Doctoral dissertation

Renalase in the skeletal muscle contributes to cell protective effect

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This doctoral thesis was revised in two of the following original papers and added the research results up to the present. Study 1 is published as the double first authors that contributes equally to this work.

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   Life Sciences, 210; 235–242, 2018. <Study 1>

2. Effect of renalase on dexamethasone-induced muscle atrophy.
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   International Journal of Analytical Bio-Science, 7(4), 2019. <Study 2>

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Ⅰ. Introduction

Renalase is a recently discovered flavin adenine dinucleotide (FAD) - dependent soluble monoamine oxidase [Xu et al. 2005]. Its primary functions include catecholamine metabolism and blood pressure regulation [Xu et al. 2005; Wu et al. 2011; Desir et al. 2012]. Renalase is predominantly expressed in the proximal tubule in the kidney. It is also expressed in other tissues, including the skeletal muscle, heart, and intestine [Xu et al. 2005]. In addition to its function in catecholamine metabolism, renalase was recently reported to inhibit apoptosis, inflammation, fibrosis, and oxidative stress [Lee et al. 2013; Wang et al. 2014, 2015, 2016; Guo et al. 2014; Du et al. 2015; Wu et al. 2017, 2018; Huang et al. 2019]. Renalase binds to its receptor, plasma membrane Ca^{2+} ATPase isoform 4b (PMCA 4b) [Wang et al. 2015] and is involved in cell survival and protection. Wang et al. (2014) reported that it makes phosphorylated protein kinase B (Akt) *in vitro* study.

Exercise is a valuable means of increasing or maitanining health and fitness [Lobero et al. 2014]. Exercise can be aerobic and anerobic, and can involve many activities, such as running, resistance exercise, and ball-related games. Skeletal muscle is mainly involved in body movement. Skeletal muscle is a highly adaptive (plastic) tissue that can increase the number and growth of muscle cells (hypertrophy) or display atrophy. The skeletal muscle comprises approximately 40% of the body mass in humans. The finding that exercise promotes better glucose uptake in the skeletal muscle in people with type 2 diabetes highlights the importance of maintain skeletal muscle in good condition [Stanford and Goodyear. 2014].

Renalase expression is affected by exercise in animal models and in humans [Czarkowska-Paczek et al. 2013; Yoshida et al. 2017]. Czarkowska-Paczek et al. (2013) examined the protein and mRNA expression of renalase in the serum and gastrocnemius muscle following exercise. The authors described mRNA expression of the white portion in gastrocnemius muscles of rats after making them run for 60 min at 28 m/min on a treadmill. An earlier study reported the significantly increased serum renalase concentration following long-term exercise (30 km running) in humans [Yoshida et al. 2017]. In addition, renalase expression in skeletal muscle and concentration in the blood were increased by acute moderate-intensity exercise in rat model [Yoshida's doctoral thesis and Tokinoya and Yoshida et al. 2020]. On the other hand, renalase mRNA expression in the other tissues including heart, liver, lung, and andrenal grand did not change after acute exercise.

However, renalase expression in the kidney was decreased after moderate-intensity exercise [Yoshida's doctoral thesis and Tokinoya and Yoshida et al. 2020]. Although the dynamics of renalase expression during transient exercise has been studied, its mechanism and physiological effects have remained unclear.

Ⅱ. Previous studies

1. Renalase

1) Gene and protein expression

Renalase is a FAD - dependent soluble monoamine oxidase. It exists mainly in kidney tissue [Xu et al. 2005]. The gene encoding renalase is located on chromosome 10 at q23.33. The gene comprises 11 exsons spanning 309,469 base pairs (bp) [Xu et al. 2005; Desir et al. 2009; Desir et al. 2012; Wang et al. 2014].

There are at least four alternatively spliced variants isoforms. The most highly expressed isoform (renalase1) is 342 amino acids in length and is encoded by exons 1–4, 6–7, and 8–10. Renalase consists of three domains: aminoxidase, FAD binding, and signal peptide sites [Milani et al. 2011]. The, molecular mass is 38 kDa.

Renalase expression is detected in plasma, kidney, heart, pancreas, skeletal muscle, and liver tissue, among others [Xu et al. 2005; Desir et al. 2009; Desir et al. 2012; Wang et al. 2014; Guo et al. 2016]. In addition, renalase is secreted from the kidney into the blood [Xu et al. 2005; Wang et al. 2014].

2) Function 1: Metabolizing catecholamine (Fig. 1-A)

Xu et al. (2005) reported firstly that renalase metabolizes circulating catecholamine in the blood in the presence of FAD, but not the other amines including serotonin, tyramine, benzyl-amine, methylamine and spermidine. The findings indicated that renalase plays a role in the regulation of cardiac function and blood pressure [Desir et al. 2009; Wu et al. 2011]. Human diseases including essential hypertension, chronic kidney disease, and preeclampsia increased the blood levels of renalase [Desir et al. 2009; Desir et al. 2012; Zbroch et al. 2013; Yılmaz et al. 2016]. Additionally, human studies have shown that high salt intake increases the urinary excretion of renalase and low salt intake increases serum renalase [Wang et al. 2014, 2015]. A positive correlation between urine renalase and serum dopamine has been described for salt diet interventions [Wang et al. 2015]. In a study that addressed the blood pressure regulation of renalase, subcutaneous administration of renalase in a rat model of hypertension resulted in circulating catecholamines and a decrease in blood pressure of approximately 15% [Wang et al. 2014; Baraka et al. 2012].

On the other hand, it has been reported that renalase does not catalyze the oxidation of

catecholamines [Beaupre et al. 2015]. In addition, Boomsma and Tipton (2007) mentioned that the rate of hydrogen peroxide (H_2O_2) generation is too low to be ascribed to enzymatic conversion of catecholamines by renalase. However, renalase knock-out (KO) mice displayed a phenotypes of increased catecholamines and hypertension [Wu et al. 2011]. Therefore, this argument must examine more detail.

3) Function 2: Protective effect (Fig. 1-B)

Considering renalase as a survival and growth factor, there is evidence that it protects cells phosphated via the plasma membrane Ca^{2+} ATPase isoform 4b (PMCA4b) receptor [Wang et al. 2014, 2015, 2017]. Renalase protects against both heart and kidney injury [Lee et al. 2013; Wang et al. 2014, 2015, 2016; Guo et al. 2014; Du et al. 2015]. Yin et al. (2016) showed that renalase protects against renal injury and cardiac remodelling after subtotal nephrectomy via inhibiting inflammation, oxidative stress and phosphorylation of extracellular signal-regulated kinase (ERK)-1/2. In the study, oxidative stress was increased by the unilateral ureteral obstruction in rats. When renalase overexpression was produced in the rats using adenovirus, the malondialdehyde level decreased while the superoxide dismutase (SOD) level increased [Wu et al. 2018]. The findings are consistent with previous studies involving other signals [Lee et al. 2013; Wang et al. 2014; Zhao et al. 2015; Wu et al. 2017, 2018; Huang et al. 2019]. Renalase peptide comprised of amino acids 220-239 (RP-220), which does not oxidize amine or NADH, ameliorated cell survival against cisplatin. In addition, histidine tagged RP-220 phosphated Akt in human renal proximal tubular (HK-2) cells [Wang et al. 2014].

4) Transcription factor

Renalase is regulated by various transcription factors. These include hypoxia-inducible factor-1-alpha (HIF-1α), nuclear factor-kappa B (NF-κB), specificity protein 1 (Sp1), signal transducer and activator of transcription 3 (STAT3), and zinc binding protein 89 (ZBP89) [Sonawane et al. 2014; Wang et al. 2014; Du et al. 2015; Hollander et al. 2016].

Sonawame et al. (2014) demonstrated the activation of Sp1, ZBP89, and STAT3 were activated by epinephrine and nicotine using various cell lines (HEK-293, IMR32, and HepG2). These transcription factors were researched using gain of function (transfected plasmid), loss of function (transfected small interference RNA), and reporter assays.

NF-κB is activated by epinephrine via α-adrenal receptor in HK-2 cells. This effect is abrogated by an inhibitor of NF-κB [Wang et al. 2014]. Du et al. (2015) reported that HIF-1α regulates renalase expression during cardiac ischaemia–reperfusion injury, and also demonstrated this using a chromatin immunoprecipitation assay.

5) Exercise and skeletal muscle

The relationship between renalase and exercise has not been extensively studied. Czarkowska-Paczek et al. (2013) reported no significant difference in the serum renalase concentration during both acute exercise and endurance training. However, renalase mRNA expression in the kidneys at rest after 6 weeks of endurance training was significantly increased compared with the expression in rats without training. In addition, there were no change of renalase expression in the red portion of gastrocnemius muscle. On the other hand, mRNA expression of renalase in the white portion of this muscle was increased by acute exercise, while protein expression was decreased. Additionally, expression in the white portion of the gastrocnemius muscle in endurance training group was significantly low compared to the untrained group. The study findings suggested the possibility that exercise and renalase levels might be related. Especially, white muscle and fast twitch fiber showed the high responses upon exercise.

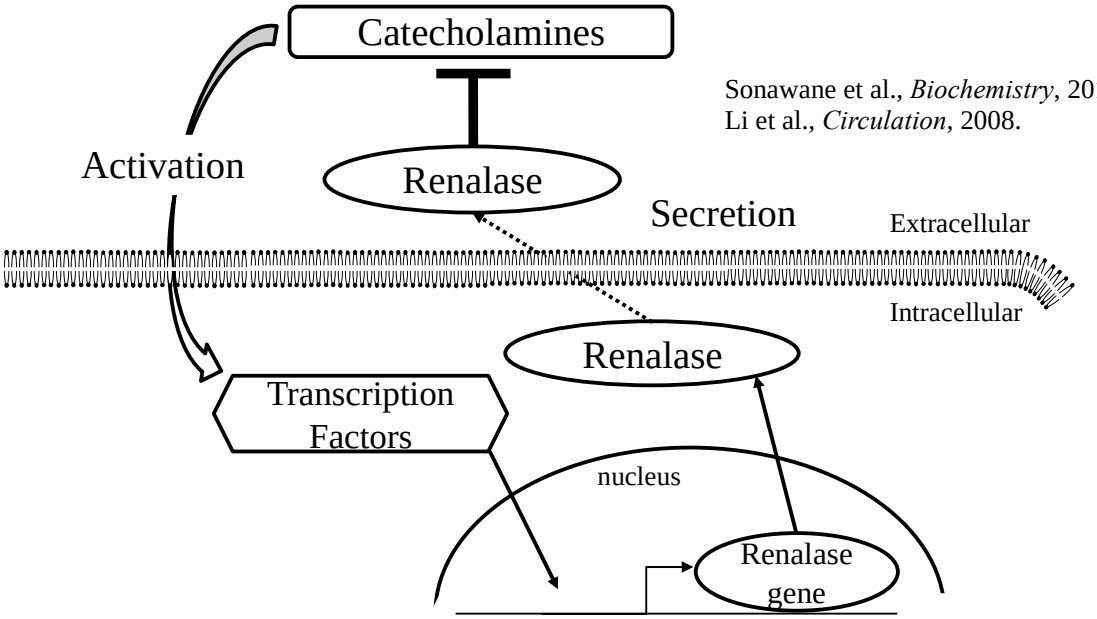
In a human experiment on exercise, amature runnners ran 30 km at 90% ventilatory threshould. The blood concentration of renalase was increased by the prolonged running [Yoshida et al. 2017]. In addition, renalase concentration was positively correlated with 2-thiobarbituric acid reactive substances (TBARS) and negatively correlated with estimated glomerular filtration value (eGFR), which is an index of kidney function caluculated from cystatin C levels. The findings indicated that prolonged exercise increased renalase in the blood.

Tokinoya and Yoshida et al. (2020) reported that moderate-intensity exercise for 60 min increases both renalase concentration in the blood and its expression in skeletal muscle. In addition, its expression in kidney was decreased by exercise. The other organs including adrenal grand, heart, lung, and liver mRNA expressions did not change after moderate-intensity exercise. The sedentary group rats were euthanized under isoflurane-induced anesthesia after resting on a treadmill for 15 min. Rats in the moderate-intensity exercise group ran for 1 h and were then euthanized under anesthesia immediately after running. An acute bout of exercise comprised treadmill running for 1

h at a speed of 20 m/min. Moreover, the mRNA expression of Sp1, ZBP-89, and STAT3 in the skeletal, soleus, plantaris, and EDL muscles were compared between the sedentary and moderate-intensity exercise groups. The expression of all three mRNA transcripts was significantly higher after exercise, only in the soleus muscle ($p < 0.05$ for all transcripts). Thus, catecholamine stimulation in the skeletal muscles, especially the soleus muscle, may influence renalase expression.

Yoshida et al. (2017) showed that renalase mRNA expression was increased by epinephrine in C2C12 mouse myotubes *in vitro*. This result is consistent with previous studies in other cell lines [Sonawane et al. 2014; Wang et al. 2014]. Thus, it is possible that renalase expression in skeletal muscle is controlled by epinephrine.

A. Metabolizing catecholamine



B. Protective effect

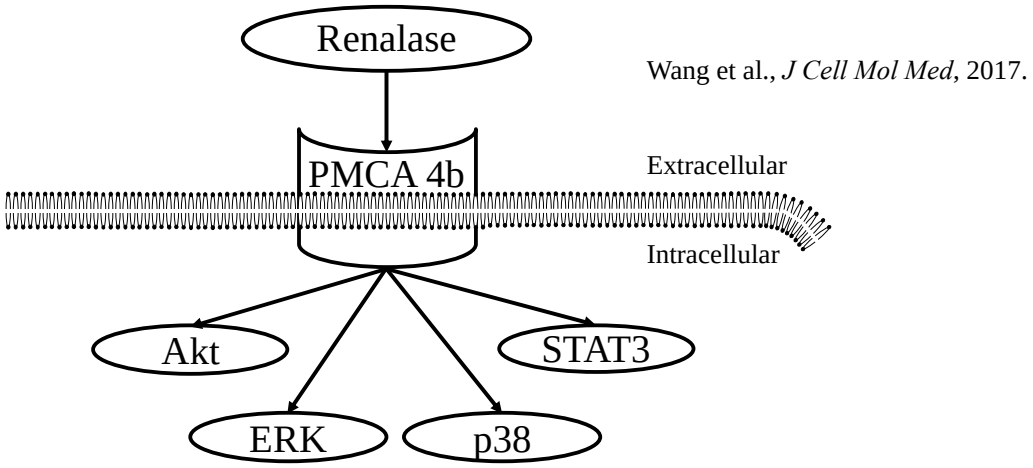


Figure 1: Physiological effects of renalase.
"A" is metabolizing catecholamine, and "B" is protective effect referred to previous studies.
PMCA 4b, Plasma membrane Ca^{2+} ATPase isoform 4b; Akt, Protein kinase B; ERK, Extracellular signal-regulated kinase; p38, p38 Mitogen-activated protein kinase; STAT3, Signal transducers and activator of transcription 3.

2. Ubiquitin proteasome pathway in the skeletal muscle

1) Ubiquitin proteasome pathway

The specificity of the muscle specific E3s regulated skeletal muscle atrophy in various pathological and physiological conditions [Rom and Reznick. 2016]. The ubiquitin–proteasome pathway involves ubiquitin-ligase enzymes (E3s), such as muscle RING finger-1 (MuRF-1) and muscle atrophy F-box (MAFbx). These factors are known as atrogenes. Atrogenes are regulated by forkhead box O (FoxO). FoxO isoforms include 1, 3a, and 4 in mammals. FoxO exists the cell nucleus when phosphorylated by activated protein kinase B (Akt).

The ubiquitin–proteasome pathway is related to muscle atrophy induced by dexamethasone (DEX), denervation, and suspension [DePinho et al. 2014; Liu et al. 2016; Son et al. 2017; Wang et al. 2017; Kim et al. 2018; Sakai et al. 2019]. The pathway is increased by resistance exercise [Yang et al. 2006; Deldicque et al. 2008; Coffey et al. 2009]. On the other hand, a comparison of skeletal muscle proteolytic genes in response to resistance or endurance exercise, although the muscle biopsy sampling in this study differed in the resistance exercise and endurance exercise groups. In the study, the resistance exercise group of subjects performed three sets of 10 repetitions at 70% of concentric one repetition maximum of bilateral knee extensions, whereas the endurance exercise group of subjects performed 30 min of treadmill running at 75% of maximum oxygen uptake [Louis et al. 2007].

2) Dexamethasone

Dexamethasone (DEX) is commonly used to treat inflammation. It induces muscle atrophy as a side effect [Liu et al. 2016; Son et al. 2017; Wang et al. 2017; Kim et al. 2018; Sakai et al. 2019].

In one study, the weight of the tibial anterior muscle was decreased by the injection of 5 mg/kg/day DEX in mice for 18 days. In addition, MuRF-1 and MAFbx expression significantly increased [Liu et al. 2016]. Other studies have been reported that DEX induced muscle atrophy features atrogenes both *in vivo* and *in vitro* [Son et al. 2017; Wang et al. 2017; Kim et al. 2018; Sakai et al. 2019].

The glucocorticoid receptor (GR) in combination with DEX regulates various factors

involving FoxO, kruppel-like factor 15 (KLF 15), and regulated in development and DNA damage responses 1 (REDD 1) [Wang et al. 2006; Shimizu et al. 2011]. Shimizu et al. (2011) showed that GR combines with KLF 15 promorter in skeletal muscle, but not liver, in the presence of DEX and regulates atrogenes. Additionally, the authors reported the increase in REDD 1 by DEX. Moreover, myostatine, which was increased by DEX [Kun et al. 2001], decreased the phosphorylation of Akt. Akt was decreased by DEX [Son et al. 2017].

3. Exercise

1) Intensity

There are two methods to determine the exercise intensity. One is the lactate threshould (LT), in which blood lactate accumulates during exercise. The other is the ventilatory threshould (VT), which is the sharp increase in ventilation or oxygen uptake during exercise. These factors increase because of the transformation in energy metabolism from aerobic to anaerobic metabolism and strongly correlated.

Soya et al. (2007) reported on the effect of exercise intensity in rats. A catheter was inserted into the jugular vein of each rat to allow sampling of blood during treadmill running. A pace of approximately 20 m/min was determined as the LT in Wistar rats based on the incremental change in blood lactate. In addition, plasma adrenocorticotrophic hormone, glucose, and osmolality were also increased by the supra-LT rate of 25 m/min compared to the sub-LT rate of 15 m/min and the sedentary control.

Ⅲ. Aim

This study hypothesized that renalase expression is increased by acute exercise to protect cells via interaction with skeletal muscle receptor. The effect of transient exercise on renalase expression was examined at different intensities. As well, the mechanism and physiological effects of renalase expression was examined using an *in vivo* and *in vitro* models.

<Study 1>

“Influence of acute exercise on renalase and its regulatory mechanism”

Exercise makes renalase in the blood increased in human experiment. However, it is unclear which organ increases renalase concentration in the blood, how physiological effect exsit. Thsu, acute exercise effects on renalase expression or concentration in tissues or blood.

<Study 2>

“Effect of renalase on dexamethasone-induced muscle atrophy”

Study 1 suggested that renalase expression in the skeletal muscle surpressed protein degradation on acute exercise for 30 min. Thus, it was examined whether renalase is related to muscle protein degradation *in vitro* using dexamethasone-induced muscle atrophy model.

This doctoral dissertation discussed effects of renalase on the skeletal muscle by above studies.

Ⅳ. Study 1

Influence of acute exercise on renalase and its regulatory mechanism

1. Aim

This study hypothesized that exercise-induced renalase expression is mediated through transcription factors such as HIF-1α, NF-κB and Sp1, particularly in the skeletal muscle. This study was aimed to clarify the effect of transient exercise with different intensities on renalase expression, and to examine its mechanism and physiological effect.

2. Materials and Methods

Ethics statement and Animals

All animal experiments were approved by the Animal Subjects Committee, University of Tsukuba, Japan, which were performed in accordance with the principles and guidelines on animal care proposed by the Physiological Society of Japan (Approval number: 17-076).

A total of 21 male Wistar rats (Japan SLC, Inc., Shizuoka, Japan) were used in this experiment (8-wk-old, 230-275 g), provided standard chow (MF 12 mm φ pellet, Oriental Yeast Co, Tokyo, Japan) and water *ad libitum*, and housed under standard laboratory conditions (20-26 ℃, 12:12 h light-dark cycle).

Experimental design

After one week of preliminary rearing, all rats were familiarized with treadmill running for 20-30 min per day for 5 days. Animals were assigned to either a low-intensity exercise group (LOW, n=7), high-intensity exercise group (HIGH, n=7) or sedentary group (SED, n=7), and rested for 48 h. Both exercise groups performed an acute treadmill exercise (described below). Rats were fasted 2 h before the exercise. After resting for 15 min on a treadmill, a running exercise was conducted for 30 min at a speed of 10 m/min in the LOW group and 30 m/min in the HIGH group. The running speed was determined by referring to Soya et al. (2007). SED group were sacrificed after 2-h fasting followed by a rest period.

Animals were sacrificed under anesthesia and euthanized by cervical dislocation. Blood was collected from the inferior vena cava and placed in a microtube with EDTA-2 Na and sodium heparin. Blood samples were centrifuged (4 ℃, 800 ×g, 5 min) and the supernatant was collected. Kidney, soleus and plantaris muscle, and the other tissues were taken and used for biochemical analysis. For these tissues, RIPA buffer (1 M Tris-HCl, 5 M NaCl, 0.5 M EDTA, 0.5% sodium dodecyl sulfate, 2.5% sodium deoxycholate, 5% NP-40, distilled water) was added and the mixture was crushed by a bead crusher (Tissue Lyser, QIAGEN). After crushing, samples were centrifuged (4 ℃, 12,000 ×g, 15 min) and the

supernatant was collected.

Western blot analysis

Bicinchoninic Acid (BCA) was used for the quantification of protein concentration. Ten microliters of sample diluted with RIPA buffer and hundred microliters of working Solution (BCA Reagent A:B, 50:1) were placed in each well of the microplate. Immediately after incubating (37 ℃, 10 min) the absorbance was measured at a wavelength of 562 nm using a microplate reader (Varioskan LUX; Thermo Fisher Scientific, Waltham, MA, USA). A calibration curve was prepared from the result of bovine serum albumin (BSA) standard (2 mg/mL) and the sample concentration was compared with the standard.

Homogenized samples of skeletal muscle and kidney tissue were used to quantify the renalase protein by western blotting. To separate renalase, 10% (m/v) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used. The sample supernatant was diluted with sample buffer (2× Laemmli Sample Buffer and 10% 2-mercaptoethanol) to a concentration of 2 mg/mL. Five microliters of each sample was applied to the gel. After the migration, isolated proteins were transferred to the polyvinylidene fluoride membrane (PVDF; 0.3 A, 60 min). Blocking buffer containing 5% (m/v) skim milk in Tris buffer containing 0.1% Tween-20 (Tris-buffered saline with Tween-20 0.1 %: TBST) was used to block the PVDF membrane for one hour at 20 – 25 ℃.

Washing was carried out three times for 5 min with TBST, and the washed PVDF membrane was reacted with anti-renalase monoclonal antibody (ab178700; Abcam, Cambridge, UK) overnight at 4 ℃. Thereafter, washing was conducted 3 times for 5 min each with TBST and reacted with a secondary antibody (L006326, Bio-rad), diluted 10000-fold with TBST, for one hour. Again, the PVDF membrane was washed three times, and luminescence was detected using a chemiluminescent reagent (GE healthcare, RPN 2235 V) and detection equipment (ImageQuant LAS - 4000, GE Healthcare Life Sciences). Signal intensities were analyzed using JustTLC software (Sweday, Sodra Sandby, Sweden).

In addition to the protein content of IκBα (4814, Cell Signaling Technology, Japan), phosphorylation (p-) IκBα (2859, Cell Signaling Technology), Akt (9272, Cell Signaling Technology), p-Akt (9271S, Cell Signaling Technology) was also measured. For phosphorylation of IκBα, the value was obtained by dividing the amount of p-IκBα by the amount of IκBα. Glyceraldehyde triphosphate dehydrogenase (GAPDH, sc-365062, Santa Cruz Biotechnology, Dallas TX, USA) was used as an internal standard in this study.

SOD assay

With reference to the method of Peskin and Winterbourn (2000), superoxide dismutase (SOD) activity was determined. Samples of plasma, soleus and skeletal muscle were used to determine the ratio of SOD inhibition against superoxide.

Absorbance at 450 nm was measured by a microplate reader (Varioskan LUX) following incubation at 37 ℃ for 20 min. SOD inhibition rate was determined by the SOD inhibition against the maximum superoxide development reacted with a solution without SOD (phosphate buffered saline, PBS buffer).

Enzyme-linked Immunosorbent Assay (ELISA)

Plasma renalase concentration was measured by a sandwich method using renalase ELISA (Enzyme-linked Immunosorbent Assay) kit (SEC845Ra, Cloud-Clone Corp, Houston, TX, USA). All methods were conducted according to the instructions. Plasma samples were diluted 100-fold with PBS buffer. Absorbance at 450 nm was measured using a microplate reader (Varioskan LUX). To quantify the sample, a calibration curve was prepared from the reaction result of the standard solution and was compared with the sample.

Quantitative real-time RT-PCR

mRNA expression of renalase, HIF-1α, Sp1 was measured by real-time RT-PCR. ~50 mg of kidney and skeletal muscle were excised. Sepasol®-RNA I Super G (Nacalai Tesque, Kyoto, Japan) was added, and the tissue was disrupted with a bead crusher (Tissue Lyser; Qiagen, Hilden, Germany). To separate the nucleic acids, chloroform was added to the sample after extraction and mixed by inversion. Samples were settled at room temperature for three minutes and centrifuged (4 ℃, 12,000 ×g, 15 min). The separated aqueous phase in the tube was removed and 2-isopropanol was added. The mixture was mixed by inversion, left standing for 10 min and centrifuged (4 ℃, 12,000 ×g, 15 min). Afterward, the supernatant was removed and 75% ethanol was added, and suspended in a vortex mixer. Again, centrifugation (4 ℃, 12,000 ×g, 5 min) was performed to remove the supernatant. Finally, RNase-free Water (9012, Takara Bio Inc., Kusatsu, Japan) was applied and incubated at 65 ℃ for 5 min.

The total RNA concentration was measured by spectrophotometer (NanoDrop, Thermo Fisher Scientific). Based on that value, samples were adjusted to 400 ng/μL, diluting with sterile water 400 ng/μL. After applying 5× PrimeScript RT Master Mix (RR 036 A, Takara Bio Inc.) and RNase free water to the diluted RNA, reverse transcription was carried out in a thermal cycler (TP 350, Takara Bio Inc.) (37 ℃, 15 min; 85 ℃, 5 sec; 4 ℃, ∞).

After completion of the reverse transcription process, KAPA SYBR® FAST qPCR Master Mix (KK 4602, Kapa Biosystems, Wilmington, MA, USA), upstream primer, downstream primer, ROX Low Reference Dye (KD 4601, Kapa Biosystems), and Ultra Pure™ Distilled Water (10977-015, Thermo Fisher Scientific) were employed, and PCR was conducted. Amplification of RT-PCR consisted of initial decomposition step at 95 ℃ for 20 sec, decomposition at 95 ℃ for 30 sec, annealing and extension at 60 ℃ for 30 sec of 40 cycles, using Real-time PCR systems 7500 Fast (Life Technologies, Applied Biosystems® 7500 Fast). The mRNA expression of GAPDH was set as the housekeeping gene.

The cycle threshold (Ct) value of the target gene was standardized by the Ct value of the housekeeping gene (ΔΔCt method). The relative expression level of the target gene was calculated as the relative value to the SED group. The sequences of the primers used in this study are shown in Table 1.

Statistical analysis

Data are shown as mean ± standard error of the mean (SEM). For all measurements, a one-way analysis of variance was used. When a significant F value was obtained, statistical significance was calculated according to Dunnet and Tukey's method. SPSS statistics 24 for Mac (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 7 software (GraphPad, Inc., La Jolla, CA, USA) were used for all statistical calculation, and the significant level was set at $p < 0.05$ for all cases.

Table 1. Primer sequences used in mRNA analyses.

Name	Forward (5' -> 3')	Reverse (5' -> 3')
Gapdh	GGAAACCCATCACCATCTTC	GTGGTTCACACCCATCACAA
Hif-1α	GGCGAGAACGAGAAGAAAAATAGG	AGATGGGAGCTCACGTTGTG
Sp1	GCTATAGCAAACACCCCAGGT	CAGGGCTGTTCTCTCCTTCTT
Tnf-α	ACAAGGCTGCCCCGACTAT	CTCCTGGTATGAAGTGGCAAATC
Pmca 4b	CATGCCGAGATGGAGCTT	GCTCCCGTCTGGAATTTGT
Trim63 (MuRF-1)	AGGACTCCTGCCGAGTGAC	TTGTGGCTCAGTTCCTCCTT
Fbxo32 (MAFbx)	GAAGACCGGCTACTGTGGAA	ATCAATCGCTTGCGGATCT

Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Hif-1α, hypoxia inducible factor-1α; Tnf-α, tumor necrosis factor-alpha; Pmca 4b, plasma membrane Ca^{2+} ATPase isoform 4b; Trim63 (MuRF-1), tripartite motif containing 63 (Muscle RING finger protein); Fbxo32 (MAFbx), F-box protein 32 (Muscle atrophy F-box).
Submitted a master's thesis of Shiromoto and accepted and modified *Life Sciences* (Tokinoya and Shiromoto et al. 2018).

3. Results

Renalase expression in each tissue and concentration in the blood

Renalase expression in the red portion of the gastrocnemius muscle significantly increased in the two exercised groups compared with that in the SED group (Fig. 2-A, $p < 0.05$, $p < 0.01$). Renalase expression in the soleus muscle significantly increased in the LOW group compared with that in the SED group (Fig. 2-C, $p < 0.01$). In contrast, renalase expression in the white portion of the gastrocnemius muscle significantly increased in the LOW group compared with that in the SED group (Fig. 2-B, $p < 0.01$). Renalase expression was significantly higher in the plantaris muscle of the HIGH group than in the SED group (Fig. 2-D, $p < 0.01$). Renalase expression in the kidney significantly decreased in the LOW and HIGH groups compared with that in the SED group (Fig. 3, $p < 0.01$). Additionally, renalase expression in the heart and liver significantly decreased in the LOW and HIGH groups compared with that in the SED group (Fig. 4-A, B, $p < 0.05$ and $p < 0.01$, respectively). Renalase expression in the blood did not change (Fig. 5).

Transcriptional regulation of renalase in skeletal muscle

Phosphate IκBα in the plantaris significantly increased in the HIGH group compared with that in the SED group ($p < 0.01$), whereas expression did not change in the soleus (Fig. 6). The mRNA expression of HIF-1α in the soleus significantly increased in the LOW group compared with that in the SED group ($p < 0.01$). In contrast, the mRNA expression of HIF-1α in the plantaris did not change (Fig. 7). The mRNA expression of Sp1 and TNF-α in these muscles did not change (Figs. 8; 9).

Inhibition rate for SOD in the plasma and skeletal muscle

The inhibition rate for SOD to super oxide in the plasma and plantaris in the HIGH group was significantly higher than that in the SED group ($p < 0.05$ and $p < 0.01$, respectively), whereas that in the soleus muscle did not change (Table 2).

PMCA 4b gene expression in the kidney and skeletal muscles

PMCA 4b gene expression was significantly higher in the soleus than in the plantaris (Fig. 10, $p < 0.01$). Additionally, the expression in the kidney was significantly higher than that in the plantaris ($p < 0.01$), but not in the soleus.

Akt, MuRF-1 (Trim63), and MAFbx (Fbxo32) signaling in the skeletal muscles

Phosphate Akt in the plantaris significantly increased in the HIGH group compared with that in the SED group ($p < 0.05$), while that in the soleus did not change (Fig. 11). Trim63 in the plantaris significantly increased in the LOW group compared with that in the SED group ($p < 0.01$), while that in the soleus did not change (Fig. 12). Fbxo32 in the plantaris significantly increased in the LOW and HIGH groups compared with that in the SED group ($p < 0.01$, $p < 0.05$). However, that in the soleus did not change (Fig. 13).

Plasma creatinine and liver function tests to evaluate kidney and liver function

Plasma creatinine significantly increased in the HIGH group compared with that in the SED group (Fig. 14, $p < 0.01$). Additionally, plasma aspartate aminotransferase and alanine aminotransferase levels significantly increased in the HIGH group compared with that in the SED group (Fig. 15, $p < 0.01$).

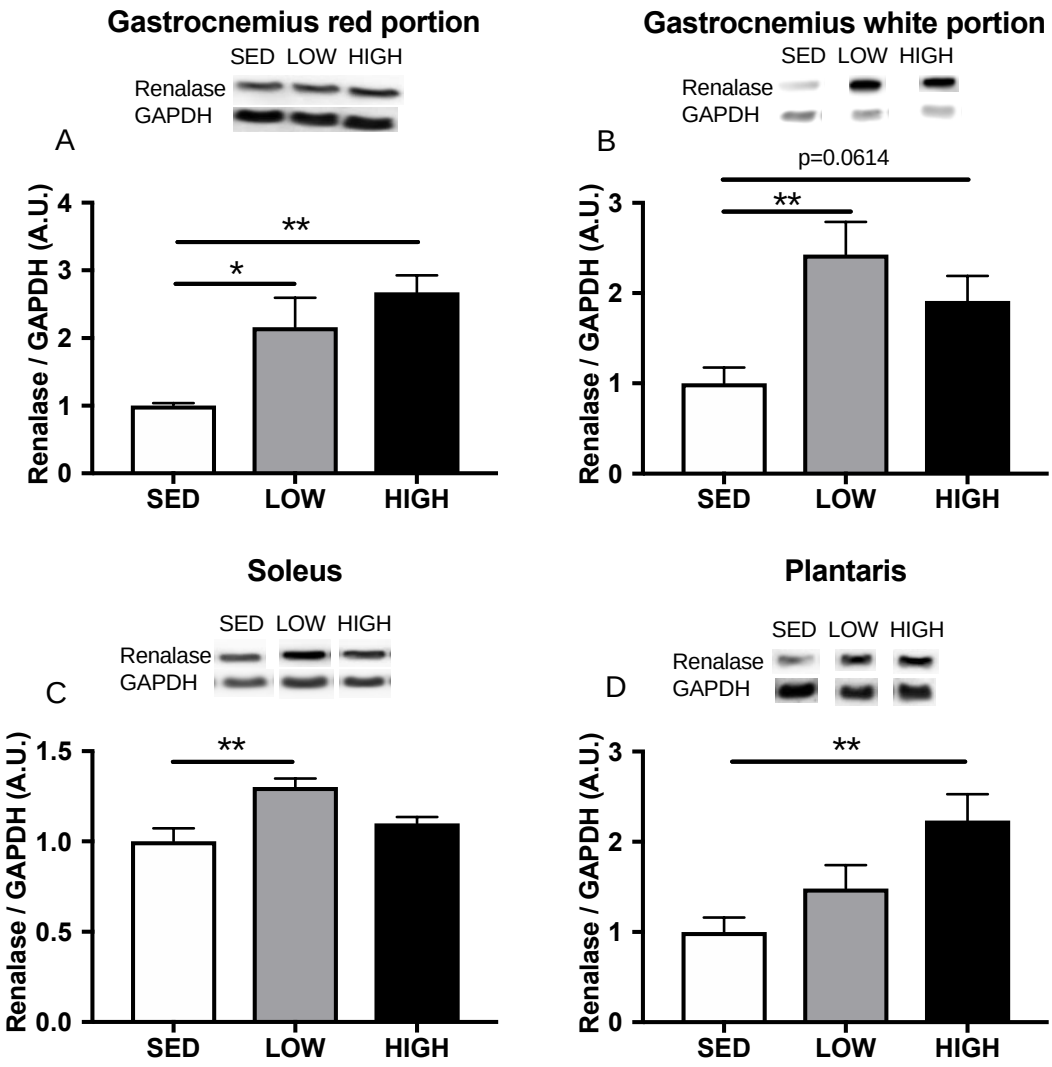


Figure 2. Renalase protein expression in skeltal muscles after acute exercise.
Renalase protein expression were assessed in skeletal muscles on acute exercise for 30 min using Western blot. Data are shown as mean ± SEM. n = 7 in each group. Data were analyzed using a one-way ANOVA. *, ** statistical difference was set at $p < 0.05$, $p < 0.01$ vs SED, respectively. SED, sedentary; LOW, low intensity exercise; HIGH, high intensity exercise. Submitted a master's thesis of Shiromoto and accepted *Life Sciences* (Tokinoya and Shiromoto et al. 2018).

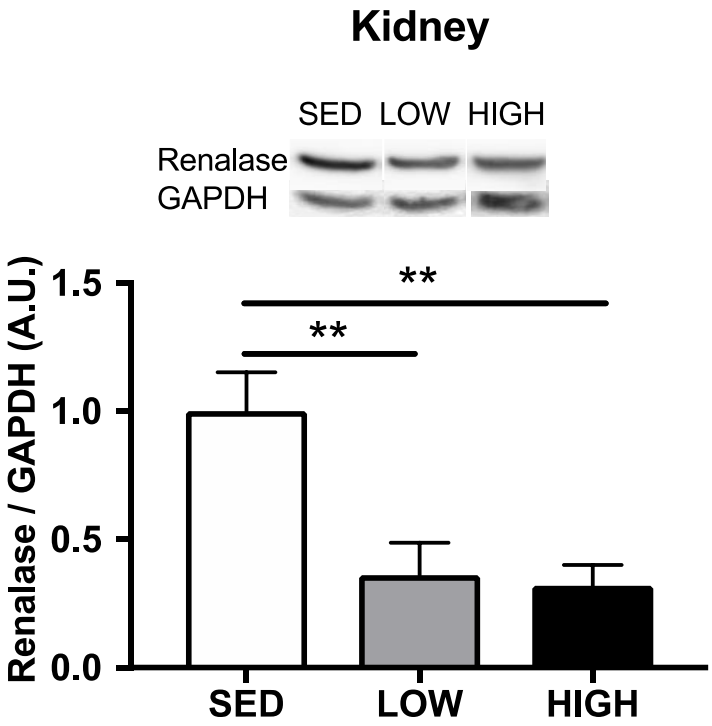


Figure 3. Renalase protein expression in kidney after acute exercise. Renalase protein expression were assessed in skeletal muscles, the other organs on acute exercise for 30 min using Western blot. Data are shown as mean ± SEM. n = 7 in each group. Data were analyzed using a one-way ANOVA. *, ** statistical difference was set at $p < 0.05$, $p < 0.01$ vs SED, respectively. SED, sedentary; LOW, low intensity exercise; HIGH, high intensity exercise. Submitted a master's thesis of Shiromoto and accepted *Life Sciences* (Tokinoya and Shiromoto et al. 2018).

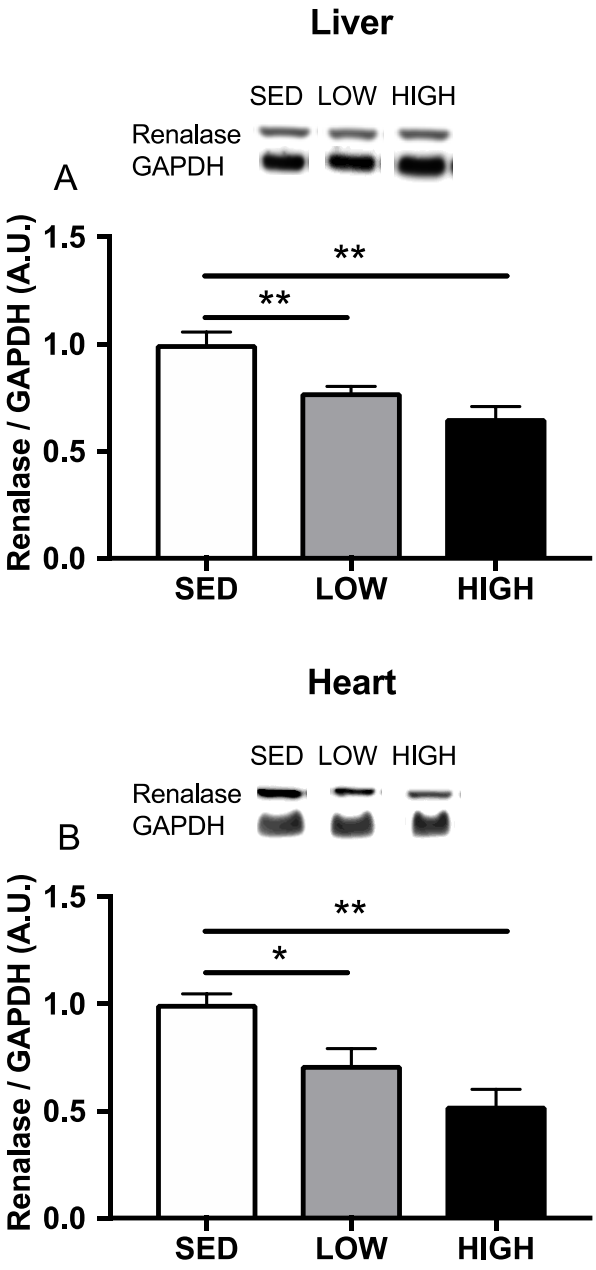


Figure 4. Renalase protein expression in liver and heart after acute exercise. Renalase protein expression were assessed in skeletal muscles, the other organs on acute exercise for 30 min using Western blot. Data are shown as mean ± SEM. n = 7 in each group. Data were analyzed using a one-way ANOVA. *, ** statistical difference was set at $p < 0.05$, $p < 0.01$ vs SED, respectively. SED, sedentary; LOW, low intensity exercise; HIGH, high intensity exercise. Accepted *Life Sciences* (Tokinoya and Shiromoto et al. 2018).

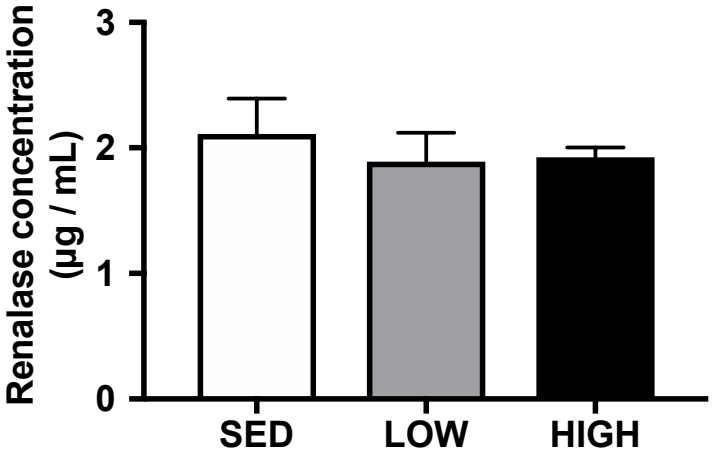


Figure 5. Renalase concentration in the blood after acute exercise.
Renalase concentration were assessed in the blood on acute exercise for 30 min using ELISA. Data are shown as mean ± SEM. n = 7 in each group. Data were analyzed using one way ANOVA. SED, sedentary; LOW, low intensity exercise; HIGH, high intensity exercise. Submitted a master's thesis of Shiromoto and accepted *Life Sciences* (Tokinoya and Shiromoto et al. 2018).

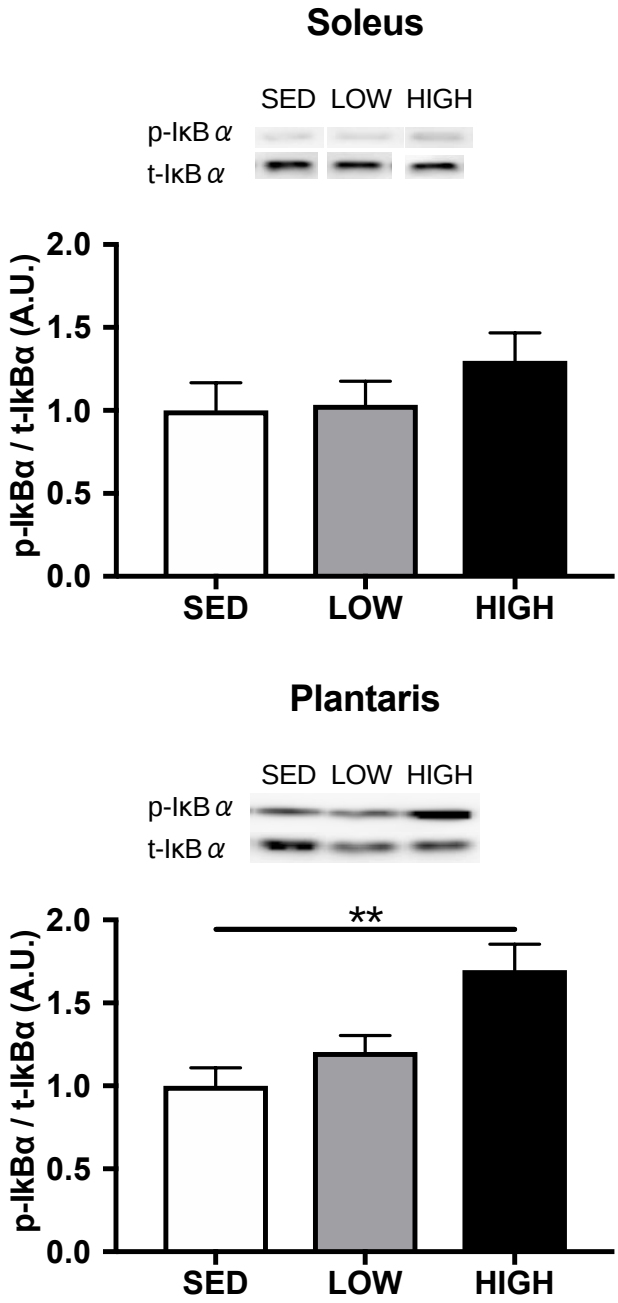


Figure 6. Phosphorylation of IkBα in soleus and plantaris after acute exercise.
Several factors were assessed in skeletal muscles on acute exercise for 30 min using Western blot . Data are shown as mean ± SEM. n = 7 in each group. Data were analyzed using one way ANOVA. ** statistical difference was set at $p < 0.01$ vs SED. SED, sedentary; LOW, low intensity exercise; HIGH, high intensity exercise. Submitted a master's thesis of Shiromoto and accepted *Life Sciences* (Tokinoya and Shiromoto et al. 2018).

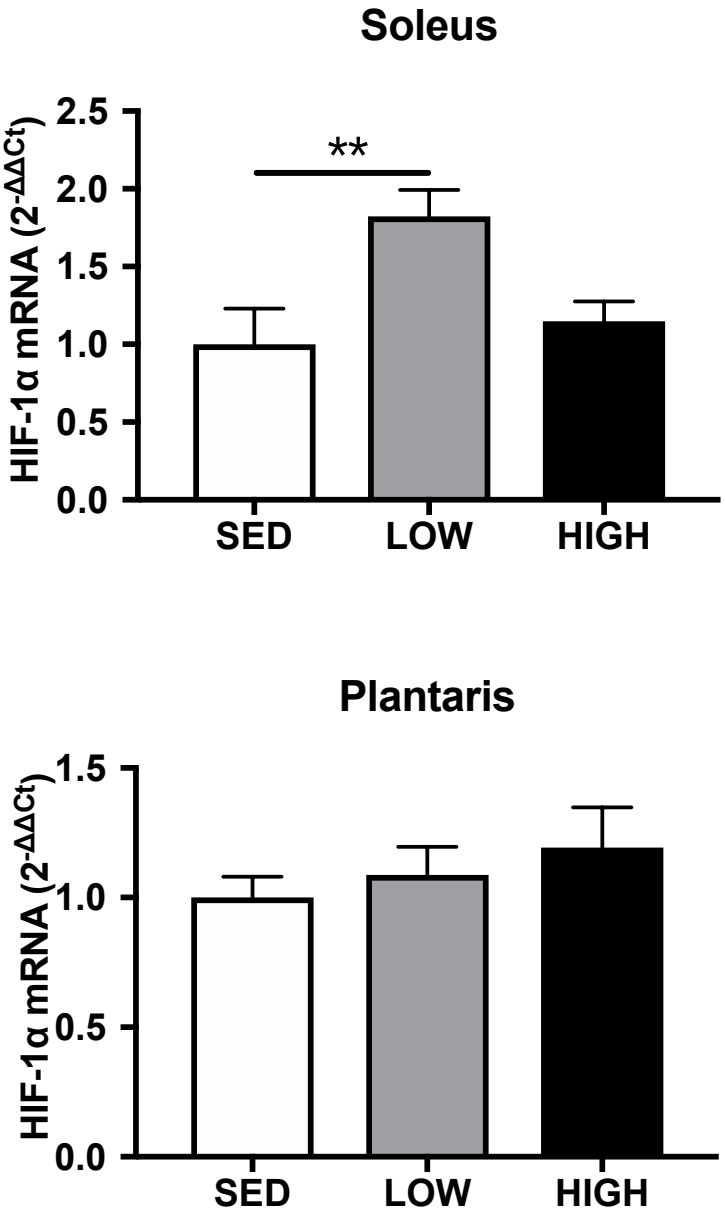


Figure 7. mRNA expression of HIF-1α in soleus and plantaris after acute exercise. Several factors were assessed in skeletal muscles on acute exercise for 30 min using RT-qPCR (ΔΔCt method). Data are shown as mean ± SEM. n = 7 in each group. Data were analyzed using one way ANOVA. ** statistical difference was set at $p < 0.01$ vs SED. SED, sedentary; LOW, low intensity exercise; HIGH, high intensity exercise. Submitted a master's thesis of Shiromoto and accepted *Life Sciences* (Tokinoya and Shiromoto et al. 2018).

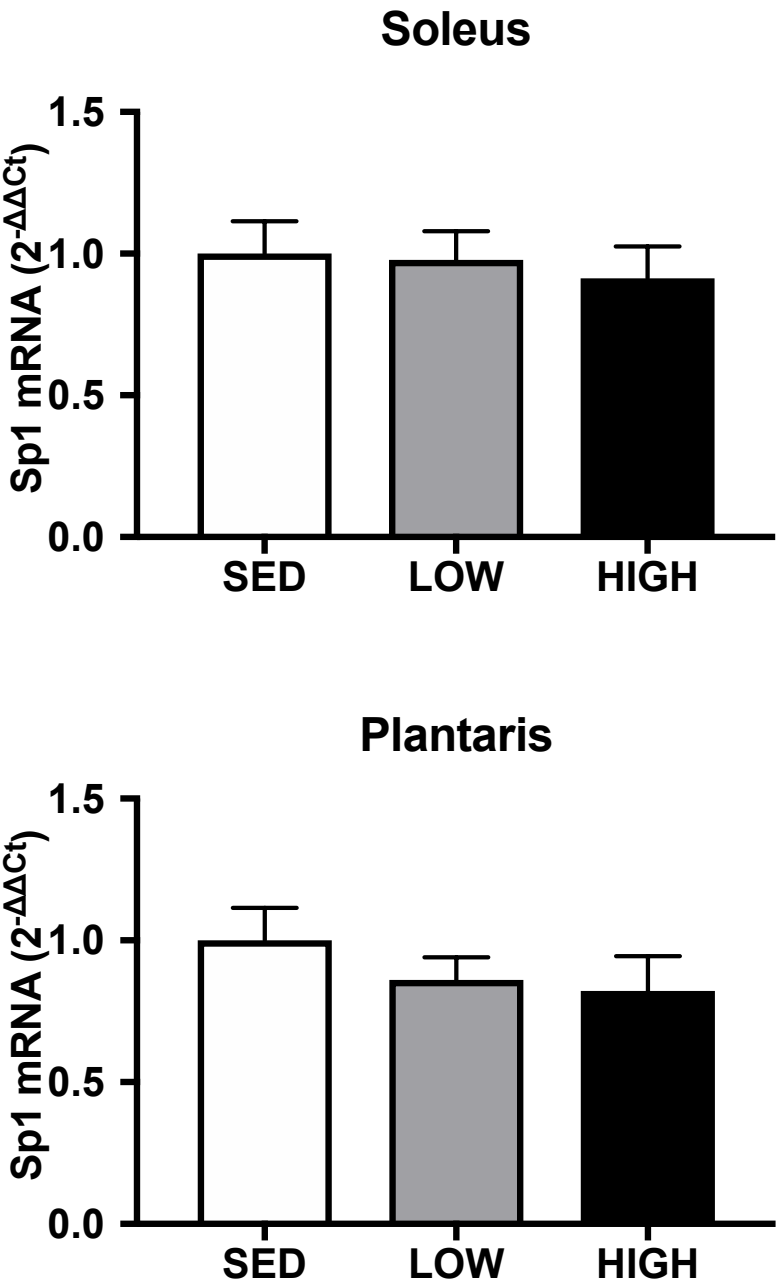


Figure 8. mRNA expression of Sp1 in soleus and plantaris after acute exercise.
Several factors were assessed in skeletal muscles on acute exercise for 30 min using RT-qPCR (ΔΔCt method). Data are shown as mean ± SEM. n = 7 in each group. Data were analyzed using one way ANOVA. ** statistical difference was set at $p < 0.01$ vs SED. SED, sedentary; LOW, low intensity exercise; HIGH, high intensity exercise. Submitted a master's thesis of Shiromoto and accepted *Life Sciences* (Tokinoya and Shiromoto et al. 2018).

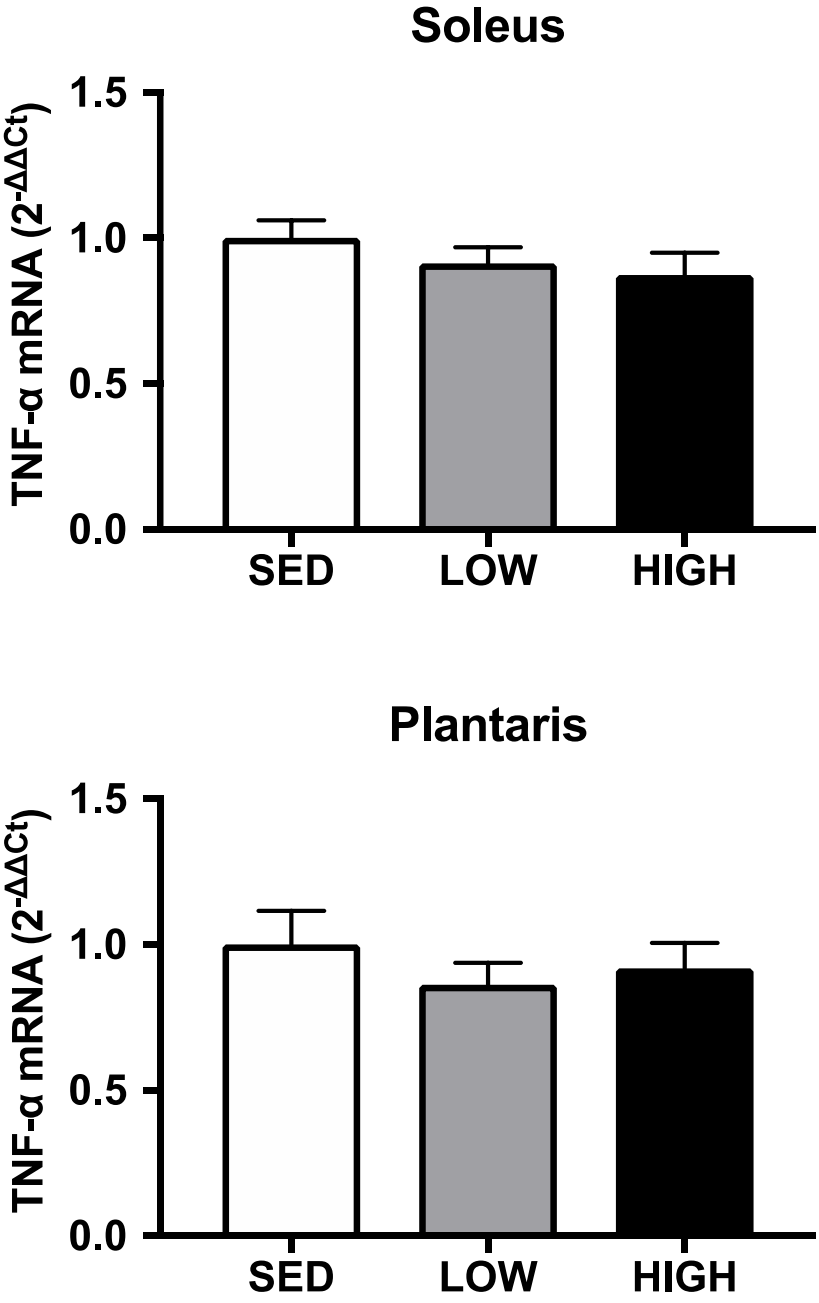


Figure 9. mRNA expression of TNF-1α in soleus and plantaris after acute exercise.

Several factors were assessed in skeletal muscles on acute exercise for 30 min using RT-qPCR (ΔΔCt method). Data are shown as mean ± SEM. n = 7 in each group. Data were analyzed using one way ANOVA. ** statistical difference was set at $p < 0.01$ vs SED. SED, sedentary; LOW, low intensity exercise; HIGH, high intensity exercise. Accepted *Life Sciences* (Tokinoya and Shiromoto et al. 2018).

Table 2. Super oxide inhibition rate for superoxide dismutase (SOD).

	SOD inhibition rate for super oxide (%)		
	SED	LOW	HIGH
Soleus	92.4±2.0	96.5±0.4	95.7±0.5
Plantaris	87.0±1.3	90.1±1.1	93.3±0.5*
Plasma	75.1±1.2	81.5±1.5**	83.7±1.1**

Values are mean ± SEM. SED, sedentary; LOW, low intensity exercise; HIGH, high intensity exercise. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with SED. Submitted a master's thesis of Shiromoto and accepted *Life Sciences* (Tokinoya and Shiromoto et al. 2018).

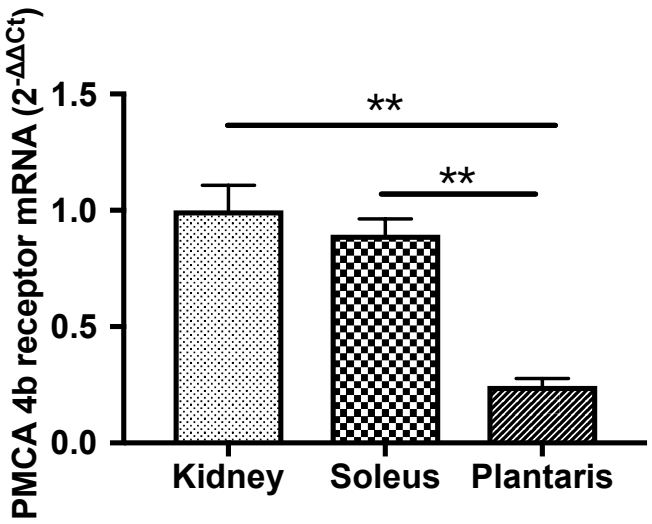


Figure 10. Plasma membrane Ca^{2+} ATPase 4b receptor mRNA expression in soleus, plantaris muscles and kidney.

Values are mean ± SEM and show relative values of kidney. PMCA 4b mRNA expression was assessed in kidney and skeletal muscles using R T-qPCR (ΔΔCt method). Data are shown as mean ± SEM. n = 7 in SED group. Data were analyzed using one way ANOVA. ** statistical difference was set at $p < 0.01$ vs Kidney. Accepted *Life Sciences* (Tokinoya and Shiromoto et al. 2018).

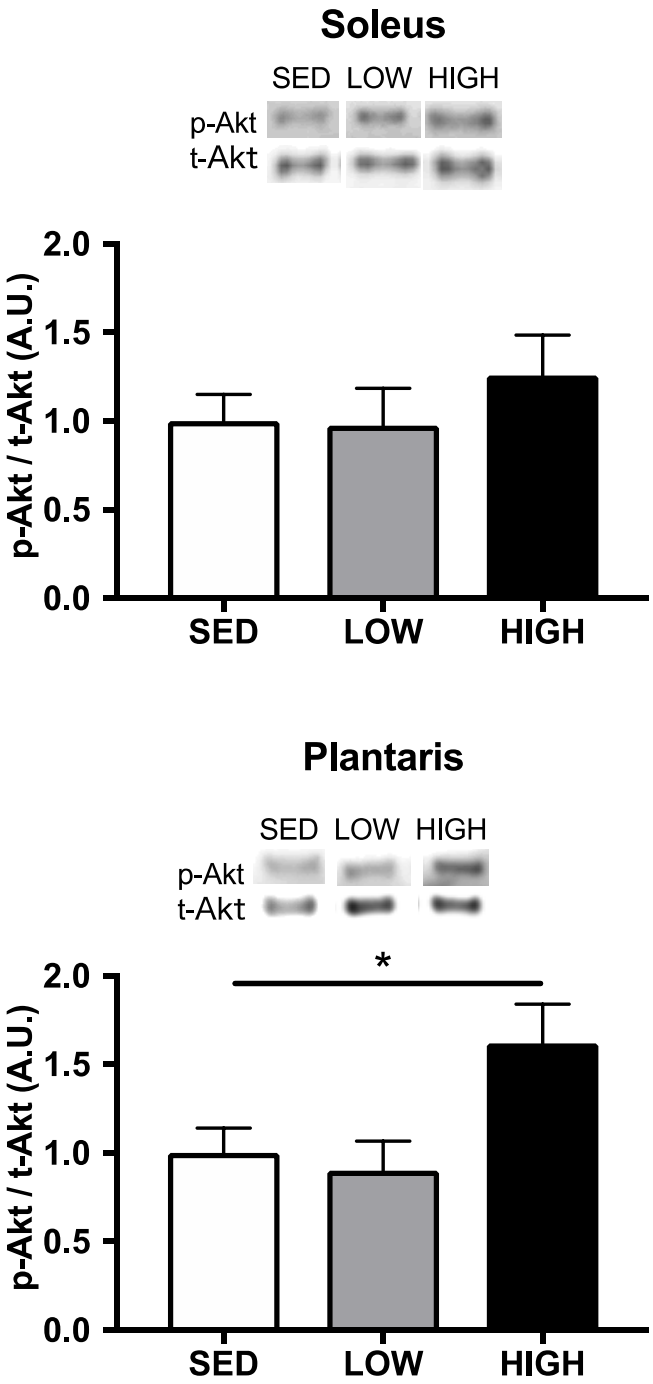


Figure 11. Phosphorylation of Akt in soleus and plantaris after acute exercise. Phosphorylation of Akt was assessed in skeletal muscles on acute exercise for 30 min using Western blot. Data are shown as mean ± SEM. n = 7 in each group. Data were analyzed using one way ANOVA. *, ** statistical difference was set at $p < 0.05$, $p < 0.01$ vs SED, respectively. SED, sedentary; LOW, low intensity exercise; HIGH, high intensity exercise. Accepted *Life Sciences* (Tokinoya and Shiromoto et al. 2018).

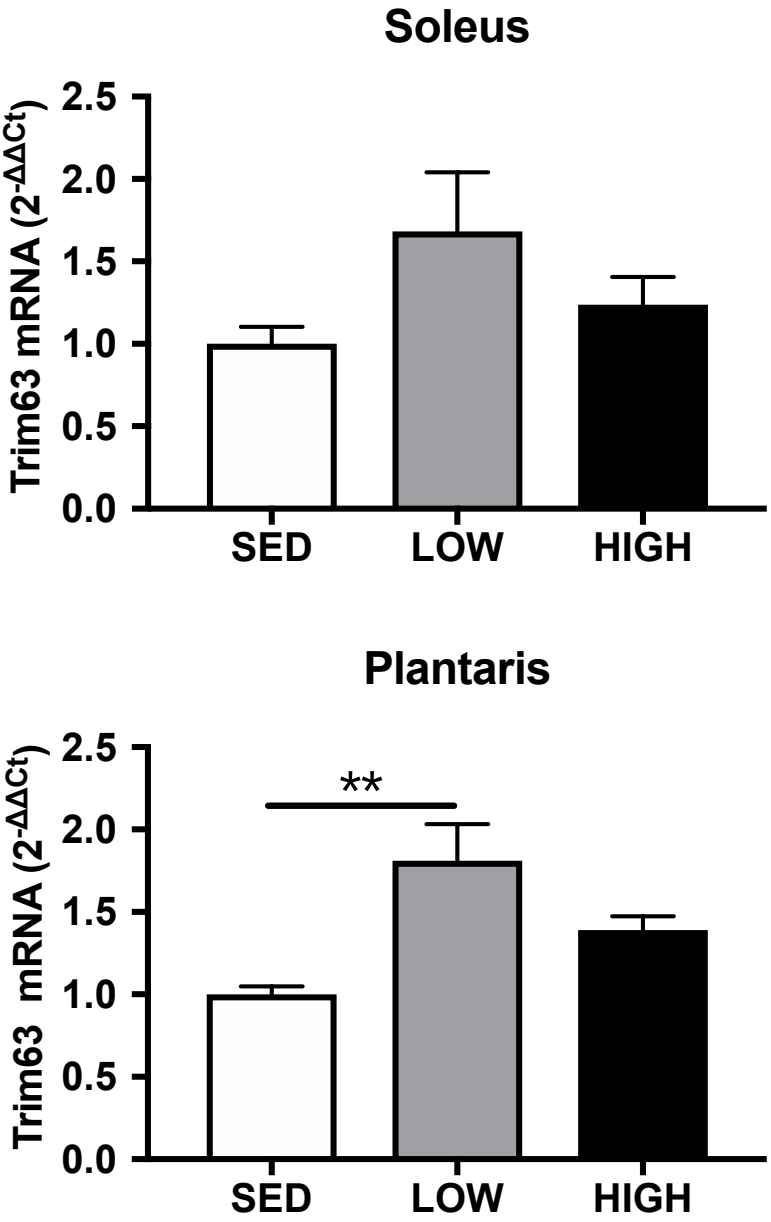


Figure 12. mRNA expression of Trim63 (MuRF-1) in soleus and plantaris after acute exercise.

Trim63 was one of the factors for protein degradation was assessed in skeletal muscles on acute exercise for 30 min using RT-qPCR (ΔΔCt method). Data are shown as mean ± SEM. n = 7 in each group. Data were analyzed using one way ANOVA. *, ** statistical difference was set at $p < 0.05$, $p < 0.01$ vs SED, respectively. SED, sedentary; LOW, low intensity exercise; HIGH, high intensity exercise. Accepted *Life Sciences* (Tokinoya and Shiromoto et al. 2018).

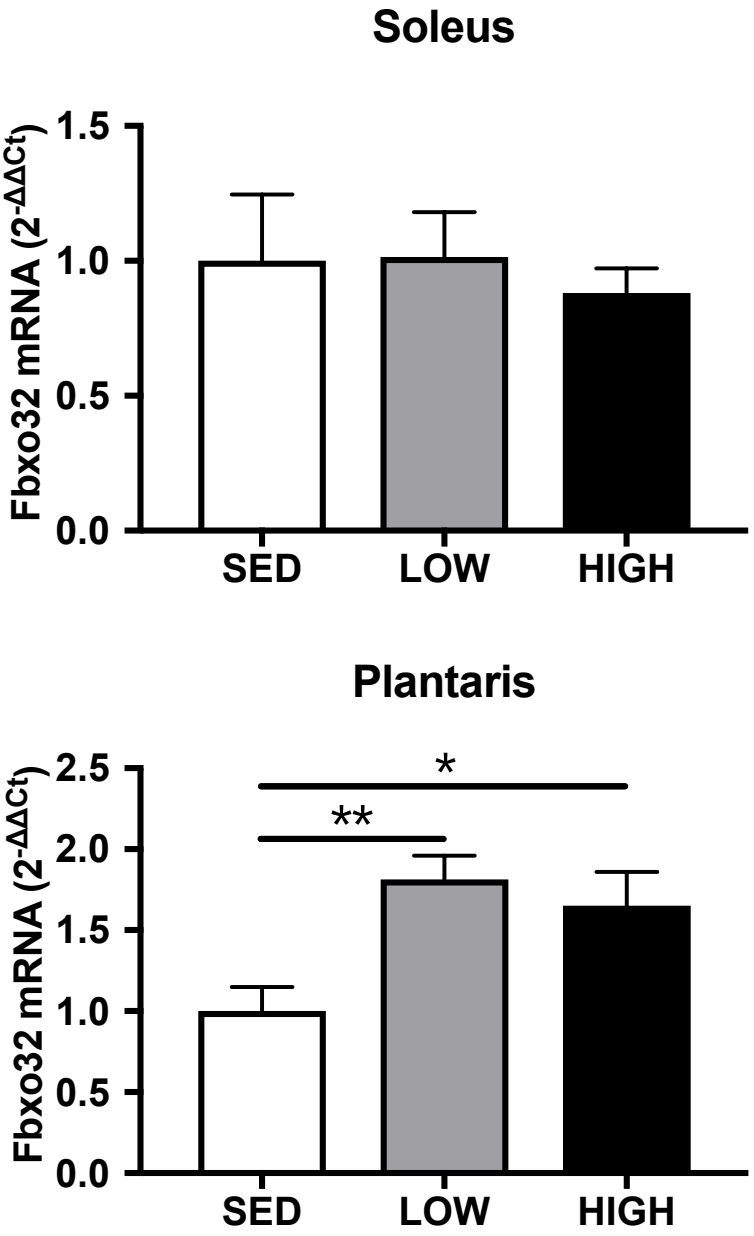


Figure 13. mRNA expression of Fbxo32 (MAFbx) in soleus and plantaris after acute exercise.

Fbxo32 was one of the factors for protein degradation was assessed in skeletal muscles on acute exercise for 30 min using RT-qPCR (ΔΔCt method). Data are shown as mean ± SEM. n = 7 in each group. Data were analyzed using one way ANOVA. *, ** statistical difference was set at $p < 0.05$, $p < 0.01$ vs SED, respectively. SED, sedentary; LOW, low intensity exercise; HIGH, high intensity exercise. Accepted *Life Sciences* (Tokinoya and Shiromoto et al. 2018).

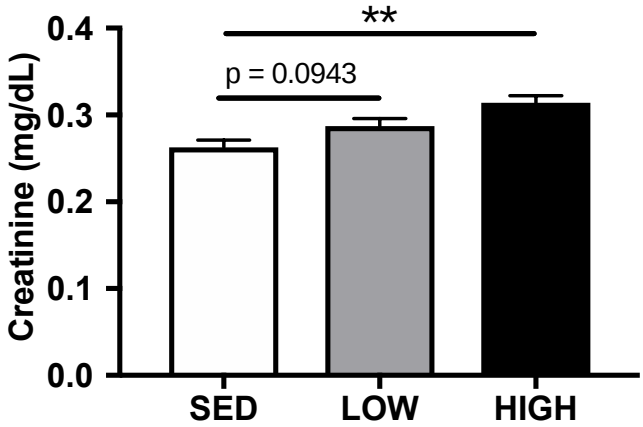


Figure 14. Plasma creatinine level to evaluate renal function after acute exercise. Kidney and liver function were assessed in skeletal muscles on acute exercise for 30 min. Data are shown as mean ± SEM. n = 7 in each group. Data were analyzed using one way ANOVA. ** statistical difference was set at $p < 0.01$ vs SED. SED, sedentary; LOW, low intensity exercise; HIGH, high intensity exercise; AST, aspartate aminotransferase; ALT, alanine aminotransferase. Accepted *Life Sciences* (Tokinoya and Shiromoto et al. 2018).

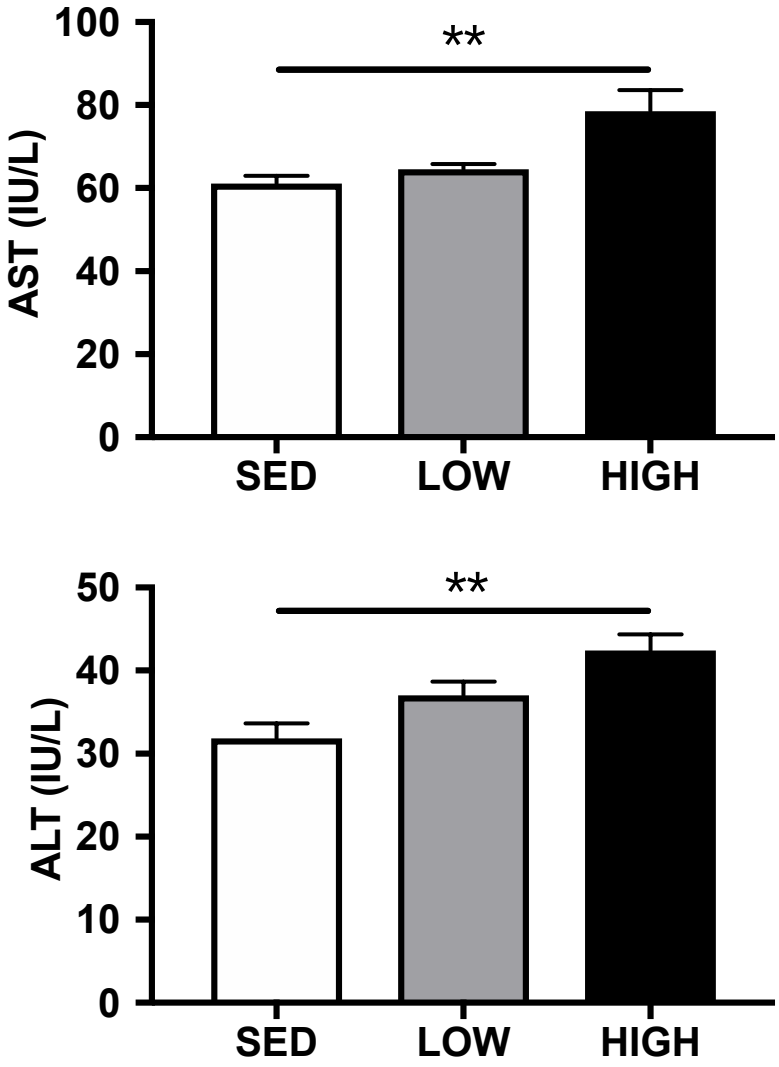


Figure 15. Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) to evaluate liver function after acute exercise.

Kidney and liver function were assessed in skeletal muscles on acute exercise for 30 min. Data are shown as mean ± SEM. n = 7 in each group. Data were analyzed using one way ANOVA. ** statistical difference was set at $p < 0.01$ vs SED. SED, sedentary; LOW, low intensity exercise; HIGH, high intensity exercise; AST, aspartate aminotransferase; ALT, alanine aminotransferase. Accepted *Life Sciences* (Tokinoya and Shiromoto et al. 2018).

4. Discussion

Renalase expression and function in different organs during exercise

My findings support previous animal studies showing no change in renalase concentration in the plasma after exercise [Czarkowska-Paczek et al. 2013]. However, a previous study observed increased renalase after 30-km running in humans [Yoshida et al. 2017]. They suggested the alternations in blood markers by the moderate intensity exercise, leads to decreased renal function. The result of this study found that plasma creatinine was increased and renalase expression in the kidney was decreased by low- and high-intensity exercise (Figs. 3; 14). Renalase in the kidney was related to kidney function in this study, as renalase knockout mice also showed an increased plasma creatinine [Lee et al. 2013]. Additionally, the heart and liver showed decreased renalase expression following exercise and liver function following only high-intensity exercise (Figs. 4; 15). Kidney and liver function may influence renalase expression because blood flow was decreased during exercise [Musch et al. 1987]. In contrast, the plasma renalase concentration remained unchanged in the present study (Fig. 5). The 30-min-running period was considered as an appropriate time to perform high-intensity exercise in this study, suggesting that the exercise ended before detecting increased renalase in the blood. Renalase expression in the skeletal muscles was increased by exercise; particularly, renalase expression increased in the soleus in the LOW group (Fig. 2-C), and in the plantaris in the HIGH group (Fig. 2-D). This suggests that renalase expression is increased in the respective main muscle fiber type recruited depending on exercise intensity. In general, the soleus is known as the slow muscle fiber type, while the plantaris is composed of the fast muscle fiber type [Armstrong and Phelps. 1984; Kuznetsov et al. 1996]. Recruitment of motor units depends on the force of movement [Sieck and Fournier. 1989]. The findings of this study suggest that the soleus is mainly used in the low-intensity exercise, while the plantaris is used in high-intensity exercise. However, renalase expression in gastrocnemius muscles was significantly increased by low- and high-intensity exercise (Fig. 2-A, B) and differed response from plantaris and soleus. It is difficult to evaluate the fiber type influences renalase because gastrocnemius muscles were collected together, although they were divided red and white portions.

Several studies reported that the PMCA 4b receptor exists for renalase and functions locally [Wang et al. 2015; Yin et al. 2016]. It is likely that renalase contributes to cell

survival in the skeletal muscle during exercise as an autocrine growth factor. The result of this study found that PMCA 4b expression differed between the soleus and plantaris, which are slow and fast twitch fibers, respectively (Fig. 10). Soleus muscles contain more mitochondria than plantaris muscle and produce more reactive oxygen species during exercise. Moreover, renalase expression may protect cells by activating Akt signaling via PMCA4b. Soleus muscle did not change with Akt signaling or MuRF-1 and MAFbx mRNA expression, which are downstream of Forkhead box O and regulated by Akt signaling [Sandri et al. 2004; Stitt et al. 2004]. In contrast, cell protective effects, or Akt, in the plantaris were increased in the HIGH group in this study (Fig. 11). Additionally, there was no difference between the SED and HIGH groups in MuRF-1 mRNA expression in the plantaris (Fig. 12). The plantaris, which contain fast twitch fibers, may be recruited by high-intensity exercise and accounted for >50% compared with 10% of slow twitch fibers in motor units [Sieck and Fournier. 1989]. Additionally, the response to Akt and NF-κB and muscle atrophy activated MuRF-1 and MAFbx occur for fast twitch fibers but not slow twitch fibers [Wang and Pessin. 2013]. Furthermore, the decreased cross-sectional area in the plantaris was earlier than that in soleus on muscle atrophy [Ohira et al. 2006]. Thus, the results of this study in the plantaris muscle support a relationship with the cell protective effects of renalase.

Transcription regulatory factors involved in renalase expression during exercise

To investigate the renalase expression signaling pathway, it was examined transcription factors that control renalase expression. Phosphorylation of IκBα, a member of the subfamily of NF-κB, was increased significantly in the plantaris muscle (Fig. 6). It was previously shown that phosphorylated IκBα protein increased immediately after exercise [Ji et al. 2014]. Furthermore, some studies reported that nuclear translocation of NF-κB occurs in skeletal muscle cells through exercise-induced oxidative stress [Aoi et al. 2004; Ji. 2008]. Renalase expression was activated by the TNF-α/NF-κB pathway [Wang et al. 2016]. In this study, however, TNF-α mRNA did not change and SOD was increased by acute exercise (Fig. 9; Tab 2). Thus, NF-κB was activated by oxidative stress while TNF-α was not. Several studies have demonstrated a relationship between renalase and NF-κB [Wang et al. 2014; 2016]. In the present study, IκBα phosphorylation in the plantaris muscle was significantly increased following high-intensity exercise and showed similar

results as renalase expression in the plantaris muscle. This suggests that renalase expression and NF-κB are related during acute exercise, with NF-κB acting as an important mediator of renalase expression. In contrast, there was no change in the soleus muscle (Fig. 6), suggesting that NF-κB contributes less to regulating renalase expression in the soleus muscle.

mRNA expression of HIF-1α in the soleus muscle was significantly increased in the LOW group following exercise (Fig. 7). HIF-1α was previously found to be increased following low-intensity exercise [Ameln et al. 2005]. My findings support HIF-1α activation in soleus muscle. In myocardial cells, renalase was found to be a hypoxia-responsive gene and correlated with HIF-1α expression [Du et al. 2015]. In this study, both renalase expression and HIF-1α mRNA expression in the soleus muscle significantly increased in the LOW group following exercise (Figs. 2-C; 7). Thus, it is likely that HIF-1α is mainly involved in regulating renalase expression in the soleus muscle during low-intensity exercise. Nevertheless, HIF-1α mRNA expression did not change in the plantaris muscle (Fig. 7), suggesting that HIF-1α contributes little regulating renalase expression in the plantaris muscle. Additionally, HIF-1α did not increase following high-intensity exercise in the soleus muscle. The soleus is a slow twitch fiber and may not greatly contribute to performance, although as exercise intensity increased, additional skeletal muscle fibers were recruited. In fact, slow twitch fiber contributed to just 10% of overall performance [Sieck and Fournier. 1989]. However, in low-intensity exercise, the load of slow twitch fibers is relatively greater because recruitment of fast twitch fibers is lower. Additionally, HIF-1α did not change by high-intensity exercise because the exercise time was set to 30 min in this study and almost muscle fibers were recruited by high-intensity exercise.

5. Summary

In this study, renalase expression in the skeletal muscle was increased by acute exercise, which was regulated by HIF-1α and NF-κB. In contrast, mRNA expression of Sp1 was unchanged in both the soleus and plantaris muscle following exercise when examining the contribution of catecholamine metabolic function in the skeletal muscle to renalase expression. In addition, renalase expression in the kidney was decreased by acute exercise.

Ⅴ. Study 2

Effect of renalase on dexamethasone-induced muscle atrophy

1. Aim

This study hypothesized that skeletal muscle atrophy would be related to renalase expression. This study was aimed at clarifying the relationship between dexamethasone-induced muscle atrophy and renalase expression.

2. Materials and Methods

Cell culture

C2C12 myoblast cells (RCB0987, Lot#: 37, RIKEN BRC, Japan) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Wako Pure Chemical Industries, Ltd., Japan) containing 10% fetal bovine serum, 1% penicillin, and streptomycin. After the cultures reached 70–80% confluence, the medium was replaced with DMEM containing 2% horse serum (differentiation medium) and changed every 2 days.

Dexamethasone-induced muscle atrophy

On the 5th day, the myotubes were treated with either dimethyl sulfoxide (DMSO; Sigma-Aldrich Co., USA) or dexamethasone (DEX; Fujifilm Co., Japan). After incubation under various conditions, samples were collected to extract protein and mRNA, and stored at −80 ℃ for subsequent analyses.

Quantitative real-time RT-PCR

Reffer to Study 1 Method.

The cycle threshold (Ct) value of the target gene mRNAs were standardized by the Ct value of the GAPDH gene (ΔΔCt method) for both DMSO and DEX groups. The primer sequences used in this study are shown in Table 3.

Western blot analysis

Reffer to Study 1 Method.

The signals were analyzed using Image J. The signals of renalase (ab178700, Abcam), Akt (9272, Cell Signaling Technology), p-Akt (13038, Cell Signaling Technology), β-actin

(sc-81178, Santa Cruz Biotechnology), and β-tubulin (sc-5274, Santa Cruz Biotechnology) were measured. Notably, β-actin or β-tubulin was analyzed as an internal standardization control.

Observation and cell counting

C2C12 myotubes were observed under a microscope (BZ-X710, Keyence, Japan), and images were taken. Five myotubes were selected randomly from each well (6-well plate) in accordance with previous studies [Son et al. 2017; Kim et al. 2018]. Myotube widths measured from these images were analyzed using Image J. Cell count was performed using Cell Counting Kit 8 (Dojin, Japan) in accordance with the manufacturer's instructions.

Statistical analysis

Data are shown as mean ± SEM. For all measurements, a one-way analysis of variance was used to evaluate significance. Statistical analyses were conducted using the GraphPad Prism 7 software (GraphPad, Inc.). The data were subjected to t-tests for the comparison between the two groups; *p* values below 0.05 were considered significant.

Table 3. Primer sequences used in mRNA analyses.

Name	Forward (5’ -> 3’)	Reverse (5’ -> 3’)
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
Myog	CCTTGCTCAGCTCCCTCA	TGGGAGTTGCATTCACTGG
Myh 4	TGCCTCCTTCTTCATCTGGT	CCATCTCAGCGTCGGAAC
Renalase	TGACCTTGTCATCCTCACCA	TCCCTCTGGCGTTCACTAAT
Trim 63	TGATTCCTGATGGAAACGCTATGG	ATTCGCAGCCTGGAAGATGTC
Fbxo 32	GACAAAGGGCAGCTGGATTGG	TCAGTGCCCTTCCAGGAGAGA
Ddit 4	CCAGAGAAGAGGGCCTTGA	CCATCCAGGTATGAGGAGTCTT
Klf 15	CGGTGCCTTGACAACTCATC	AAATGCACTTTCCCAGGCTG

Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Myog, Myogenin; Myh 4, Myocine heavy chain 4; Trim63 (MuRF-1), tripartite motif containing 63 (Muscle RING finger protein); Fbxo32 (MAFbx), F-box protein 32 (Muscle atrophy F-box); Ddit 4 (REDD 1), DNA-damage-inducible transcript 4 (regulated in DNA damage and development 1); Klf 15, Krüppel-like factor 15.
Modified *International Journal of Analytical Bio-Science* (Tokinoya and Takekoshi. 2019).

3. Results

Differentiation of C2C12 myotube

Confirmation of myotubes showed greater myogenin and myosin heavy chain expression levels on day 5 of the C2C12 differentiation than those is the before differentiation (Fig. 16).

Time course and concentration of DEX on renalase and artrogenes

The renalase protein expression was significantly increased by 10 μmol/L DEX upon incubation for 24 h with DEX as compared to that with DMSO (Fig. 17). In addition, the mRNA expression of renalase and atrogenes such as MuRF-1 and MAFbx were increased by the condition (Figs. 17; 19). On the other hand, phosphorylation of Akt was decreased by the condition (Fig. 18).

Forty-eight hours was the optimal time course as the mRNA expression of renalase and atrogenes (MuRF-1 and MAFbx) was significantly increased by DEX (Figs. 20; 21).

Renalase and factors related to DEX expression, cell death, and width on the condition

The renalase protein expression was elevated by treatment with 10 μmol/L DEX for 48 h (Fig. 23) although the myotube width and cell survival ratio were lowered (Fig. 22). The mRNA expression of REDD 1 and KLF 15 was increased (Fig. 25), and phosphorylation of Akt was decreased by the condition (Fig. 24).

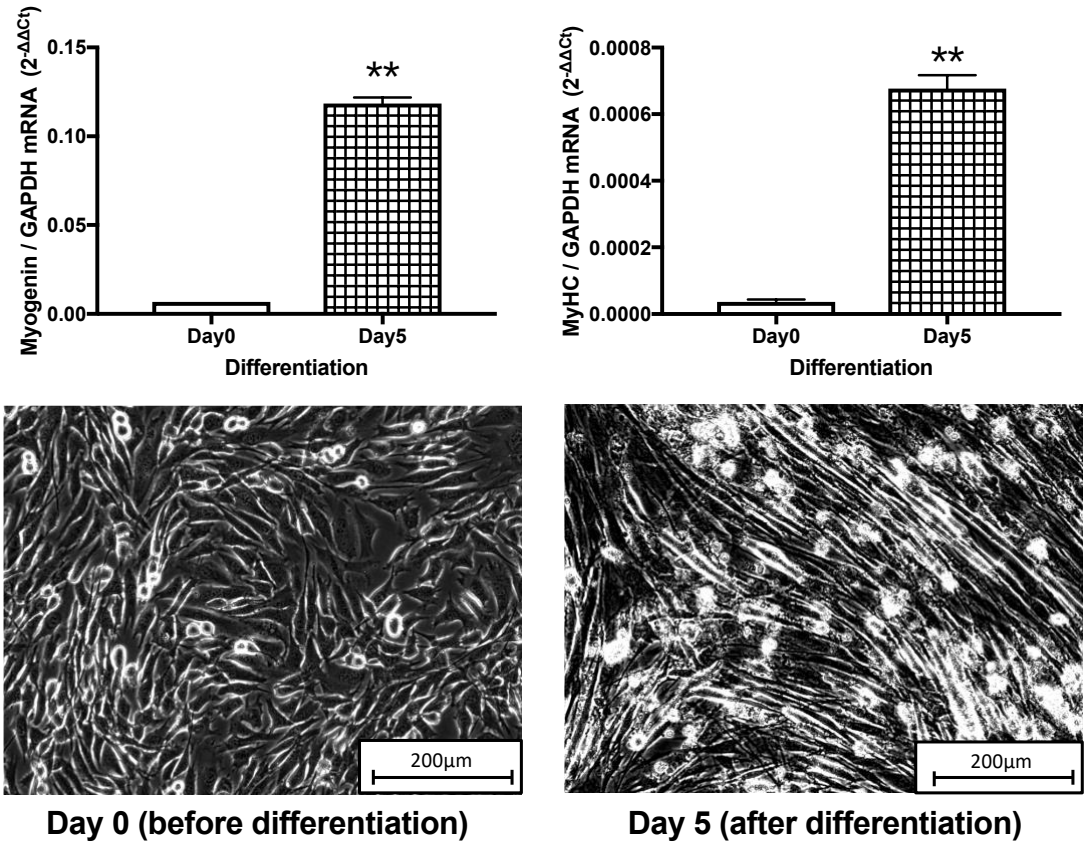


Figure 16: Differentiation markers of the C2C12 cells.
C2C12 myoblasts differentiated into myotubes by 2% horse serum medium. The asterisk shows a significant difference as compared with Day 0 (**$p < 0.01$). Data are shown as mean ± SD. n = 3 in each group. Modified *International Journal of Analytical Bio-Science* (Tokinoya and Takekoshi. 2019).

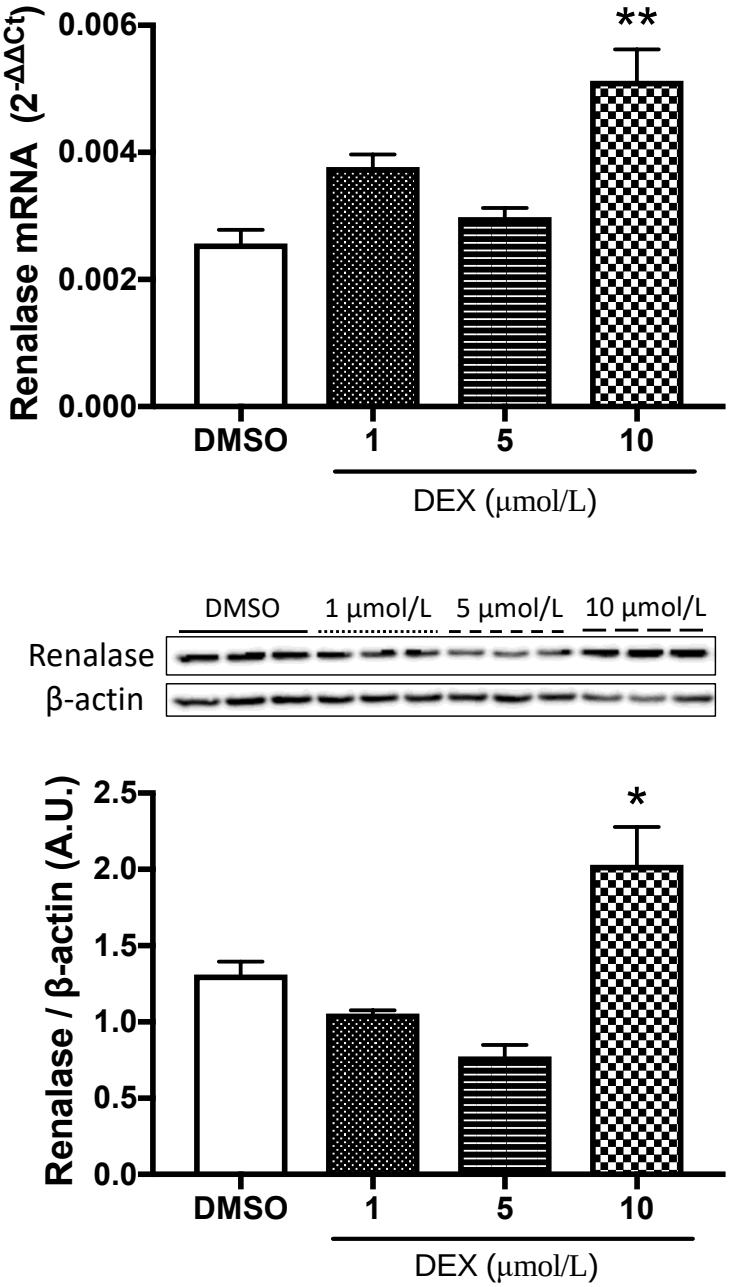


Figure 17: Renalase protein and mRNA expression levels under the various concentrations for 24 h.

C2C12 myotubes were stimulated by various concentrations of DEX for 24 h. The asterisk shows a significant difference as compared with DMSO (*$p < 0.05$, **$p < 0.01$). Data are shown as mean ± SD. n = 3 in each group. DMSO, dimethyl sulfoxide; DEX, dexamethasone. Modified *International Journal of Analytical Bio-Science* (Tokinoya and Takekoshi. 2019).

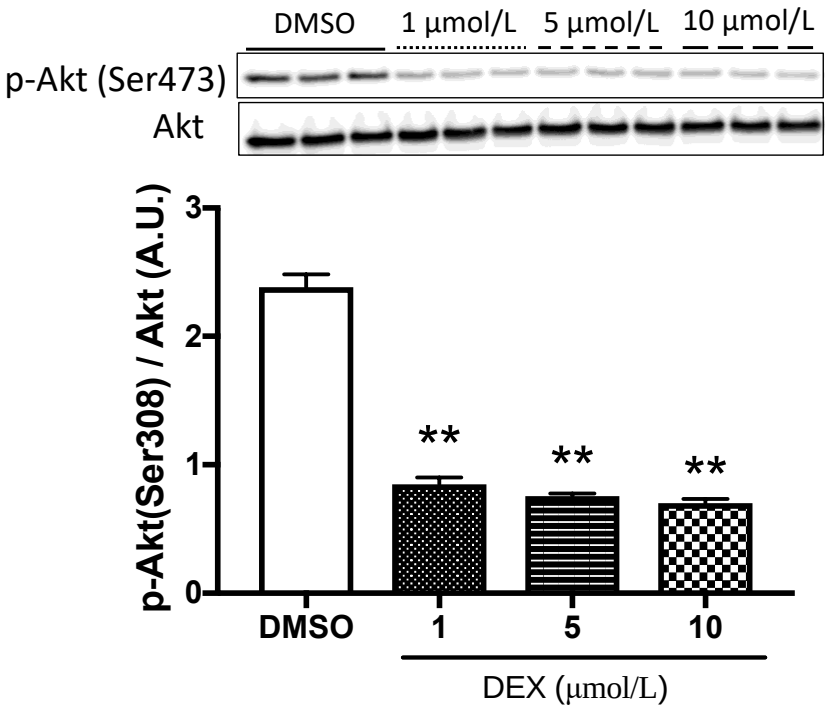


Figure 18: Phosphorylation of Akt under the various concentrations for 24 h.

C2C12 myotubes were stimulated by various concentrations of DEX for 24 h. The asterisk shows a significant difference as compared with DMSO (**$p < 0.01$). Data are shown as mean ± SD. n = 3 in each group. DMSO, dimethyl sulfoxide; DEX, dexamethasone. Modified *International Journal of Analytical Bio-Science* (Tokinoya and Takekoshi. 2019).

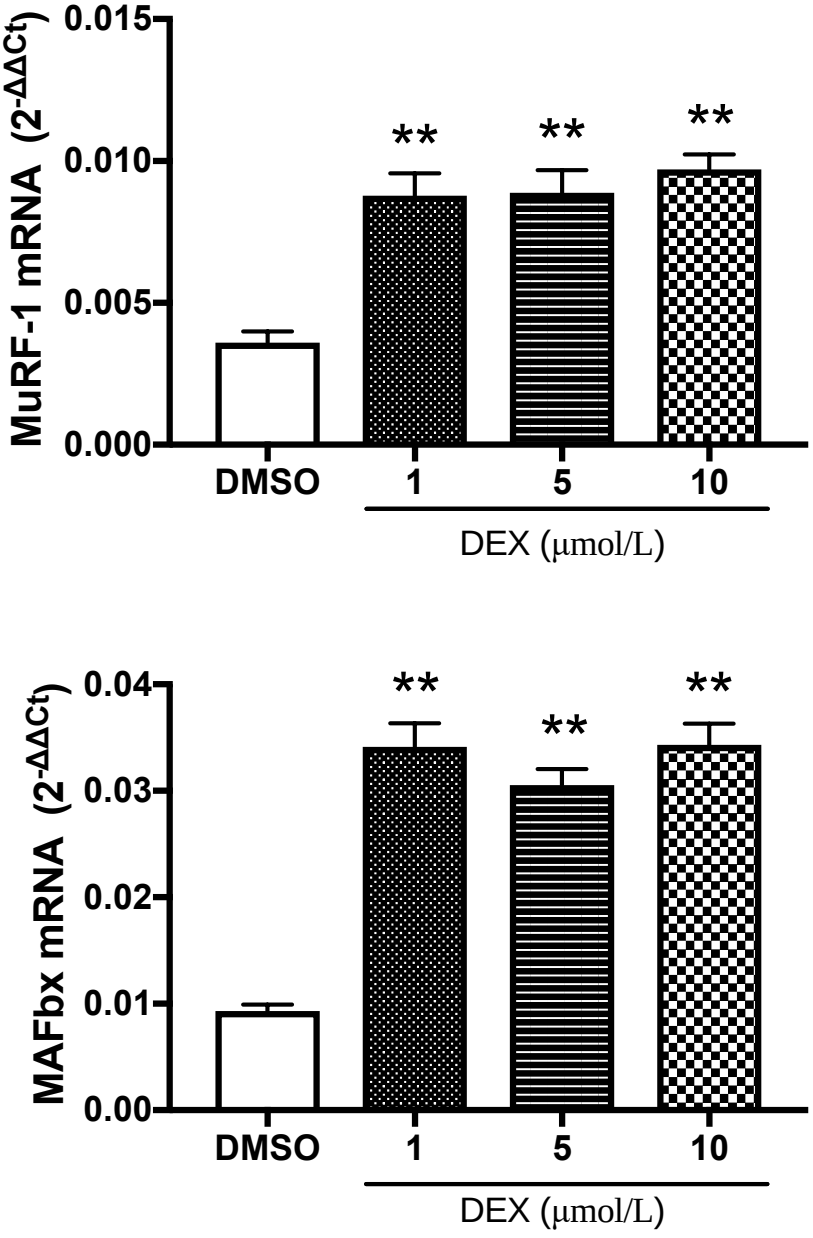


Figure 19: MuRF-1 and MAFbx mRNA expression levels under the various concentrations for 24 h.

C2C12 myotubes were stimulated by various concentrations of DEX for 24 h. The asterisk shows a significant difference as compared with DMSO (**p < 0.01). Data are shown as mean ± SD. n = 3 in each group. DMSO, dimethyl sulfoxide; DEX, dexamethasone. Modified *International Journal of Analytical Bio-Science* (Tokinoya and Takekoshi. 2019).

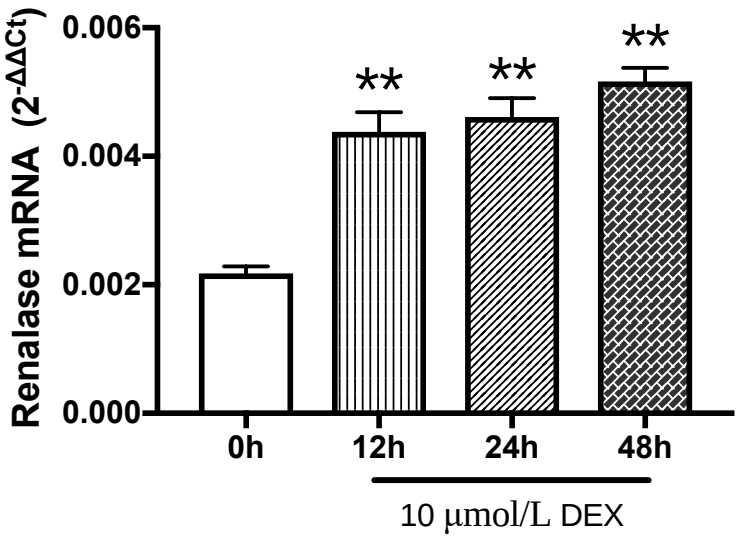


Figure 20: Renalase mRNA expression level was increased at various time courses with 10 μmol/L DEX.

C2C12 myotubes were stimulated at various time courses with 10 μmol/L DEX. The asterisk shows a significant difference as compared with 0 h incubation (**$p < 0.01$). Data are shown as mean ± SD. n = 6 in each group. DMSO, dimethyl sulfoxide; DEX, dexamethasone. Modified *International Journal of Analytical Bio-Science* (Tokinoya and Takekoshi. 2019).

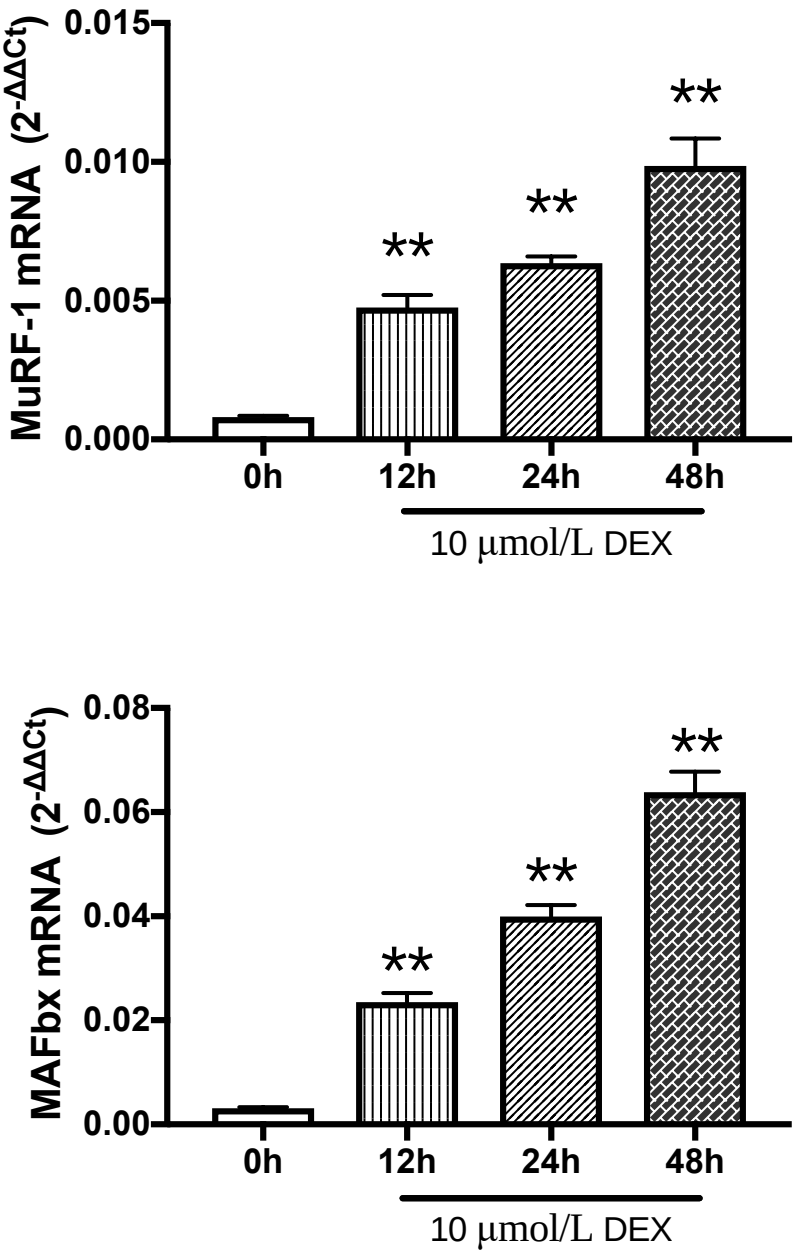


Figure 21: Atrogene mRNA expression levels were increased at various time courses with 10 μmol/L DEX.

C2C12 myotubes were stimulated at various time courses with 10 μmol/L DEX. The asterisk shows a significant difference as compared with 0 h incubation (**$p < 0.01$). Data are shown as mean ± SD. n = 6 in each group. DMSO, dimethyl sulfoxide; DEX, dexamethasone. Modified *International Journal of Analytical Bio-Science* (Tokinoya and Takekoshi. 2019).

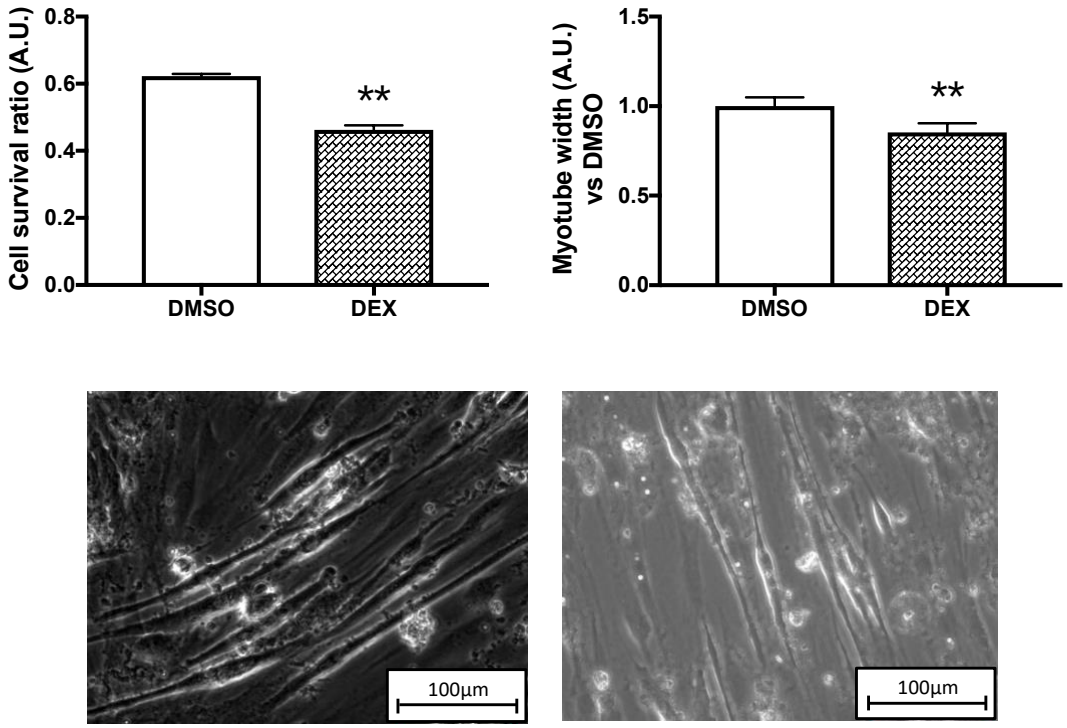


Figure 22: Changes in cell survival ratio and myotube width by 10 μmol/L DEX for 48 h.

C2C12 myotubes were stimulated by 10 μmol/L DEX for 48 h. Myotube widths were quantified in 30 randomly selected fields. The asterisk shows a significant difference as compared with DMSO (**$p < 0.01$). Data are shown as mean ± SD. n = 6 in each group. DMSO, dimethyl sulfoxide; DEX, dexamethasone. Modified *International Journal of Analytical Bio-Science* (Tokinoya and Takekoshi. 2019).

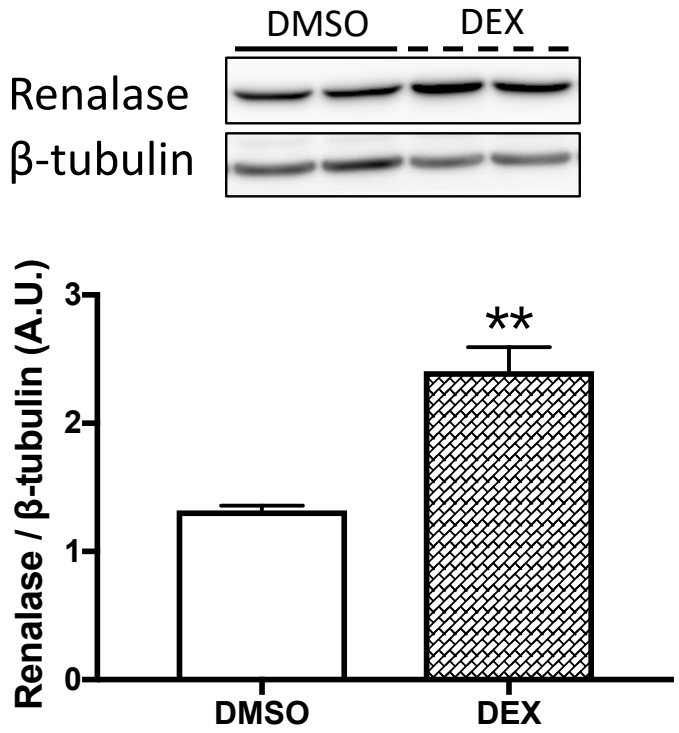


Figure 23: Renalase protein expression was increased by 10 μmol/L DEX for 48 h.

C2C12 myotubes were stimulated by 10 μmol/L DEX for 48 h. The asterisk shows a significant difference as compared with DMSO (**$p < 0.01$). Data are shown as mean ± SD. n = 6 in each group. DMSO, dimethyl sulfoxide; DEX, dexamethasone. Modified *International Journal of Analytical Bio-Science* (Tokinoya and Takekoshi. 2019).

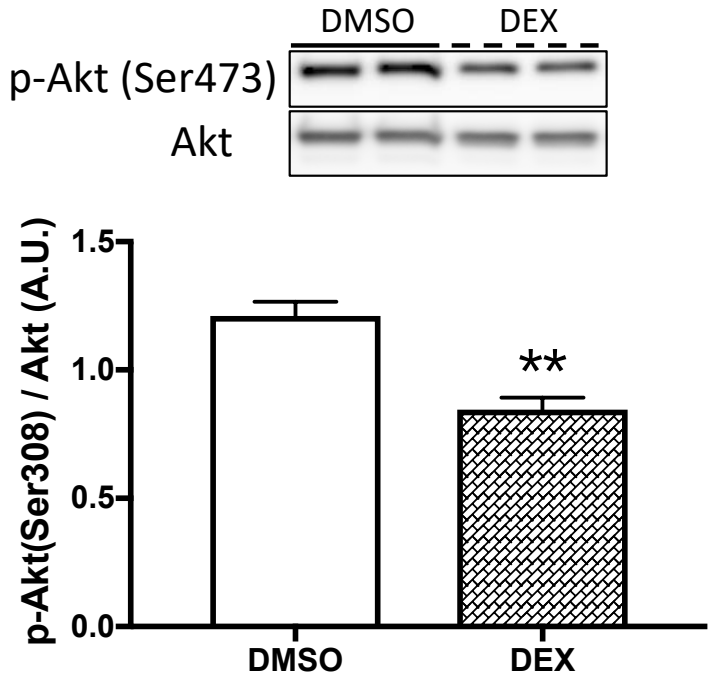


Figure 24: Phosphorylation of Akt was decreased by 10 μmol/L DEX for 48 h.

C2C12 myotubes were stimulated by 10 μmol/L DEX for 48 h. The asterisk shows a significant difference as compared with DMSO (**$p < 0.01$). Data are shown as mean ± SD. n = 6 in each group. DMSO, dimethyl sulfoxide; DEX, dexamethasone. Modified *International Journal of Analytical Bio-Science* (Tokinoya and Takekoshi. 2019).

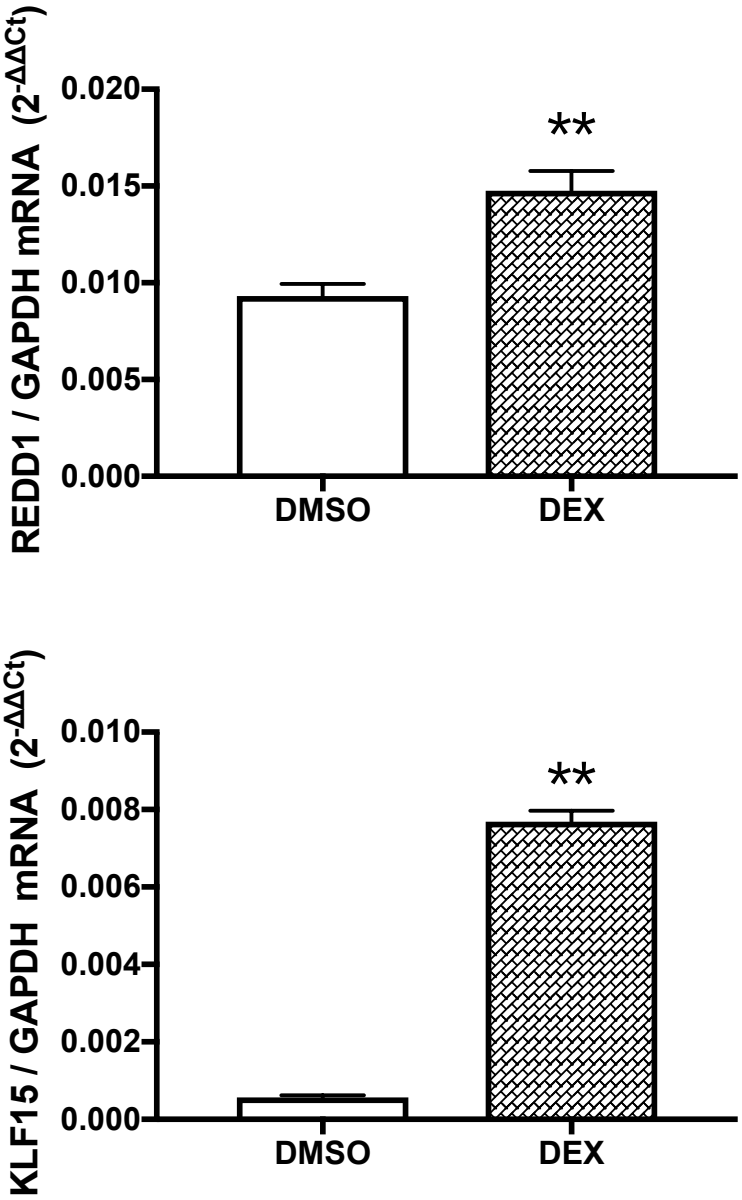


Figure 25: Changes in muscle atrophy factors by 10 μmol/L DEX for 48 h.
C2C12 myotubes were stimulated by 10 μmol/L DEX for 48 h. The asterisk shows a significant difference as compared with DMSO (**$p < 0.01$). Data are shown as mean ± SD. n = 6 in each group. DMSO, dimethyl sulfoxide; DEX, dexamethasone. Modified *International Journal of Analytical Bio-Science* (Tokinoya and Takekoshi. 2019).

4. Discussion

The purpose of this study was to investigate the effect of renalase expression on dexamethasone-induced muscle atrophy. The model used is the most common model for muscle atrophy *in vitro*. C2C12 cell differentiation was assessed first. The differentiation of C2C12 myotubes was more pronounced than that of myoblasts based on the differentiation factors (Fig. 16). Therefore, subsequent experiments on the conditions of differentiation focused on C2C12 myotubes.

The mRNA expression levels of MuRF-1 and MAFbx increased for all concentrations of DEX during 24 h. Phosphorylation of Akt was decreased by the condition (Fig. 18). These results agree with those in previous studies [Liu et al. 2016; Son et al. 2017; Wang et al. 2017; Kim et al. 2018; Sakai et al. 2019]. Moreover, the protein and mRNA expression levels of renalase were increased by the condition. C2C12 myotubes were stimulated by 10 μmol/L DEX for some time course. Notably, 48 h was the optimal time course because the mRNA expression levels of renalase and atrogenes (MuRF-1 and MAFbx) were significantly increased by DEX, although mRNA expression levels of renalase and atrogenes were increased at all the times examined. The use of 10 μmol/L DEX for 48 h was determined to be the optimal condition for subsequent experiments.

When C2C12 myotubes were stimulated by 10 μmol/L DEX for 48 h, the myotube width and cell survival were decreased. The findings indicated DEX-induced muscle atrophy. Previous studies described decreased myotube width upon DEX treatment [Son et al. 2017; Kim et al. 2018]. In addition, the expression levels of REDD 1 and KLF 15, which restrain the mTOR and regulate atrogenes and mRNA expressions, respectively, were increased by the condition (Fig. 25). The results supported previous studies and indicate the influence of GR [Shimizu et al. 2011; Kuo et al. 2012]. The level of expression of renalase protein was significantly increased by the condition. Tokinoya et al. (2018) suggested that increases in the expression of renalase in skeletal muscle are related to protein degradation that occurs via the surpression of MuRF-1 when Akt is phospharylated. The phosphorylation of Akt was decreased by DEX in this study, as the signaling was decreased by DEX in previous studies [Son et al. 2017; Sakai et al. 2019]. In addition, myostatin, which was increased by DEX [Kun et al. 2001], decreased the phosphorylation of Akt. Thus, there are many factors which surpressed Akt in this model. Although the renalase expression level was increased by DEX-induced muscle atrophy, the effect on atrogenes remains unknown.

Tokinoya et al. (2018) suggested that the renalase expression level in the skeletal muscle can be increased by oxidative stress and that the level of expression of MuRF-1 is reduced during acute exercise. Acute exercise results in the production of reactive oxygen sepsis (ROS). Akt is known to be a potent activator of ROS [Shaw et al. 1998]. Therefore, the findings might be a problem of the signaling related to Akt because many factors activate the phosphorylation of Akt in an acute exercise model, whereas the DEX model in this study inhibited the phosphorylation of Akt by various factors. However, renalase induces the phosphorylation of Akt to protect cells from acute kidney disease [Wang et al. 2014]. It is possible that renalase activates the phosphorylation of Akt via the receptor, since the phosphorylation of Akt had recovered by 48 h compared with 24 h (Figs. 18; 24). Future studies should verify the effect of renalase overexpression or knock down on DEX-induced muscle atrophy. In conclusion, this study suggests that the renalase expression level restrains DEX-induced muscle atrophy. The effect of overexpression and KO of renalase on muscle atrophy remain unclear.

5. Summary

Renalase expression restrains dexamethasone-induced muscle atrophy via the Akt pathway. Phosphorylation of Akt recovered by 48 h compared with 24 h.

Ⅵ. Whole discussion

1. New knowledge

Renalase expression in the skeletal muscle was increased by acute exercise and dexamethasone-induced muscle atrophy. On the other hand, its expression in the kidney and other tissues decreased on acute exercise. In addition, renalase in the skeletal muscle contributed to cell protective effect via MuRF-1 of ubiquitine proteasome pathway by restraint of Akt signaling.

Renalase expression in skeletal muscle was increased by 30-min of acute exercise performed at low or high intensity. The exercise time set initially set as 30 min in Study 1 because rats cannot reproducibly run for more 30 min at the speed of 30 m/min on a treadmill. Renalase expression in the kidney was decreased by exercise performed at these intensities. The renalase expression in the kidney decreased after acute exercise in this study is consistent with result of previous study [Yoshida's doctoral thesis and Tokinoya and Yoshida et al. 2020]. It was considered some factors that might result in this expression pattern. The decreased blood flow in the kidney might be related to microRNAs (miRNA). The increased levels of miR-29b can decrease renalase expression in mice with hypertension [Kalyani et al. 2015]. It is possible that miR-29b expression in the kidney might be increased after acute exercise because blood pressure was increased by exercise in previous study [Katch et al. 2011]. However, it is unclear whether miR-29b in kidney is increased by exercise. In addition, this phenomenon might not occur in the skeletal muscle because of increased renalase expression in skeletal muscle on exercise. Other tissues showed decreased renalase expression following acute exercise. Nevertheless, renalase concentration in the blood was increased by 60 min of acute exercise in the previous study (Fig. 26) [Yoshida's doctoral thesis and Tokinoya and Yoshida et al. 2020]. Acute exercise decreased renalase expression in the kidney. Renalase in the kidney is secreted by epinephrine [Wang et al. 2014]. In present and previous studies, tissues including liver, heart, lung, and kidney, except skeletal muscles, showed unchanged or decreased renalase expression on acute exercise. Thus, it is possible that renalase was also secreted from the other tissues. In other words, the skeletal muscle may secrete renalase into the blood. Myokine is a physiologically active substance which secreted by skeletal muscles. Myokine influences the body [Febbraio and Pedersen. 2005]. Renalase might also be a myokine because renalase expression in the skeletal muscle was increased upon exercise.

Renalase in the soleus muscle only increased after low-intensity exercise for 30 min, whereas it in the plantaris muscle it increased only after high-intensity exercise. Exercise intensities influence the recruitment of skeletal muscle fibers [Sieck and Fournier. 1989]. Renalase mRNA expression in soleus showed higher than that in plantaris on mice (Fig. 27) [Tokinoya and Yoshida et al. 2020]. The soleus muscle is a red twitch fiber, while the plantaris muscle is a white twitch fiber. Red and white twitch fibers are recruited mainly by low-intensity and high-intensity exercise, respectively. Therefore, renalase might be affected by skeletal muscle contraction. In this study, it is suggested that renalase in plantaris influenced on cell protective effect after acute exercise for 30 min. However, physiological effect of renalse in soleus remained unclear in this study.

Transcription factors including HIF-1α, NF-κB, Sp1, STAT3, and ZBP-89 have been related to renalase [Sonawane et al. 2014; Wang et al. 2014; Du et al. 2015; Hollander et al. 2016]. In this study, the relationship of HIF-1α and NF-κB was evident after 30 min of exercise. On the other hand, Sp1, STAT3, and ZBP-89 were related following 60 min of exercise [Yoshida's doctoral thesis and Tokinoya and Yoshida et al. 2020]. Whether these factors actually activate renalase expression in the skeletal muscle remains unclear, however, because reporter assays were not done. Exercise activates various factors that include oxidative stress, hypoxia, and hormones [Aoi et al. 2004; Ameln et al. 2005; Soya et al. 2007]. Therefore, it is possible that renalase in the skeletal muscle is regulated by these transcription factors on acute exercise.

Protein degradation in the skeletal muscle can be surpressed the ubiquitin proteasome pathway by renalase following acute exercise. MuRF-1, which is reduced by phosphorylated FoxO when phosphorylation of Akt occurs, showed no significant change between sedentary and high-intensity exercise on acute exercise in the plantaris muscle in this study. In addition, DEX-induced muscle atrophy increased renalase expression. Especially, the phosphorylation of Akt at 48 h was recovered compared with 24 h despite the decreased phosphorylation of Akt by DEX-induced factors. Wang et al. (2014) showed that renalase peptide activates Akt, and its activation can be abrogated by Akt inhibitor. Thus, renalase phosphorylates Akt in the skeletal muscle. The *in vitro* results of this study suggested that renalase work cell protective effect via ubiquitin proteasome pathway by phosphorylated Akt.

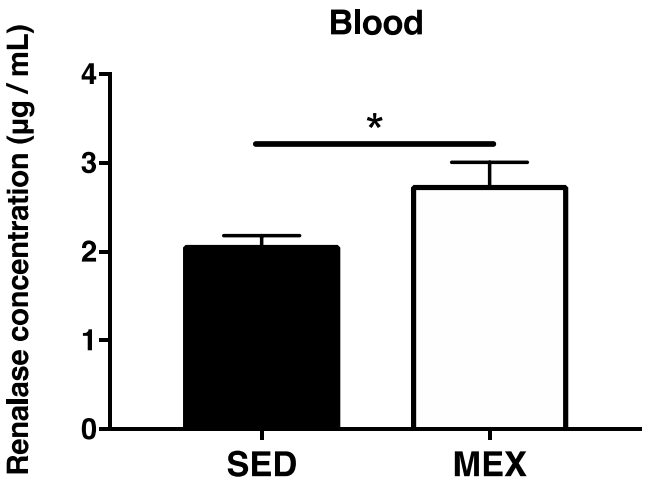


Figure 26. Renalase concentration in the blood after moderate intensity exercise. Rats ran for 60 min at moderate-intensity exercise on a treadmill. The plasma concentration of renalase was measured by ELISA. Data are shown as mean ± SEM. n = 5 or 6 in each group. Data were analyzed using t test. * statistical difference was set at $p < 0.05$ between SED and MEX group. SED, sedentary; MEX, moderate intensity exercise. Submitted a doctoral thesis of Yoshida and modified *FEBS Open Bio* (Tokinoya and Yoshida et al. 2020).

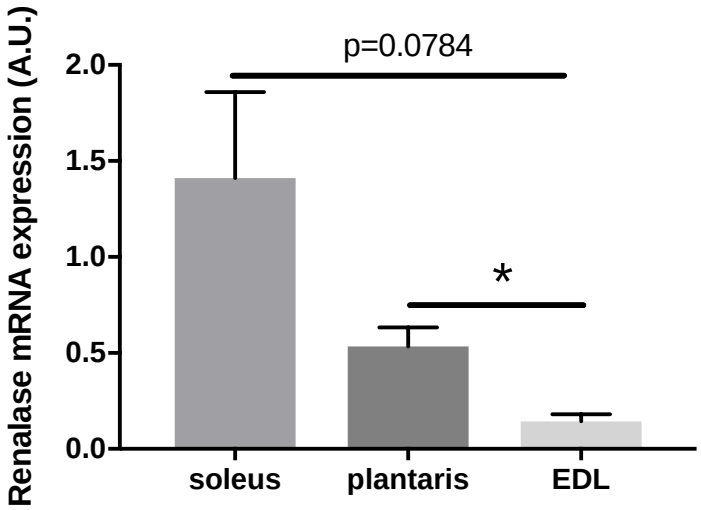


Figure 27. Renalase mRNA expression in skeletal muscle fibers. Renalase mRNA expression was assessed in skeletal muscles using RT-qPCR (a standard curve method). Data are shown as mean ± SEM. n = 6 in the SED group. Data were analyzed using one way ANOVA in "B", not "A". * statistical difference was set at $p < 0.05$. EDL, extensor digitorum longus. Modified *FEBS Open Bio* (Tokinoya and Yoshida et al. 2020).

2. Limitation

This study can not clearly determine the physiological effect of renalase in the skeletal muscle because it did investigate the effect of the overexpression or KO of renalase. Renalase expression in the skeletal muscle was actually increased by acute exercise and DEX-induced muscle atrophy. For the exercise model, it remains unclear whether renalase expression in the skeletal muscle increased after acute exercise because there are many transcription factors that are affected by various causes in renalase.

3. Perspective

The influence of exercise on downstream cell survival pathways after renalase-PMCA4b binding involves various factors. Further *in vitro* studies are needed to clarify these effects. Whether renalase in the skeletal muscle works via the PMCA 4b receptor must be investigated. Additional studies should investigate PMCA 4b or renalase and its downstream effects using small interfering RNA (siRNA) or recombinant renalase in skeletal muscle cells. In previous studies, knockdown of renalase with siRNA reduced cell viability [Guo et al. 2016; Hollander et al. 2016].

Further research is necessary using renalase KO mice to elucidate physiological effects more clearly. The cell protective effect in some organs using renalase KO mice has not been reported. Regarding skeletal muscles in renalase KO mice, there is the only report of skeletal muscle weight. However, from the results it is not clear which skeletal muscle is involved and whether fast twitch or slow twitch fibers are involved. Future studies need to examine how renalase is related to muscle protein degradation using a muscle atrophy model involving suspension, denervation, and DEX *in vivo*. It may be that renalase KO mice have worse muscle protein degradation compared to wild type mice because renalase cannot protect cells via a receptor.

Ⅶ. Conclusion

Renalase in the skeletal muscle contributes to protect cells by surpressing protein degradation. This study clarifies the involved molecular pathway (Fig. 28). Renalase is regulated by various transcription factors and depends on exercise intensities and muscle fiber types. Renalase may reduce protein degradation in the skeletal muscle.

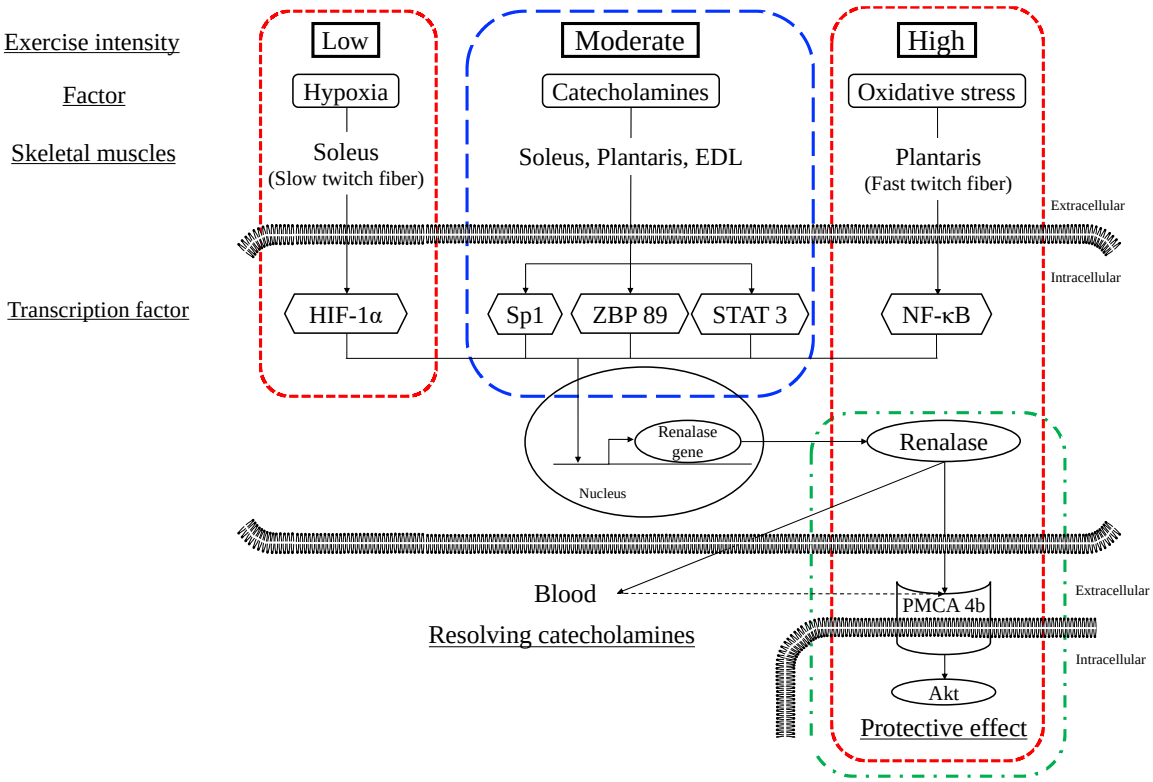


Figure 28. The molecular mechanism of renalase in the skeletal muscle to protect cell.
Doctoral research showed that a short broken line (Red) is Study 1, a short and long broken line in turn (Green) is Study 2, a long broken line (Blue) is previous study (Tokinoya and Yoshida et al. 2020) in conclusion. EDL, extensor digitorum longus.

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