESULTS

Body weight, tissue weight, and cross-sectional area of the muscle fibers. Body weight was not significantly different between all animal groups at the start of the experimental period. The increase in body weight during the 7-wk experimental period was lower in 5/6Nx rats than in sham rats (Table 1). The soleus muscle weight and the mean cross-sectional area

Table 1. *Body weight and muscle weights for 7-wk experimental period*

	Sham-LPD (n = 6)	Sham-BD (n = 6)	Sham-LPD + Ex (n = 6)	Sham-BD + Ex (n = 8)	5/6Nx-LPD (n = 10)	5/6Nx-BD (n = 10)	5/6Nx-LPD + Ex (n = 11)	5/6Nx-BD + Ex (n = 10)	ANOVA P Value
Body wt, g	378 ± 8	373 ± 11	360 ± 8	357 ± 7	335 ± 10[e]	343 ± 7	334 ± 8[e]	340 ± 7	<0.005
Gastrocnemius muscle wt, g	2.03 ± 0.03	1.91 ± 0.05	2.11 ± 0.09	2.04 ± 0.02	1.95 ± 0.05	1.87 ± 0.04[g]	2.03 ± 0.04	2.02 ± 0.03	<0.05
Soleus muscle wt, mg	131 ± 2.8	127 ± 3.2	152 ± 10.8[g]	146 ± 5.1	124 ± 2.7[b,h]	131 ± 3.2	151 ± 3.3[c,f,i]	154 ± 4.0[a,c,d,e]	<0.001
CSA of soleus muscle fiber, μm^2	2,195 ± 103	2,144 ± 100	2,806 ± 96	2,533 ± 240	2,049 ± 126[g]	1,976 ± 107[g]	2,135 ± 186	2,290 ± 70	<0.05

Values are means ± SE. LPD, low-protein diet; BD, branched-chain amino acid diet; Ex, exercise; CSA, cross-sectional area. [a]$P < 0.01$ vs. sham-BD; [b]$P < 0.01$ vs. sham-LPD + Ex; [c]$P < 0.01$ vs. /5/6Nx-LPD; [d]$P < 0.01$ vs. 5/6Nx-BD; [e]$P < 0.05$ vs. sham-LPD; [f]$P < 0.05$ vs. sham-BD; [g]$P < 0.05$ vs. sham-LPD + Ex; [h]$P < 0.05$ vs. sham-BD + Ex; [i]$P < 0.05$ vs. 5/6Nx-BD.

Table 2. *Laboratory data for 7-wk experimental period*

	Sham-LPD ($n = 6$)	Sham-BD ($n = 6$)	Sham-LPD + Ex ($n = 6$)	Sham-BD + Ex ($n = 8$)	5/6Nx-LPD ($n = 10$)	5/6Nx-BD ($n = 10$)	5/6Nx-LPD + Ex ($n = 11$)	5/6Nx-BD + Ex ($n = 10$)	ANOVA *P* Value
SBP, mmHg	136 ± 4	135 ± 5	143 ± 5	143 ± 6	169 ± 5[a,b,g,h]	179 ± 6[a,b,c,d]	177 ± 5[a,b,c,d]	170 ± 5[a,b,g,h]	<0.001
BUN, mg/dl	1.85 ± 0.22	1.63 ± 0.15	1.22 ± 0.15	1.71 ± 0.13	6.29 ± 1.21	6.38 ± 0.88	9.00 ± 1.40[a,b,c,d]	6.77 ± 1.43[f]	<0.001
Plasma Cr, mg/dl	0.39 ± 0.05	0.42 ± 0.07	0.57 ± 0.04	0.52 ± 0.03	1.05 ± 0.10[a,b,h]	1.06 ± 0.11[a,b,h]	1.28 ± 0.13[a,b,c,d]	1.23 ± 0.11[a,b,c,d]	<0.001
Urinary protein excretion, mg/day	3.3 ± 0.7	4.6 ± 0.8	3.8 ± 0.7	3.8 ± 0.3	49.3 ± 9.0[a,b,c,d]	54.8 ± 7.9[a,b,c,d]	57.6 ± 8.7[a,b,c,d]	49.6 ± 4.9[a,b,c,d]	<0.001
Urinary Cr excretion, mg/day	6.12 ± 0.49	6.47 ± 0.65	8.79 ± 0.67	7.48 ± 0.43	7.51 ± 0.54	7.06 ± 0.35	7.95 ± 0.48	7.88 ± 0.65	n.s.
Ccr, l/day	1.67 ± 0.21	1.88 ± 0.48	1.62 ± 0.20	1.45 ± 0.12	0.80 ± 0.11[b,e,f]	0.73 ± 0.08[a,b,f]	0.68 ± 0.07[a,b,c,g]	0.71 ± 0.11[a,b,f]	<0.001

Values are means ± SE. LPD, low-protein diet; BD, branched-chain amino acid diet; Ex, exercise; SBP, systolic blood pressure; BUN, blood urea nitrogen; Cr, creatinine; Ccr, creatinine clearance. [a]$P < 0.01$ vs. sham-LPD; [b]$P < 0.01$ vs. sham-BD; [c]$P < 0.01$ vs. sham-LPD + Ex; [d]$P < 0.01$ sham-BD + Ex; [e]$P < 0.05$ vs. sham-LPD; [f]$P < 0.05$ vs. sham-BD; [g]$P < 0.05$ vs. sham-LPD + Ex; [h]$P < 0.05$ vs. sham-BD + Ex.

Table 3. *Plasma branched-chain amino acids concentration for 7-wk experimental period*

	Sham-LPD ($n = 8$)	Sham-BD ($n = 8$)	Sham-LPD + Ex ($n = 7$)	Sham-BD + Ex ($n = 8$)	5/6Nx-LPD ($n = 10$)	5/6Nx-BD ($n = 10$)	5/6Nx-LPD + Ex ($n = 11$)	5/6Nx-BD + Ex ($n = 10$)	ANOVA *P* Value
Valine, μmol/l	94.2 ± 6.3	94.1 ± 4.4	95.5 ± 10.0	115.1 ± 7.4	84.8 ± 5.4	100.2 ± 4.3	86.4 ± 5.2	130.7 ± 12.7[a,b,c,d,e]	<0.001
Isoleucine, μmol/l	46.3 ± 3.9	47.5 ± 2.9[e]	46.0 ± 5.7	56.0 ± 2.7	42.8 ± 3.5	59.2 ± 1.8	47.5 ± 3.2	68.7 ± 5.4[a,b,c,d,e]	<0.001
Leucine, μmol/l	78.8 ± 7.0	107.7 ± 6.5	82.0 ± 10.3	110.7 ± 6.1[a,d,e]	69.1 ± 5.1	112.3 ± 3.4[a,d,e]	67.6 ± 6.4	129.7 ± 8.6[a,c,d,e]	<0.001
BCAA, μmol/l	219.3 ± 17.0	249.4 ± 12.9	223.5 ± 25.5	281.8 ± 15.3[d]	196.8 ± 13.7	271.8 ± 8.3[d]	201.5 ± 14.1	329.1 ± 26.3[a,b,c,d,e]	<0.001

Values are means ± SE. BCAA, branched-chain amino acids; LPD, low-protein diet; BD, branched-chain amino acid diet; Ex, exercise. [a]$P < 0.05$ vs. sham-LPD; [b]$P < 0.05$ vs. sham-BD; [c]$P < 0.05$ vs. sham-LPD + Ex; [d]$P < 0.05$ vs. 5/6Nx-LPD; [e]$P < 0.05$ vs. 5/6Nx-LPD + Ex.

of the soleus muscle fiber were increased by exercise in both sham and 5/6Nx groups compared with sedentary groups. However, the effect of exercise on the weight of the gastrocnemius muscle was relatively small in both sham and 5/6Nx groups. On the other hand, the 5/6Nx had a substantial effect on muscle mass in both exercised and sedentary groups. The cross-sectional area of the soleus muscle fiber tended to be lower in 5/6Nx groups than in sham groups, although a significant difference was observed only in a few groups. The effect of BCAA supplementation on muscle mass was negligible in all experimental conditions.

Renal function. BUN, plasma Cr, and urinary protein excretion increased and Ccr decreased in all 5/6Nx groups compared with sham groups after the 7-wk experimental period (Table 2). However, these parameters were not significantly different among the four groups of 5/6Nx rats, suggesting that there were no harmful effects of BCAA supplementation and exercise on renal function in the present study.

Plasma BCAA concentration. Plasma leucine concentrations were significantly lower in 5/6Nx rats fed LPD. BCAA supplementation resulted in increased plasma BCAA levels in sham and 5/6Nx groups after the 7-wk experimental period (Table 3).

Markers of protein synthesis in the soleus muscle. Changes in p70S6 kinase expression in the soleus muscle are shown in Fig. 1. Without exercise, BCAA supplementation did not increase the phosphorylation of p70S6 kinase in either sham or 5/6Nx rats. On the other hand, exercise significantly increased the levels of phosphorylated p70S6 kinase expression in both sham and 5/6Nx groups. In 5/6Nx rats fed LPD, the exercise stimulated the p70S6 kinase phosphorylation to about half the level of p70S6 kinase phosphorylation in exercised sham rats fed LPD. However, BCAA supplementation to exercise-trained 5/6Nx rats fed LPD restored the phosphorylation of p70s6 kinase to a level similar to that in exercise-trained sham groups. In contrast, exercise and BCAA supplementation did not increase the phosphorylation of Akt in either sham or 5/6Nx rats (data not shown).

The levels of phosphorylated 4E-BP1 are shown in Fig. 2. Exercise and BCAA supplementation had an inverse effect on the phosphorylation of 4E-BP1 expression compared with the effect on the phosphorylation of p70S6 kinase.

Muscle protein synthesis and degradation rates. The protein synthesis rate in incubated soleus muscles tended to be higher in exercise-trained sham and 5/6Nx than in nonexercised rats (Fig. 3); however, these differences were not statistically significant. BCAA supplementation had no effect on the muscle protein synthesis in any group.

The muscle protein degradation rate tended to be higher in 5/6Nx than in sham rats and appeared to be suppressed by exercise.

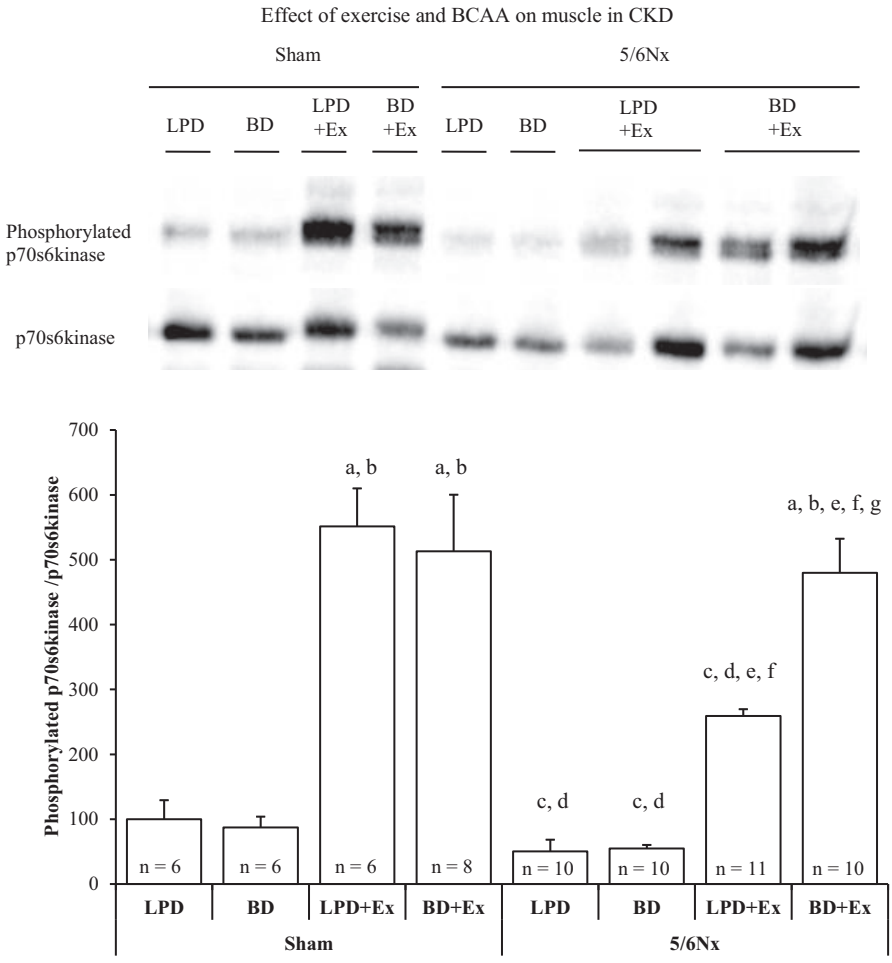

Fig. 1. The effects of treadmill exercise and BCAA on the phosphorylated p70S6 kinase of the soleus muscle. Although phosphorylation of p70S6 kinase was significantly greater in the exercise-trained sham and 5/6Nx rats, the 5/6Nx-LPD + Ex group was significantly less phosphorylated in p70S6 kinase than in the other 3 trained groups. [a]$P < 0.01$ vs. Sham-LPD; [b]$P < 0.01$ vs. Sham-BD; [c]$P < 0.01$ vs. Sham-LPD + Ex; [d]$P < 0.01$ vs. Sham-BD + Ex; [e]$P < 0.01$ vs. 5/6Nx-LPD; [f]$P < 0.01$ vs. 5/6Nx-BD; [g]$P < 0.01$ vs. 5/6Nx-LPD + Ex. Data are expressed as means ± SE. LPD, low-protein diet; BD, low-protein diet supplemented with BCAA; 5/6Nx, 5/6 nephrectomized; Ex, exercise.

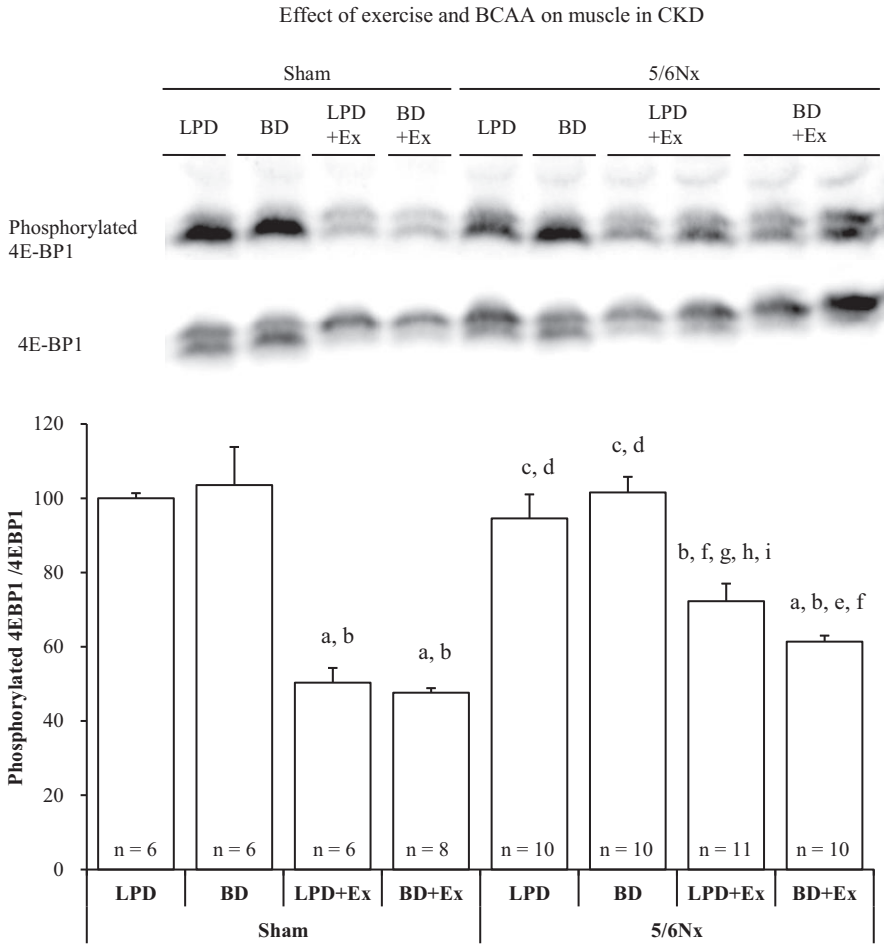

Fig. 2. The effects of treadmill exercise and BCAA on the phosphorylated 4E-BP1 of the soleus muscle. Phosphorylation of 4E-BP1 significantly decreased in the exercise-trained sham and 5/6Nx rats. [a]$P < 0.01$ vs. Sham-LPD; [b]$P < 0.01$ vs. Sham-BD; [c]$P < 0.01$ vs. Sham-LPD + Ex; [d]$P < 0.01$ vs. Sham-BD + Ex; [e]$P < 0.01$ vs. 5/6Nx-LPD; [f]$P < 0.01$ vs. 5/6Nx-BD; [g]$P < 0.05$ vs. Sham-LPD; [h]$P < 0.05$ vs. Sham-BD + Ex; [i]$P < 0.05$ vs. 5/6Nx-LPD. Data are expressed as means ± SE. LPD, low-protein diet; BD, low-protein diet supplemented with BCAA; 5/6Nx, 5/6 nephrectomized; Ex, exercise.

Expression of IL-6 gene in the soleus muscle and plasma IL-6 concentration. The expression of *IL-6* mRNA increased more than threefold in the soleus muscle of exercise-trained sham and 5/6Nx rats compared with nonexercised groups (Fig. 4). The plasma IL-6 concentrations were below the detection limit of the used IL-6 measurement kit in all samples from sham and 5/6Nx groups.

DISCUSSION

In the present study, we examined the effect of treadmill exercise and BCAA supplementation on cell signaling leading to muscle protein synthesis, muscle protein metabolism, and muscle mass in CKD model rats fed an LPD. The results demonstrated that the activation of p70S6 kinase by treadmill exercise was attenuated in 5/6Nx rats compared with sham rats. However, the supplementation of BCAA to exercised 5/6Nx rats restored the activation of p70S6 kinase to the levels elicited by the exercise in exercised sham rats. Although the nutrient-sensitive mTOR pathway is directly activated by BCAA, the supplementation of BCAA alone had no effect on the activation of p70S6 kinase in either sham rats or 5/6Nx rats in our experimental setting.

The need for BCAA in muscle protein synthesis is greatest during or after exercise. BCAA was previously shown to stimulate phosphorylation of p70S6 kinase synergistically with exercise (2, 6, 27), whereas there has been controversy as to whether exercise alone stimulates the phosphorylation of p70S6 kinase. Apró and Blomstrand (2) showed that phosphorylation of p70S6 kinase in a human leg muscle was unaffected by resistance exercise alone. In contrast, two other human studies demonstrated that resistance training leads to a robust increase in phosphorylation of p70S6 kinase at 1 or 2 h after the exercise without supplementation of BCAA (6, 27). Although the exact reason for this difference remains unknown, factors such as meal time, amount of food, exercise time, duration, or type might have affected the results.

CKD patients and animals are reportedly characterized by abnormal concentrations of free amino acids in the plasma, including low BCAA levels (4, 23). LPD was fed to 5/6Nx rats to prevent the progression of renal failure in the present study, and this study revealed that plasma BCAA levels, especially leucine levels, were actually decreased in 5/6Nx rats fed LPD. Uremia might also be resistant to the mTOR pathway activation, as shown by McIntire et al. (32). These might explain the lower p70S6 kinase phosphorylation in exercised 5/6Nx rats in the absence of BCAA administration.

In contrast to increasing phosphorylation of p70S6 kinase, the phosphorylation of 4E-BP1, another downstream effector of mTOR, decreased in both exercised sham and 5/6Nx rats, and the phosphorylation of p70S6 kinase was inversely correlated with the phosphorylation of 4E-BP1. Such contrasting responses of downstream effectors of mTOR have been reported during and immediately after resistance exercise (15). Indeed, the muscle tissues were harvested immediately after

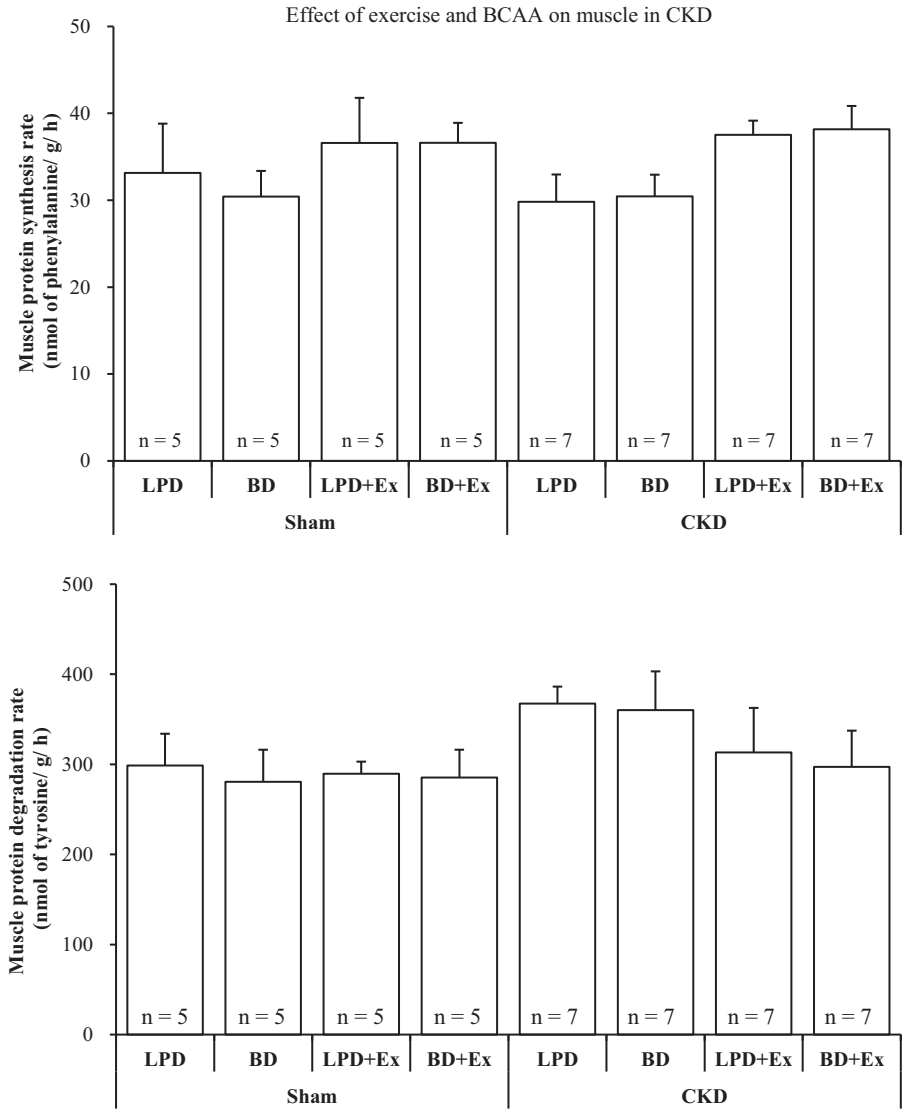

Fig. 3. Muscle-protein synthesis and degradation rates in incubated soleus muscle from sham and 5/6Nx rats. The protein synthesis rate (*top* panel) tended to be higher in exercise-trained sham and 5/6Nx than in nonexercised rats; however, this effect was not statistically significant. The muscle-protein degradation rate (*bottom* panel) tended to be higher in 5/6Nx than in sham rats, and appeared to be suppressed by exercise. Data are expressed as means ± SE. LPD, low-protein diet; BD, low-protein diet supplemented with BCAA; 5/6Nx, 5/6 nephrectomized; Ex, exercise.

treadmill exercise in our study. This phenomenon suggests that the regulation of downstream mTOR signaling is complex and may also be controlled by other mechanisms (24, 29, 30).

Phosphorylation of Akt, an upstream component of mTOR signaling pathway, was also determined in this study. We found that the phospho-Akt (Ser473) levels were comparable in all groups. Several previous studies have shown that the phosphorylation of Akt was not altered after exercise. Bernard et al. (5) showed that chronic aerobic exercise did not lead to increased phosphorylation of Akt in normal (non-CKD) rats. Furthermore, Camera et al. (7) demonstrated that Akt phosphorylation did not increase immediately after treadmill exercise. In their paper, however, the phosphorylation of mTOR and the downstream effector p70s6 kinase increased, whereas the phosphorylation of 4E-BP1 decreased immediately after treadmill exercise. These responses of Akt/mTOR signaling are consistent with the results of our study. On the other hand, Chen et al. (11) showed that muscle overloading leads to an increase in phospho-Akt levels, resulting in p70S6K and mTOR phosphorylation in CKD. Another study by Wang et al. (46) suggested that treadmill running did not stimulate the phosphorylation of p70S6K or mTOR, although this exercise slightly increased phospho-Akt levels. Although the reason for the differences in these results remains unknown, they might be associated with the type of exercise and the timing of muscle collection after the exercise.

IL-6 is a cytokine with pleiotropic functions that positively impacts the proliferation and differentiation of muscle satellite cells during the process of muscle regeneration, muscle hypertrophy, and myotube formation after exercise (21, 48). IL-6 is released by either infiltrating inflammatory cells or the muscle compartment after a prolonged exercise, and thus serves as a myosin (35, 41). It promotes muscle growth via autocrine and paracrine mechanisms. In contrast, IL-6 is principally defined as a proinflammatory cytokine, and elevated systemic IL-6 levels are associated with muscle wasting in some chronic diseases, including CKD. Plasma IL-6 levels have been reported to increase and be associated with protein-energy wasting in patients with end-stage renal disease as well as those on hemodialysis (3, 26, 37). In the present study, we evaluated the expression of IL-6 in the soleus muscle, as well as the plasma IL-6 concentrations. *IL-6* mRNA levels were elevated more

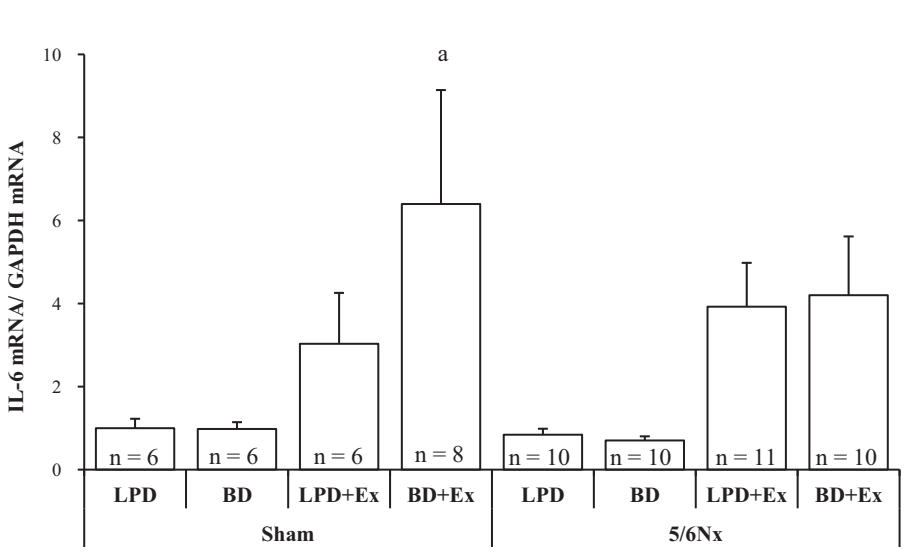

Fig. 4. IL-6 mRNA expression in the soleus muscle. The expression of IL-6 mRNA was increased over 3-fold in the soleus muscle of exercise-trained sham and 5/6Nx rats compared with untrained rats. $^{a}P < 0.05$ vs. 5/6Nx-BD group. Data are expressed as means ± SE. LPD, low-protein diet; BD, low-protein diet supplemented with BCAA; 5/6Nx, 5/6 nephrectomized; Ex, exercise.

than threefold after exercise in sham and 5/6Nx rats, although plasma IL-6 was not detected in any experimental groups. From these results, we concluded that IL-6 might play a role in muscle reproduction in the present study. However, the precise role of IL-6 in muscle protein synthesis remains to be investigated in future studies.

Although the combination of treadmill exercise and BCAA supplementation significantly stimulated the mTOR signaling pathway in both sham and 5/6Nx rats, the increase in muscle mass was marginal and remained insignificant. These findings are supported by a previous study where muscle mass was not increased by leucine supplementation and work overload in 5/6Nx rats despite a significant stimulation of the mTOR signaling (12). Moreover, the observed increase in the muscle protein synthesis rate in the present study persisted to a limited extent after exercise and BCAA supplementation. Overall, the results of the present study indicate that treadmill exercise and BCAA supplementation stimulate signaling via the mTOR pathway; however, it may be premature to conclude that this stimulation increases the muscle synthesis rate and muscle mass in CKD-model rats.

The type of exercise might be another reason for the discrepancy between the response of mTOR signaling pathway and muscle mass or muscle protein synthesis. Wang et al. (46) reported that the treadmill exercise was insufficient to improve muscle protein synthesis, whereas resistance training stimulated muscle protein synthesis in CKD model rats. It has been established that the resistance training stimulates muscle protein synthesis in association with enhanced mTOR signaling (11, 46) and that it improves muscle strength and physical activity even in CKD (18). The endurance exercise can also improve muscle strength and physical function, although the extent of the improvement (8) and the cell signaling response elicited by this exercise might differ somewhat from those elicited by resistance training. In clinical practice, a recumbent cycle ergometer has been used for endurance exercise training during hemodialysis, and this exercise was shown to improve muscle strength and physical performance in maintenance hemodialysis patients (43).

Treadmill exercise and BCAA supplementation did not decrease Ccr in 5/6Nx rats. Acute strenuous exercise has been reported to result in a significant and transient decline in the glomerular filtration rate and renal plasma flow (9, 36). Furthermore, acute and chronic exercise has been shown to worsen the course of glomerular nephritis (13, 42). However, many studies also indicated that chronic exercise training with moderate intensity could ameliorate progressive renal disease in rats with subtotal nephrectomy (19, 31, 34).

Nevertheless, dietary protein or amino acid supplementation might facilitate the progression of renal dysfunction in rats with 5/6 nephrectomy. Because our preliminary experiment showed that the supplementation of 3% BCAA to LPD induced a decline in renal function, we employed supplementation with 1% BCAA.

Our study has several limitations. First, the optimal timing of BCAA administration was not investigated in our study. It has been previously reported that both the muscle protein synthesis rate and BCAA requirement may be increased during the muscular recovery phase after exercise. Therefore, if BCAA supplementation was administered after exercise, its effects on muscle synthesis may have been much more pronounced than under the experimental conditions of the present study, wherein BCAA was provided ad libitum. Second, the results of the present study might have been affected by the paired feeding method. Although all rats were pair-fed for protein intake, the energy intake was increased only in the exercised groups to compensate for energy consumption by exercise. However, the additional energy intake might not be enough to compensate for energy consumption in the present study, and thus the muscle protein synthesis rate and muscle mass might not be increased despite the significantly increased muscle signal transduction in the exercised groups.

In conclusion, the results of the present study demonstrate that treadmill exercise incompletely activated mTOR signaling leading to muscle protein synthesis in CKD rats fed LPD. Furthermore, BCAA supplementation enhanced this effect, such that the level of mTOR activity in BCAA-supplemented, exercise-trained CKD rats was equivalent to that observed in

non-CKD rats. Although the combination of treadmill exercise and BCAA supplementation restored signaling, leading to muscle protein synthesis to normal levels, it remains to be elucidated whether this improved cell signaling is directly associated with muscle protein metabolism and/or muscle hypertrophy.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

T.Y. and H.K. conceived and designed research; T.Y., S.K., Y.T., and M.S. performed experiments; T.Y. and S.K. analyzed data; T.Y., S.M., and H.K. interpreted results of experiments; T.Y. and Y.T. prepared figures; T.Y. and H.K. drafted manuscript; T.Y., S.M., and H.K. edited and revised manuscript; T.Y., S.K., Y.T., M.S., S.M., and H.K. approved final version of manuscript.

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