Studies on fluctuation of renalase in blood, organs, and skeletal muscle during exercise.

(レナラーゼの運動時における血中、臓器、
および骨格筋の変動に関する研究)

A Dissertation Submitted to the Graduate School of Comprehensive Human Sciences

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Yasuko Yoshida

Two of the following original papers in this doctoral thesis were revised and the recently obtained research results were included in the manuscripts.

1. Transient changes in serum renalase concentration during long-distance running: The case of an amateur runner under continuous training
   Yasuko Yoshida, Takehito Sugasawa, Masaya Hoshino, Katsuyuki Tokinoya, Keisuke Ishikura, Hajime Ohmori, and Kazuhiro Takekoshi. (2017) The Journal of Physical Fitness and Sports Medicine, VOL.6, NO.3, P159-P166.

2. Epinephrine upregulates renalase expression in cultured C2C12 muscle cells
   Yasuko Yoshida, Takehito Sugasawa, Katsuyuki Tokinoya, Shunji Namba, and Kazuhiro Takekoshi. (2017) International Journal of Analytical Bio-Science, 31 Dec 2017, 5 (4): P53-P56.

✦ Changes in gene expression of renalase during medium intensity exercise: The case of animal model. Yasuko Yoshida, Katsuyuki Tokinoya, Takehito Sugasawa, Hajime Ohmori, and Kazuhiro Takekoshi. (Planned to submit a paper)

Table of Contents

List of Tables

List of Figures

I. Introduction

1. Renalase

1) Structure of renalase

Renalase is a recently discovered flavin adenine dinucleotide (FAD)-dependent soluble monoamine oxidase (Xu et al, 2005).

Gene.

The human renalase gene, located on chromosome 10 at q23.33, encompasses 309469 base pairs (bp), and has 11 exons (Xu et al, 2005; Desir et al, 2009; Desir et al, 2012; Wang et al, 2014).

Protein.

There is evidence regarding the existence of at least four alternatively spliced isoforms. The most highly expressed isoform (renalase1) is 342 aa long, and is encoded by exons 1–4, 6–7, 8, 9, and 10. The structure of renalase consists of three domains, a single peptide moiety, a bindeadenine dinucleotide binding moiety, and an amine oxidase moiety. The human renalase protein (hRenalase) has been detected in plasma, kidney, heart, skeletal muscle, and liver (Xu et al, 2005; Desir et al, 2009; Desir et al, 2012; Wang et al, 2014).

2) Function of renalase

There are two main functions of renalase.

One function is to metabolize circulating catecholamines (Xu et al, 2005; Desir et al, 2009; Desir et al, 2012; Wang et al, 2014). Because catecholamines are not metabolized when the renalase level decreases, it is thought that blood pressure increases as the level of catecholamines increases. Therefore, it is reported that the role of renalase is to regulate cardiac function and blood pressure (Desir et al, 2009). Human diseases in which the tissue and blood levels of renalase are decreased include essential hypertension, chronic kidney disease, and preeclampsia (Desir et al, 2009; Desir et al, 2012; Zbroch et al, 2012; Yılmaz et al, 2016). In addition, the renalase level decreases even when salt intake is high or there is a lack of potassium intake (Wang et al, 2014). The abovementioned diseases or lifestyle habits have hypertensive symptoms that are one of the clinical symptoms or health risks. In a study related to the regulation of renalase by via blood pressure levels, the subcutaneous administration of renalase in hypertensive model rats was performed. The administration of renalase resulted in a decrease in circulating catecholamines and a decrease in blood pressure of about 15% (Wang et al, 2014; Baraka et al, 2012).

Another function of renalase, unlike enzymes, is to function as a survival and growth factor. Therefore, it has the function of protecting cells. It reportedly protects against both heart and kidney injury (Lee et al, 2013; Wang et al, 2014, 2015, 2016; Du et al, 2015;

Guo et al, 2014). One study showed that renalase expression was elevated and that it attenuated cardiac injury in mice challenged with cardiac ischemia/reperfusion injury. A similar study was done in the kidney (Wang et al, 2016).

3) Preceding studies

There was merely one study that examined the relationship between renalase and exercise. There was no significant difference in the serum renalase concentrations during both acute exercise and endurance training, but renalase mRNA expression in the kidneys of rats at rest after being subjected to 6 weeks of endurance training was significantly increased, as compared to that in rats that were not subjected to training (Czarkowska-Paczek et al, 2013). This study suggested the possibility that exercise and renalase levels might be related.

II. The study purpose and design

Renalase regulates blood pressure and reportedly protects cells from oxidative stress. However, there has been no report investigating the association between exercise and renalase in humans. Therefore, in this study, we focused on the dynamics of renalase during exercise and hypothesized about the involvement of organs and skeletal muscles.

There is no report about the dynamics of renalase at the time of exercise; it seems that research on this topic would be useful not only in the exercise field, but also in the medical field, because there are many reports even with regard to pathological conditions.

●Research task 1: Human subject research

“Transient changes in the serum renalase concentration during long-distance running.”

There was only one report regarding the relationship between renalase and exercise, in which there was no significant difference in the blood renalase concentration. In addition, because research on human subjects has not been conducted, it cannot be concluded that the matter has been sufficiently examined. Therefore, in research task 1, it was hypothesized that the concentration of renalase in the blood fluctuated because of exercise, and this was investigated experimentally.

●Research task 2: Animal model research

“Changes in renalase levels observed using a rat moderate treadmill running model.”

In research task 1, the concentration of renalase in the blood was increased because of exercise. The main organ in which renalase is expressed is the kidney. However, in research task 1, there was a negative correlation between renalase concentration in the blood and renal function. Therefore, in research task 2, it was hypothesized that the increased concentration of renalase in the blood during exercise might occur owing to the expression of the renalase gene or protein in the skeletal muscle.

●Research task 3: Cell culture experiment

“Epinephrine upregulates renalase expression in cultured C2C12 muscle cells.”

In research task 2, a significant increase was observed in both mRNA expression and protein expression in skeletal muscles.

Therefore, in research topic 3, it was hypothesized that catecholamines were involved in the induction of increased expression of the renalase gene in skeletal muscles because of exercise.

●Research task 4: Expression factor of renalase

“Expression factor of catecholamine-induced renalase secretion in skeletal muscle tissues of the animal model of transient medium intensity exercise.”

In research task 3, it was shown that the addition of catecholamines significantly increased renalase expression in skeletal muscle cells. Therefore, in research task 4, it was hypothesized that an increase in the STAT 3, Sp 1, and ZBP 89 (factor regulating catecholamines) levels were related to the expression of the renalase gene in skeletal muscles.

III. Research task 1: Human subject research

"Transient changes in the serum renalase concentration during long-distance running."

1) Aim

Only one study conducted by Czarkowska-Paczek et al in 2013 has investigated the relationship between renalase levels and exercise in rats. Though there were no significant differences in the serum renalase concentrations during both acute exercise and endurance training, the level of renalase mRNA expression in the kidneys of mice at rest after 6 weeks of endurance training was significantly increased, as compared with that in rats that did not undergo training (Czarkowska-Paczek et al, 2013). This study suggested the possibility that exercise and renalase levels might be related. However, the association between exercise and renalase levels in humans has not been investigated yet.

The present study is the first to investigate the relationship between exercise and renalase levels in amateur runners undergoing continuous training. To measure the serum renalase concentration, a marker indicating the general condition of the body, continual exercise for more than 60 minutes was required, and measurements were performed during and after exercise; the protocol for measuring the renalase level was not reported by Czarkowska-Paczek et al. We also examined the association between renalase levels and

kidney function, as renalase is mainly expressed in the kidney, and also investigated its relationship with oxidative stress.

2) Materials and Methods

Subjects

Eleven young men who were continuously training and running about 10 to 20 km per week were recruited. One of them ceased exercise because he experienced cramping in the lower extremity at the 20 km end; hence, he was excluded from the analysis (n = 10). The characteristics of the subjects are detailed in Table 1. Before starting the experiment, we explained the background, purpose, and hazards of the study to all subjects, and obtained written consent. This study was conducted with the approval of the sports ethics committee of the Graduate School of Comprehensive Human Sciences, University of Tsukuba (Approval number: 27–149).

Table 1. Characteristics of study participants

	AVG		SD
Age (year)	22.4	±	4.0
Height (cm)	170.8	±	5.9
Weight (kg)	60.1	±	6.4
VO2max (ml/kg/min)	61.7	±	6.6
BMI (Kg/m2)	20.6	±	1.3

AVG; average
SD; standard deviation
BMI; body mass index
VO2max; maximal oxygen intake

Procedures

We set up a 5-km course at the University of Tsukuba for conducting this study. The total distance covered after 6 round trips was 30 km. Measurements were performed before running, and at distances of 10 km, 20 km, and 30 km. The conditions under which measurements before running were taken were the same as those on the day of study. The measurements before running were performed on the day before, as it otherwise becomes a physical burden to the subject. We performed a cardiopulmonary exercise test in advance using a treadmill, calculated the ventilatory threshold (VT), and set 90% VT as the running speed. The total time taken for subjects to complete 5 km laps is shown in Table 2. They ate a 400 kcal breakfast (protein: 8 g, lipid: 21.9 g, carbohydrate: 41.7 g) comprised of solid nutritional supplements, which would prevent hypoglycemic symptoms and enable the maintenance of a constant running pace, 1 hour before the test (Fuminori et al, 2014; Nagai et al, 2005; Sengoku et al, 2008; Kong et al, 2008). They then ate jelly, which provided 180 kcal (carbohydrate 45.2 g) at the 15-km point, to prevent hypoglycemic disorder and to minimize digestive activity during running from a nutritional point of view. They had free access to a sports drink every 5 km for staying hydrated, the sodium and potassium content in the drink was 0.54 / l g and 0.2 / l g, respectively. The amount of sports drinks that subjects drank are shown in Table 3, and the hematocrit values of the subjects are shown in Table 4.

Table 2. The lap time and the total time of each subject

Subject	5 km	10 km	15 km	20 km	25 km	30 km	Total	AVG	SE
A	25:00	25:00	29:31	25:14	30:05	25:13	2:40:03	26:41	01:00
B	23:00	24:31	23:29	23:00	24:00	31:47	2:29:47	24:58	01:23
C	27:29	25:53	31:17	28:40	38:22	37:19	3:09:00	31:30	02:08
D	29:04	28:57	31:39	28:48	32:30	28:30	2:59:28	29:55	00:42
E	26:14	26:40	30:44	26:35	30:42	26:41	2:47:36	27:56	00:53
F	30:47	30:37	34:45	31:02	36:21	35:18	3:18:50	33:08	01:04
G	29:55	30:29	33:29	30:07	33:46	31:16	3:09:02	31:30	00:42
H	22:46	23:01	27:11	23:05	26:37	22:49	2:25:29	24:15	00:51
I	32:30	33:26	37:55	33:01	37:15	32:38	3:26:45	34:27	01:00
J	23:41	23:24	23:55	26:56	20:24	30:59	2:29:19	24:53	01:29
AVG	27:03	27:12	30:24	27:39	31:00	30:15	2:53:32		
SE	01:05	01:06	01:27	01:03	01:52	01:25	0:07:00		

AVG; average. SE; Standard Error. Unit; [minute: second] or [hour: minute: second].

Table 3. The amount of sports drink taken by each subject

Subject	5 km	10 km	15 km	20 km	25 km	30 km	Total	AVG	SE
A	20.7	19.9	24.6	73.1	75.5	57.1	270.9	45.2	10.8
B	35.3	48.1	98.5	26.8	82.4	34.6	325.6	54.3	11.9
C	13.3	57.1	54.0	37.8	56.0	107.0	325.3	54.2	12.6
D	27.5	25.0	32.6	46.9	63.7	70.3	266.0	44.3	7.9
E	36.6	40.2	44.9	32.7	32.4	29.3	216.0	36.0	2.4
F	118.3	92.0	107.9	230.7	146.8	137.4	833.1	138.8	20.1
G	55.7	76.8	74.7	49.1	65.2	34.7	356.2	59.4	6.6
H	8.1	14.0	13.1	20.5	17.5	14.9	88.2	14.7	1.7
I	35.7	33.4	44.4	63.8	54.5	102.0	333.8	55.6	10.4
J	14.8	24.0	35.2	15.7	51.2	53.5	194.3	32.4	7.0
AVG	36.6	43.0	53.0	59.7	64.5	64.1	320.9	-	-
SE	10.1	8.1	9.9	19.9	11.0	12.6	62.4	-	-

AVG; average. SE; Standard Error. Unit; [ml]

Table 4. The hematocrit value of each subject

Subject	0 km	10 km	20 km	30 km	AVG	SE
A	46.2	48.6	47.6	48.3	47.7	0.5
B	42.5	45.8	46.3	47.3	45.5	1.0
C	45.2	48.8	47.4	46.4	47.0	0.8
D	39.2	39.2	41.2	40.6	40.1	0.5
E	45.7	48.0	47.5	47.4	47.2	0.5
F	44.6	47.8	48.4	48.5	47.3	0.9
G	44.2	49.4	49.7	49.9	48.3	1.4
H	46.0	48.6	49.4	49.1	48.3	0.8
I	45.9	47.7	48.0	48.2	47.5	0.5
J	50.6	49.0	48.3	49.1	49.3	0.5
AVG	45.0	47.3	47.4	47.5	-	-
SE	0.9	1.0	0.8	0.8	-	-

AVG; average. SE; Standard Error. Unit; [%]

Data analysis

A fingertip puncture was performed and the whole blood sample was immediately analyzed using a point-of-care test (LUCOCARD MyDIA, ARKRAY, Inc., Kyoto, Japan) for measuring the blood glucose level (GLU). For all other measurements, the antecubital vein was punctured at the measurement point without using an indwelling needle, and sampling was performed. To perform measurements before running, blood was collected after the subject had been sitting for more than 30 min, after resting for 1 h after breakfast. Blood was collected within 1 min after the subjects had covered distances of 10, 20, and 30 km. The collected blood was allowed to stand at room temperature for 30 min, after which it was centrifuged at 3000 rpm for 10 min to obtain serum. It was stored at -80 °C until measurement. The serum was used for measuring the concentrations of creatine kinase (CPK), urea nitrogen (BUN), creatinine (CRE), cystatin C (CysC), 2-thiobarbituric acid reactive substances (TBARS), and renalase. Measurements were performed at the Tsukuba i-Laboratory Limited Liability Partnership (Ibaraki, Japan) using the Japan Society of Clinical Chemistry (JSCC) standardization corresponding method for CPK, urease ultraviolet Method for BUN, and enzymatic method for CRE. The CysC level was measured by using the human cystatin C enzyme linked immunosorbent assay (ELISA) (BioVendor Laboratory Medicine, Inc., Brno, Czech Republic). The TBARS level was

measured using the thiobarbituric acid method. The renalase level was measured using the FAD-Dependent Amine Oxidase ELISA Kit (Cloud-Clone Corp, Houston, USA). In addition, the estimated glomerular filtrating ratio (eGFR) was calculated using the renal function presumption formula and serum CRE and serum CysC concentrations to evaluate kidney function while running. The eGFR was calculated from serum CRE concentrations using the modification of diet in renal disorder (MDRD) equation for Japanese individuals.

$$eGFR\ (ml/min/1.73\ m^2) = 194 \times [Concentration\ of\ serum\ CRE\ (mg/dl)]^{-1.094} \times [Age]^{-0.287}$$

The body surface area (BSA) was calculated using the formula put forward by Du Bois as follows:

$$BSA\ (m^2) = body\ weight\ (kg)^{0.425} \times height\ (cm)^{0.725} \times 0.007184$$

eGFR was calculated from the serum CysC concentration using the equation for Japanese individuals, as shown in the CKD clinical practice guidelines (Japanese Society of Nephrology, CKD clinical practice guide 2012). The eGFR was similarly calculated using the following formula:

$$eGFR\ (ml/min/1.73\ m^2) = (104 \times [Concentration\ of\ serum\ CysC\ (mg/dl)]^{-1.019} \times 0.996^{(Age)}\text{-}8$$

Statistical analysis

Statistical analysis was performed using SPSS software (IBM SPSS Statistics Version 22, SPSS, Tokyo, Japan). The Kolmogorov–Smirnov normality test was used to compare the values before and after running. One-way repeated-measures analysis of variance and the post hoc test were used if values had a normal distribution, and the Kruskal–Wallis test was used when values did not have a normal distribution. Furthermore, after verifying the significance of the correlation analysis, in order to clarify the correlation between the running distance and serum renalase concentration, the Pearson product-moment correlation coefficient or Spearman's rank correlation coefficient was calculated. Values were considered to be statistically significant if $P < 0.05$.

3) Results

Physiological response during running

The rate of increase in serum CPK concentration is shown in Figure 1. The one-way repeated-measures analysis of variance ($P < 0.01$) showed a significant difference in values. The glomerular filtration value was estimated (eGFR-CysC) using the concentration of serum CysC, which showed normality ($P > 0.05$); therefore, one-way analysis of variance was performed by taking repeated measurements; it revealed a significant difference in the values ($P < 0.001$, Figure 2). Figure 3 shows the comparison between GFR-CRE and eGFR-CysC, based on the running distance. Though both

parameters evaluate kidney function, they showed different trends. The rate of increase of TBARS levels is shown in Figure 4. Because the rate of increase of TBARS levels did not show a normality of distribution ($P < 0.0001$), the Kruskal–Wallis test was used for analysis, which revealed a significant difference in values ($P < 0.01$). Because the rate of increase of GLU did not show a normal distribution ($P = 0.031$), the Kruskal–Wallis test was used for analysis, which revealed that there were no significant differences ($P = 0.051$).

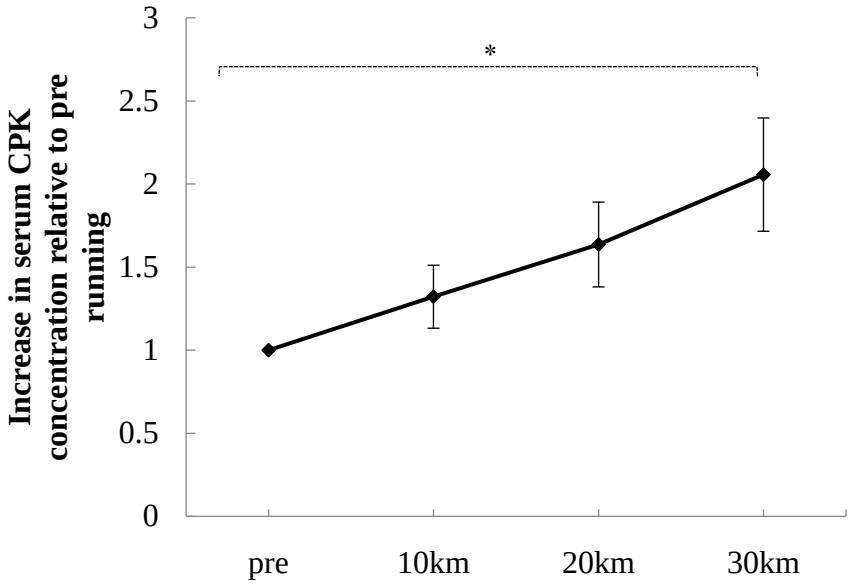


Figure 1. Rate of increase in serum CPK concentration

The rate of increase in serum creatine kinase (CPK) concentration. The one-way repeated-measures analysis of variance method is used for analysis. *P < 0.01

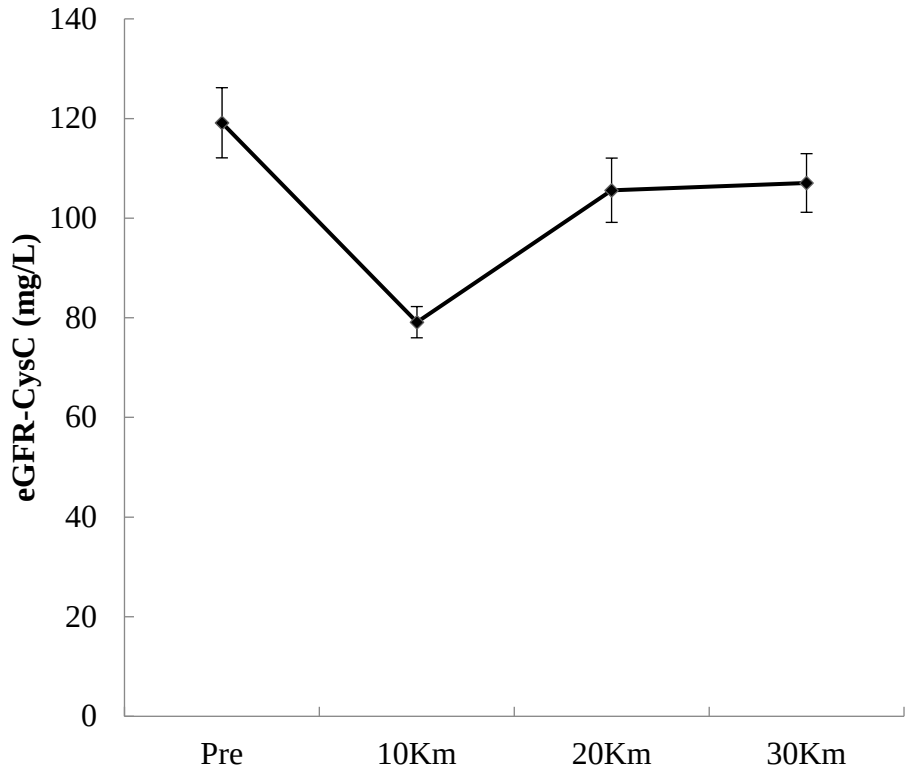


Figure 2. Changes in eGFR-CysC

eGFR-CysC; the estimated glomerular filtrating ratio (eGFR), determined using the serum cystatin C (CysC) concentration. The one-way analysis of variance method is used for analysis. *P < 0.001

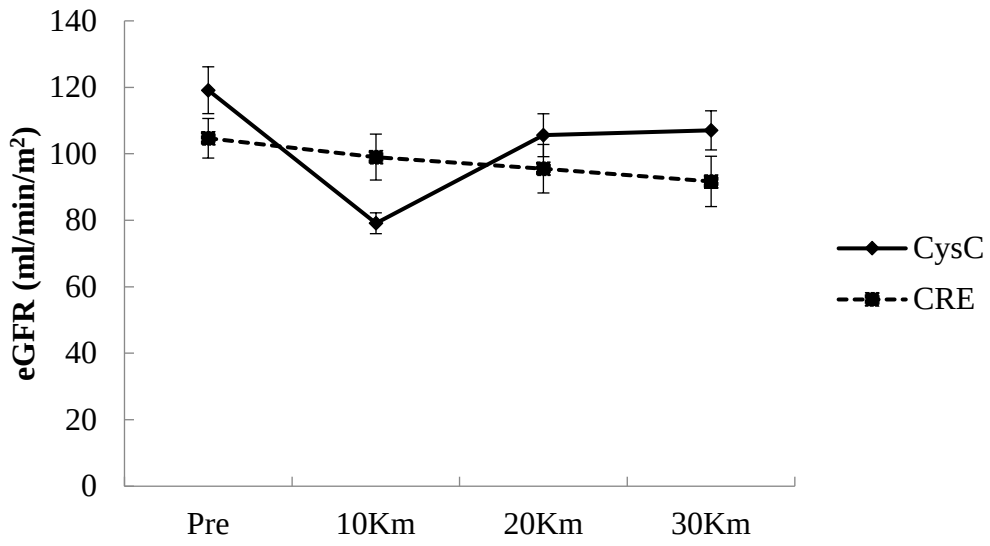


Figure 3. Comparison of eGFR-CRE and eGFR-CysC

Comparison between the estimated glomerular filtrating ratio determined using the serum cystatin C (eGFR-CysC) concentration and the estimated glomerular filtration ratio determined using the serum creatinine (eGFR-CRE) concentration.

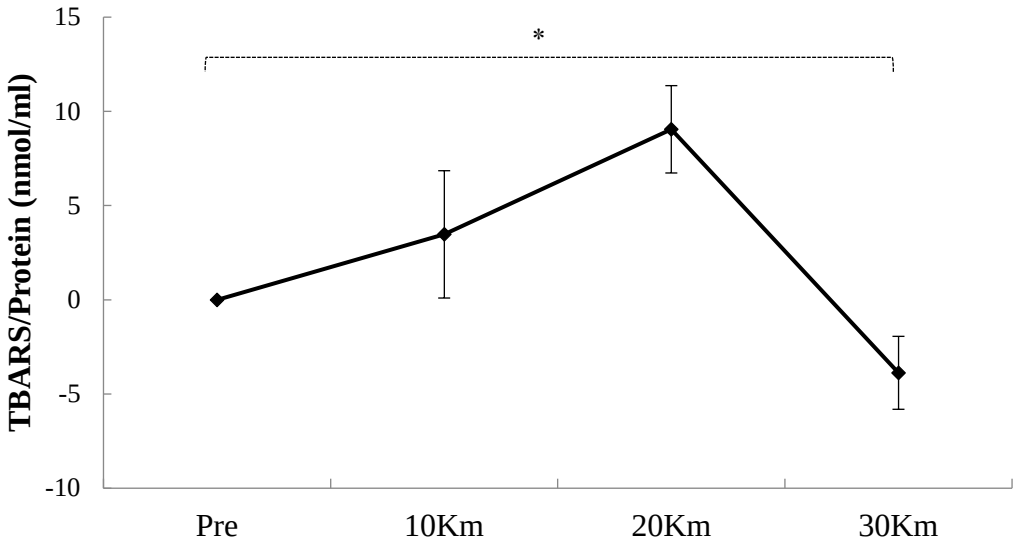


Figure 4. Rate of increase of TBARS

The rate of increase of 2-thiobarbituric acid reactive substances (TBARS). The Kruskal-Wallis test is used for analysis. *P < 0.01

Change in serum renalase concentration.

Changes in serum renalase levels with different running distances are shown in Figure 5. Because the serum renalase concentration showed normality of distribution ($P > 0.05$), one-way analysis of variance was performed using repeated measurements, which showed a significant difference in values ($P < 0.01$). In addition, the post hoc test revealed a significant increase in renalase levels at 10 km and 20 km compared with that before exercise ($P < 0.01$ and $P < 0.01$, respectively).

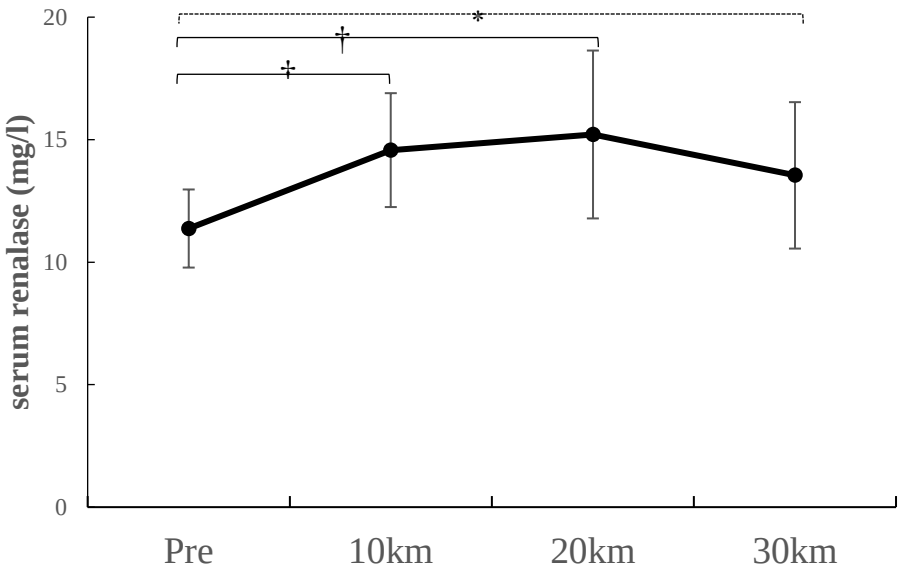


Figure 5. Concentration of serum renalase

Changes in serum renalase concentrations according to different running distances. The one-way repeated-measures analysis of variance (*P < 0.01) and post hoc test are used for analysis. (†P < 0.05)

Relationship between serum renalase level, renal function, and oxidative stress.

We examined the correlation between the serum renalase concentration, eGFR-CRE, and eGFR-CysC. A significant correlation was seen only with eGFR-CysC (Figure 6) (P >0.05 and P >0.01, r = −0.479, respectively). To determine the association between serum renalase concentration and oxidative stress, the correlation between the serum renalase concentration and rate of increase of the TBARS level was analyzed. There was no correlation between the results before running and after running 30 km, but a significant positive correlation was observed between the results obtained during the period before the subjects ran the 10-km run to that after running, and the period before the subjects ran the 20-km run to that after running (Table 5).

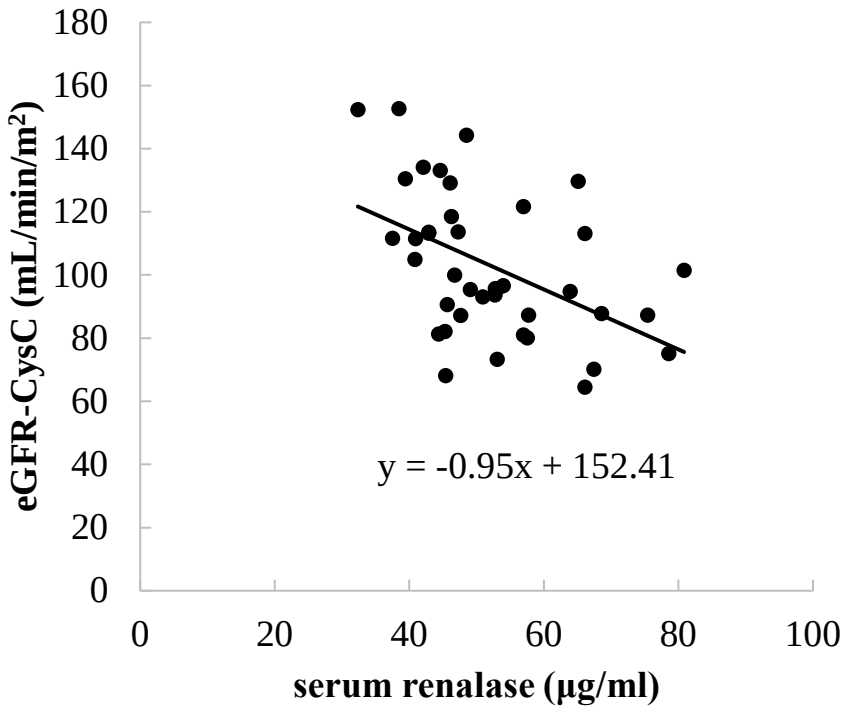


Figure 6. Correlation between renalase and eGFR-CysC

Correlation between serum renalase concentration and the estimated glomerular filtrating ratio using the concentration of serum cystatin C (eGFR-CysC). The Spearman's rank correlation coefficient was used for analysis. P (probability) < 0.01, r (correlation coefficient) = -0.479. There was a significant negative correlation between serum renalase concentration and eGFR-CysC.

Table 5. Correlation between renalase and TBARS levels (Spearman's rank correlation coefficient)

	0 - 10 km	0 - 20 km	0 - 30 km
significance probability (two - sided test)	0.003*	0.009*	0.081
correlation coefficient	0.621	0.468	-

Correlation between serum renalase and 2-thiobarbituric acid reactive substances (TBARS) levels based on the running distance. The Spearman's rank correlation coefficient is used for analysis. *P < 0.01

4) Discussion

The present study is the first to investigate the change in serum renalase concentration during exercise in humans. In a previous study conducted using rats (Czarkowska-Paczek et al, 2013), exercise was performed at a speed of 28 m/min for 60 min; no significant change was observed in the serum renalase concentration. On the other hand, in this study, the serum renalase concentration increased significantly while running long distances of 10 km and 20 km, with the exercise intensity set to 90% VT. Distance running for a long period of time might have caused the serum renalase concentration to significantly increase in in this study, which could not be demonstrated in the study by Czarkowska-Paczek et al (Czarkowska-Paczek et al, 2013). In other words, unless the exercise involves a certain amount of physical burden, changes in serum renalase concentration might not be observed because of homeostasis.

In the *in vitro* study performed by Wang et al, the addition of catecholamines to renal cells increased renalase secretion and mRNA expression (Wang et al, 2014). In addition, in the *in vivo* studies conducted by Li G, the parenteral administration of catecholamines to chronic kidney disease model rats significantly increased renalase activity and blood renalase levels (Li G et al, 2008). In these previous studies, the increase in catecholamine levels increased blood renalase levels because of a physiological response. In the present study, it was presumed that the catecholamine level increased due to exercise, which

suggests that the serum renalase concentration increased. In addition, catecholamine levels might contribute to the lack of a significant difference in the serum renalase concentration after 30 km relative to that prior to exercising. At around 20 km, the catecholamine level might not increase; it might have stabilized at a certain level, or declined with exercise. Catecholamines might have been mobilized to degrade liver glycogen. Renalase plays a role in the metabolism of catecholamines as monoamine oxidase; on the other hand, the expression and secretion of renalase is promoted by catecholamines. Although the mechanism of action is still unknown, renalase and catecholamine levels are controlled and they appear to regulate homeostasis. Therefore, if renalase is deficient in a subject, catecholamines are not metabolized, and their levels become excessive, which is thought to raise the blood pressure (Desir et al, 2012). However, the increase in blood pressure is not only due to the excessive level of catecholamines that were not degraded. If an increase in catecholamine levels does not induce renalase expression or secretion, it might result in a problem. Alternatively, a substance might inhibit the expression and secretion of renalase, as reported by Li G. It was not possible to measure the parameters related to the plasma catecholamine concentration and blood pressure; hence, the relationship between renalase and catecholamines during exercise is unclear. We need to clarify this point in research

conducted in the future. The serum CPK concentration, which is an indicator of tissue damage after exercise, was significantly increased with an increase in the running distance. Similarly, the serum CRE concentration also increased significantly with an increase in the running distance. However, serum CRE and CysC concentrations, which are indicators of renal function, did not show a similar trend. CRE is synthesized from the metabolism of creatine, which is the energy source for muscles; therefore, its level increases with exercise in a manner similar to that of the serum CPK concentration. Although the serum CRE concentration indicates the kidney function when the subject is at rest, it cannot be said that the process of CRE production indicates the kidney function at the time of exercise. Conversely, CysC is a serum protein that is produced and secreted by somatic cells, and the amount produced is constant. In addition, it is less susceptible to extracellular influences such as inflammation because it encodes a house-keeping gene. Because CysC secreted extracellularly is reabsorbed only in the proximal renal tubules of the kidney, the concentration of CysC in the blood depends on the glomerular filtration value (Freije et al, 1991). Therefore, it is a measurable parameter that is suitable for evaluating kidney function during exercise. A similar opinion was also expressed in a previous study that evaluated kidney function during exercise (Shlipak et al, 2005; Coll et al, 2000; Pucci et al, 2007). In this study, a significant negative correlation was

observed between the serum renalase concentration and eGFR-CysC, i.e., as the serum renalase concentration increased, the eGFR-CysC value, which is an indicator of kidney function, decreased. A significant positive correlation was found between the rate of increase in TBARS level and serum renalase concentration in the period before the subjects ran the 10-km run to that after running, and the period before the subjects ran the 20-km run to that after running. Though the TBARS level increased at a certain rate and serum renalase level decreased, no correlation was found in the period after the subjects ran the 20-km run to that after they ran the 30-km run. Although the effect of antioxidants could not be measured in this study, it could be a possible cause of the decrease in the TBARS level (Kayatekin et al, 2002; Tong et al, 2016). Li et al showed that there was a significant increase in renalase expression with the increase in oxidative stress in a mice model of ischemia–reperfusion injury, and that it was suppressed by antioxidants (Li et al, 2016). In addition, it has been reported that the renalase level increases during ischemia–reperfusion and has a protective effect on organs (Lee et al, 2016; Yin et al, 2016; Wu et al, 2011). There was also a significant increase in oxidative stress in this study, which is presumed to be because of ischemia of an organ or organ damage due to exercise (Niemelä et al, 2016). Therefore, it is speculated that an increase in the serum renalase concentration due to exercise might also contribute to protection against organ damage due to exercise.

IV. Research task 2: Animal model research

"Changes in renalase levels observed using a rat moderate treadmill running model."

1) Aim

The results of research task 1 show that the running exercise load significantly increases the concentration of renalase in the blood. Therefore, animal model studies were conducted with the purpose of clarifying the mechanism by which the renalase level fluctuates from the perspective of genetic expression.

Wistar male rats were used as animal models, because they could withstand the running exercise load. Then, the running exercise load was set to medium strength, and it was equivalent to that used for human subject research in research task 1.

The kidney is the main expression organ of renalase (Xu J et al, 2005). However, in research task 1, the exercise load caused deterioration in kidney function, and there was a negative correlation between renalase concentration in the blood and renal function.

Therefore, in research task 2, the following hypothesis was formulated: The expression of the renalase gene, which causes the renalase concentration in the blood to increase because of the exercise load, occurs not only in the main expression organ, the kidney, but also in the skeletal muscles.

2) Materials and Methods

Animals

Male Wistar rats (Japan SLC, Inc, Shizuoka, Japan) were used for the experiments. The rats were brought in at a weight of 166 – 187 g (8 weeks). On arrival, rats were housed in a room at 20 – 26 °C, 40 – 60% humidity, and a 12 h:12 h light-dark cycle. Animals were fed a normal chow diet (MF 12 mm φ pellet, Oriental Yeast Co, Tokyo, Japan), and given water ad libitum. The Animal Ethics Committee of The University of Tsukuba approved all experimental protocols in accordance with the principles and guidelines on animal care put forward by the Physiological Society of Japan.

Moderate Exercise

All rats were familiarized with a motor-driven horizontal treadmill for 30 min (FVRO.4E9S-6, Fuji Medical Science Co., Ltd, Chiba). By giving mild electric shocks (0.8 mA) to the rats at the rear end of the treadmill, they were made to complete a 6-day long program (Table 6). The rats were distributed into two groups, i.e., the control (COT, n = 6) and moderate-intensity exercise (MEX, n = 6) groups, 48 h after the exercise practice was completed. There was no significant weight difference between the two groups of rats (Table 7). The rats were fasted 2 hours before exercise, and rested on a treadmill for 15 minutes. The COT rats were killed under the influence of anesthesia (cervical dislocation after isoflurane aspiration). The MEX rats were subjected to an acute

bout of exercise consisting of a 60-min run on the treadmill at a rate of 20 m/min. Immediately after the rats were killed, samples from the muscle, kidney, heart, liver, lung, and plasma were collected, added to EDTA, and stored at -80 °C for subsequent analyses.

Table 6. The plan for practicing rats to run

Day	Speed and Time
Day 1	rest 10 minutes, 5 m/min 10 minutes, and 10 m/min 10 minutes
Day 2	rest 5 minutes, 5 m/min 10 minutes, 10 m/min 10 minutes, and 15 m/min 10 minutes
Day 3	rest
Day 4	rest 5 minutes, 10 m/min 10 minutes, 15 m/min 10 minutes, and 20 m/min 10 minutes
Day 5	rest 5 minutes, 15 m/min 10 minutes, 20 m/min 10 minutes, and 25 m/min 10 minutes
Day 6	rest 5 minutes, 15 m/min 10 minutes, 20 m/min 10 minutes, and 25 m/min 10 minutes

Table 7. The weight of the rats at the time of experiment

	1	2	3	4	5	6	AVG	SE	P
COT	257	253	244	236	266	257	252.2	4.3	0.59
MEX	261	234	270	239	253	230	247.8	6.5	

AVG; average value, SE; standard error, P; probability value, Unit; g
COT; control group, MEX; Moderate - intensity exercise group
There was no significant difference in body weight between control group (COT) and Moderate - intensity exercise group (MEX).

Blood concentration of Renalase

The concentration of renalase in the blood was measured using plasma samples. The FAD-Dependent Amine Oxidase ELISA Kit (Cloud-Clone Corp, Houston, USA) was used for measurement.

Real-time PCR

The mRNA was isolated using the following method. The skeletal muscle, kidney, heart, liver, and lung tissues were homogenized in a Tissue Lyser bead mixer (Qiagen, Germany) at a frequency of 25 Hz for 2 – 5 min. Total mRNA isolation was performed using the Sepasol-RNA I Super G solution (Nacalai, Kyoto, Japan), according to the manufacturer's instructions. Total RNA concentrations were measured at 260 nm, using the ND-1000 Spectrophotometer (NanoDrop Thermo Fisher Scientific Inc., Massachusetts, USA). Samples were then frozen and stored at -80 °C for further analyses.

Reverse transcription was performed using the following method. Total RNA was reverse transcribed into cDNA using PrimeScript RT Master Mix (Perfect Real Time; TAKARA BIO INC., Siga, Japan). To quantify gene expression levels, PCR was carried out using a KAPA SYBR FAST qPCR kit (Kapa Biosystems, Wilmington, USA) and the Applied Biosystems 7500/7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, USA), according to the manufacturer's instructions. The designed primer

sequences have been shown below.

Gene (rat) renalase

Forward: 5'-TGCCAACAGTCCTCATAATCC -3'

Reverse: 5'-TCCTTCCTTCACTTCCATTCC-3'

Gene (rat) GAPDH

Forward: 5'-GGAAACCCATCACCATCTTC-3'

Reverse: 5'-GTGGTTCACACCCATCACAA -3'

The cycling program involved preliminary denaturation at 95 °C for 20 seconds, followed by 40 cycles of denaturation at 95 °C for 3 seconds, and annealing and elongation at 60 °C for 3 seconds. Then, it was confirmed by melting curve analysis that non-specific by-products were not contained in the PCR product. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal normalizer control for mRNA. The measured values were analyzed by using the calibration curve method.

Western Blotting

The BCA assay kit (TAKARA BIO INC., Japan) was used for the quantitation of the protein content. Ten microliters of the sample (skeletal muscle samples were diluted 30 –

50 times and kidney samples were diluted 50 times) diluted in RIPA lysis buffer, and 100 μl of working solution containing reagent A: reagent B mixed in the 50: 1 ratio were added to each well of the microplate. Absorbances were measured at 562 nm after incubation for 10 min at 37 °C using a microplate reader (Varioskan LUX, Thermo Fisher Scientific, Japan). Proteins were separated by using SDS- PAGE, and transferred to a polyvinylidene fluoride membrane (GE Healthcare Life science, Germany). The membranes were blocked with 5% skim milk in TBS-T (0.1% Tween 20) added to 5% Blocking One (Nacalai Tesque, Japan) solution for ~30 – 180 min, and were incubated with some primary antibodies overnight at 4 °C while shaking. The membranes were washed for 5 min thrice in TBS-T, and incubated with HRP-conjugated secondary antibodies at room temperature (25 °C) for 60 min. The membranes were washed for 5 min thrice in TBS-T, treated with chemiluminescent reagent (PerkinElmer, NEL103001EA), and before the analytes were detected using ImageQuant LAS-4000 (GE Healthcare Life science, Japan). The signals were analyzed using JustTLC (Sweday). All primary and secondary antibodies were diluted 1: 1000 and 1: 10000 times, respectively, using TBS-T added to 5% Blocking One. Renalase (Abcum, ab178700; Cloud-Clone Corp., LAC845Hu71) and GAPDH (SANTA CRUZ, sc-365062) levels were measured. GAPDH was used as an internal standardization control and analyzed.

Statistical analysis

Statistical analysis was conducted using SPSS statistical software (version 24.0; SPSS Inc., Chicago, Illinois, USA). The data were subjected to the t-test for comparing the two groups. P values below 0.05 were considered to be significant.

3) Results

Change in plasma renalase concentration

The concentration of renalase in the plasma collected from rats was measured. The results are shown in Figure 7. The control (COT group) and the medium intensity exercise (MEX group) groups were compared using the t-test. The results showed a significant increase in the renalase concentration in the MEX group, as compared to that in the COT group ($P = 0.047$).

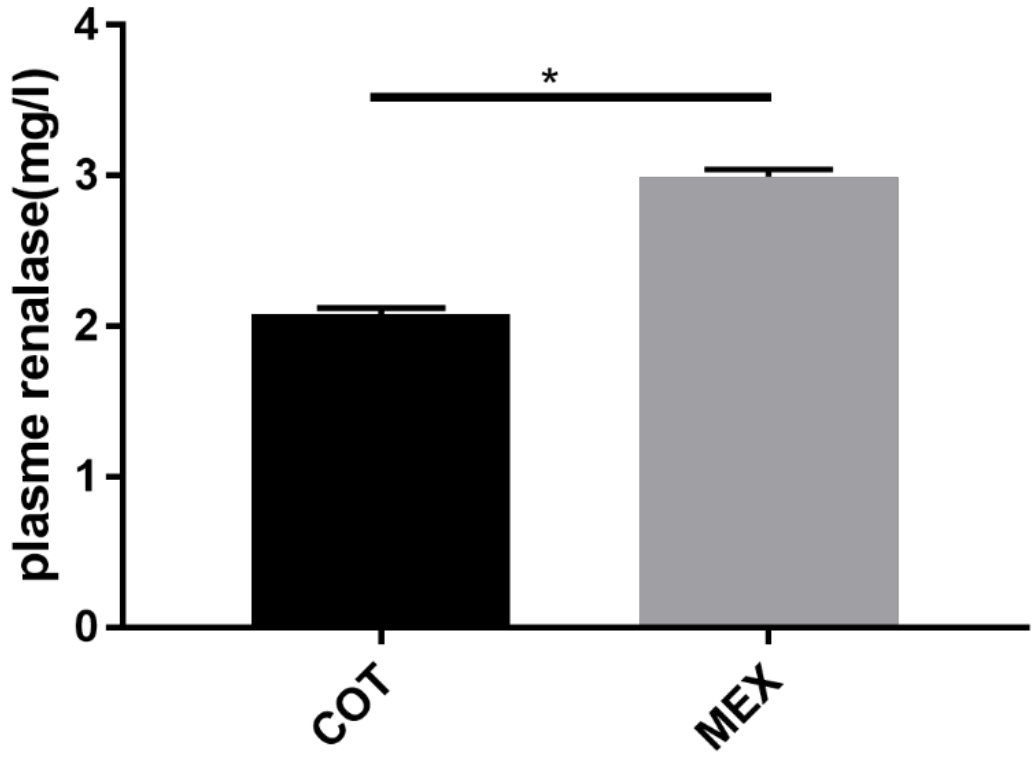


Figure 7. Renalase concentration in plasma
Comparison of renalase concentrations in plasma obtained from moderate-intensity exercise (MEX) and control (COT) groups. The t-test is used for analysis. There was a significant difference in the values (P = 0.047).

Expression of renalase mRNA

Initially, the levels of renalase mRNA expressed in the kidney, heart, liver, lung, adrenal gland, and skeletal muscle were examined in COT group rats using real-time PCR. Real-time PCR is a type of quantitative RT-PCR, and the analysis was performed by using the calibration curve method. The results showed that mRNA expression levels of the renalase gene differed in each organ and skeletal muscle (Figure 8).

Next, the change in the mRNA expression of the renalase gene, owing to the exercise load, was investigated. The levels of the COT and MEX groups were compared in the kidney, heart, liver, lung, adrenal gland, and skeletal muscle. The results showed that there were no significant differences in the heart (Figure 9), liver (Figure 10), lung (Figure 11), adrenal glands (Figure 12), and visceral organs. However, in the kidney, there was a significant decrease in the mRNA expression level in the MEX group, as compared to the COT group (Figure 13). With regard to the skeletal muscles, there were no significant differences observed in the soleus (Figure 14) and plantaris muscles (Figure 15). However, there was a significant increase in the renalase mRNA level in the extensor digitorum longus muscle in the MEX group, as compared to that in the COT group (Figure 16).

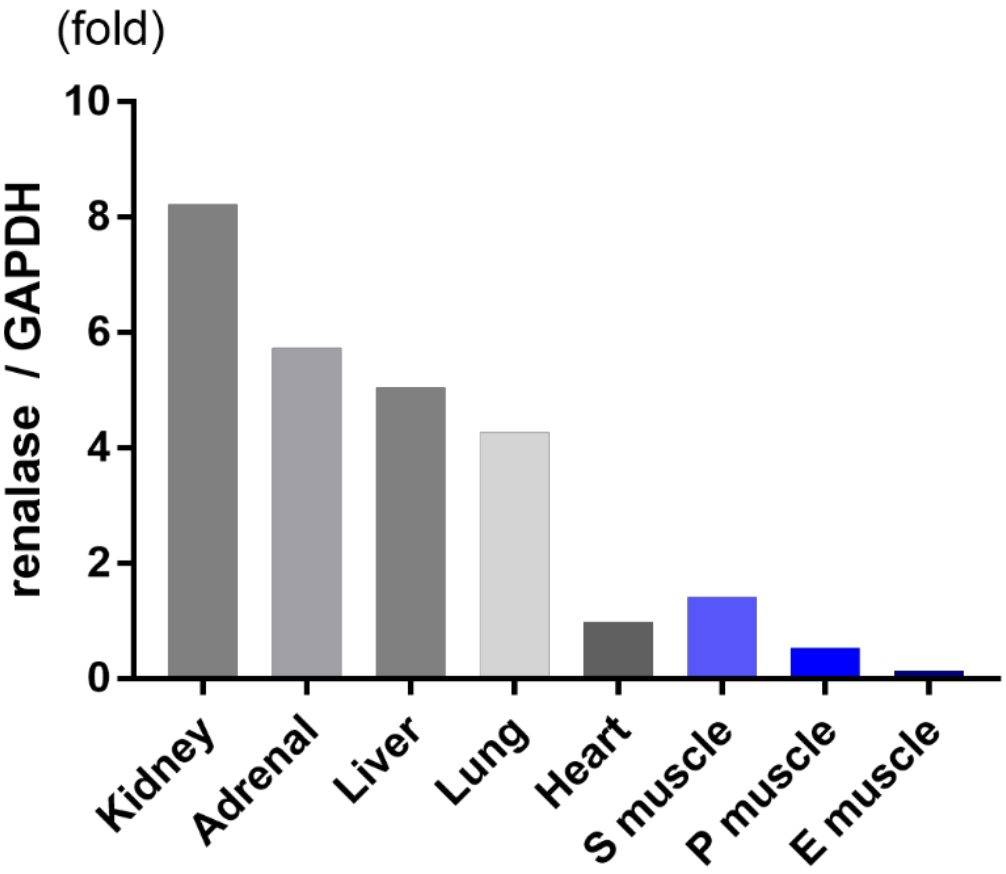


Figure 8. Renalase mRNA expressed by each organ and skeletal muscle
Differences in renalase mRNA expression were compared between organs and skeletal muscles, and the differences were confirmed.
S muscle: Soleus muscle, P muscle: Plantaris muscle, E muscle: Extensor digitorum longus muscle.

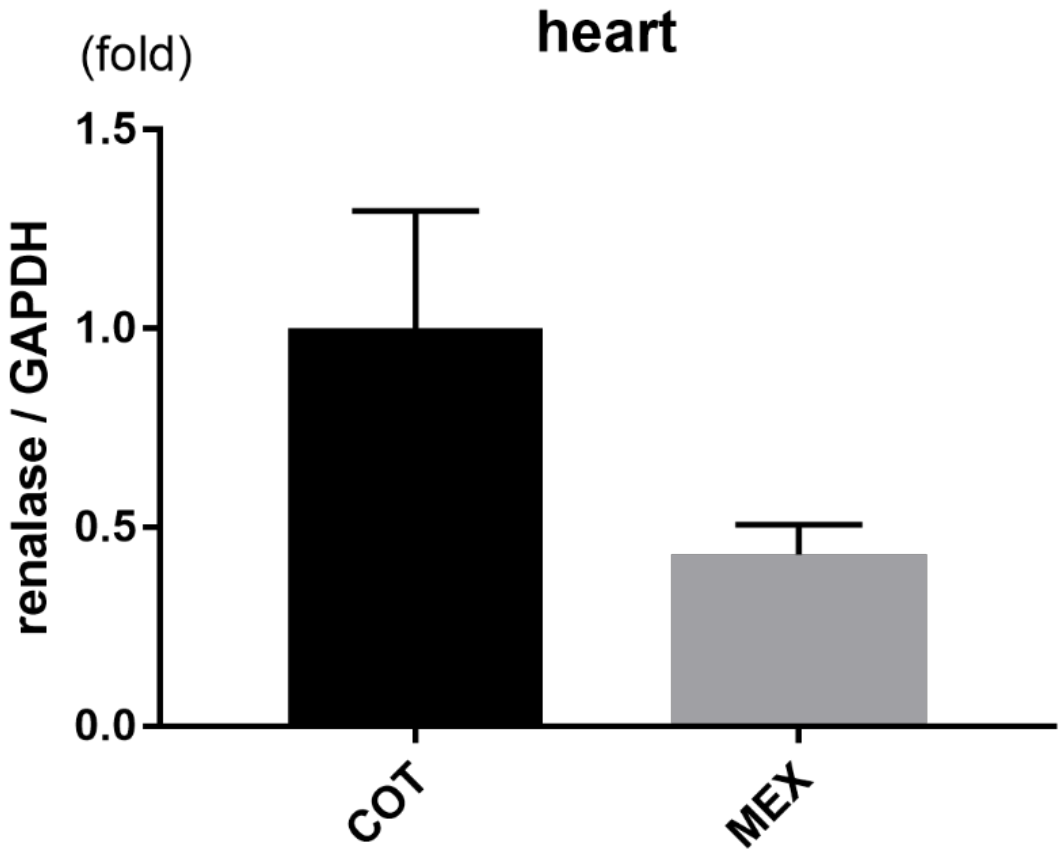


Figure 9. Renalase mRNA expression in the heart
Comparison of the renalase mRNA expression level in the hearts of rats in the moderate-intensity exercise (MEX) and control (COT) groups. The t-test (*P < 0.05) is used for analysis. No significant differences are observed (P = 0.092).

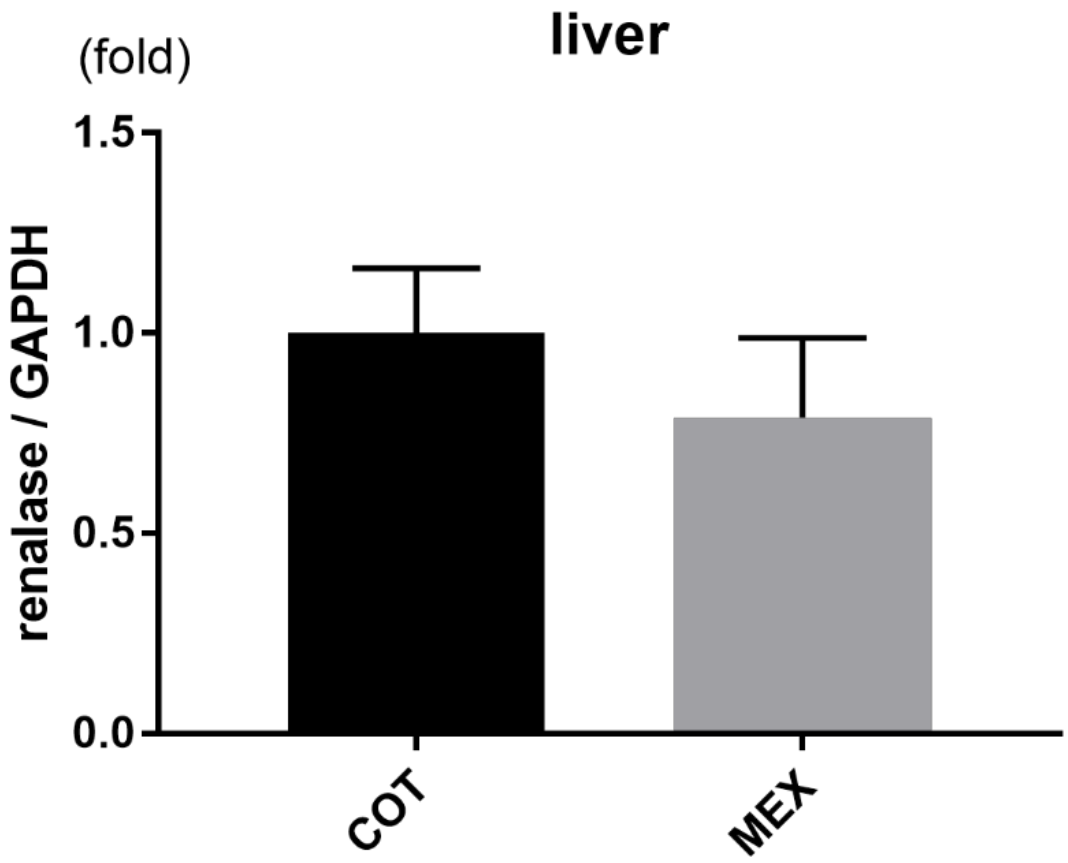


Figure 10. Renalase mRNA expression in the liver
Comparison of renalase mRNA expression in the liver of rats in the moderate-intensity exercise (MEX) and control (COT) groups. The t-test (*P < 0.05) is used for analysis. No significant differences are observed (P = 0.434).

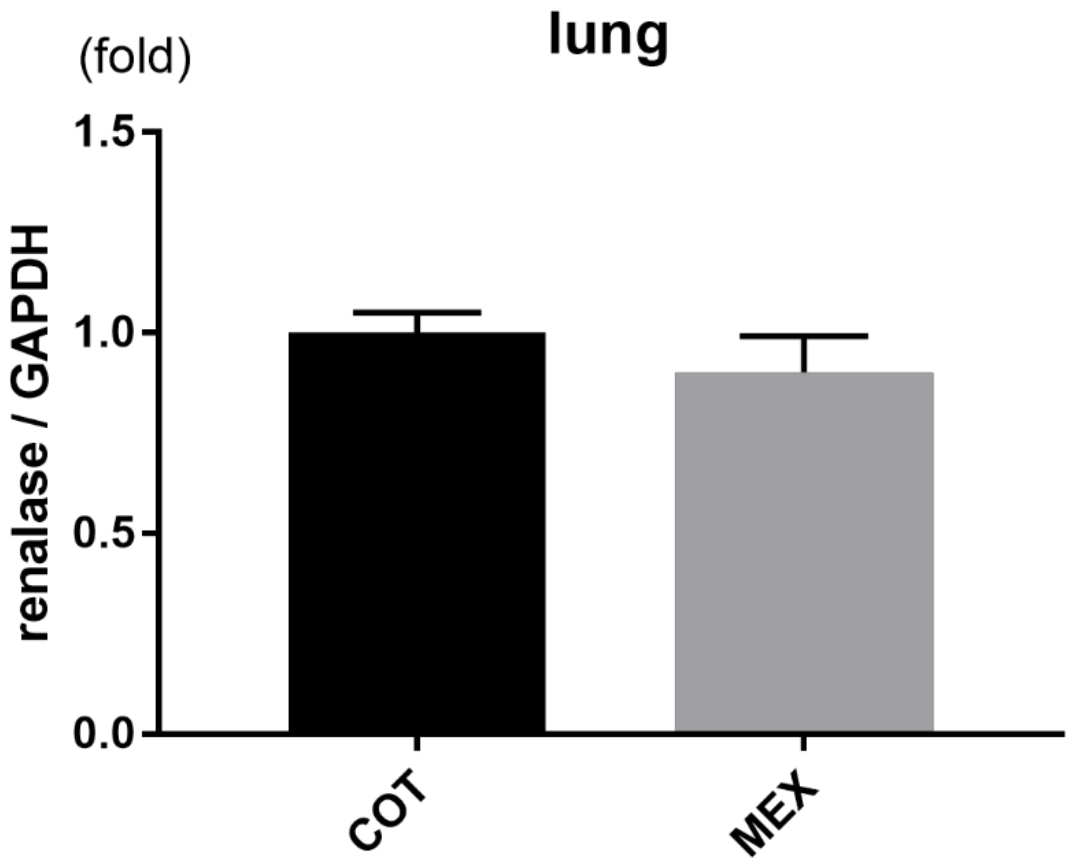


Figure 11. Renalase mRNA expression in the lung
Comparison of renalase mRNA expression in the s of rats in the moderate-intensity exercise (MEX) and control (COT) groups. The t-test (*P < 0.05) is used for analysis. No significant differences are observed (P = 0.368).

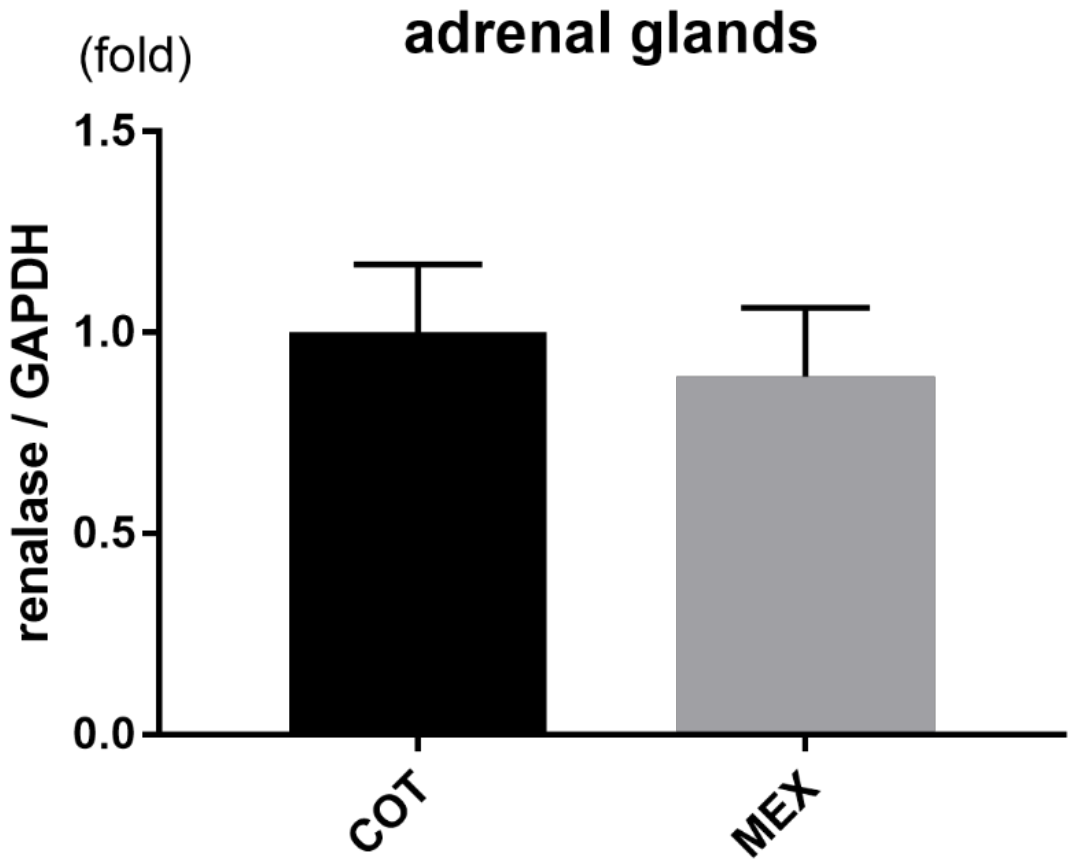


Figure 12. Renalase mRNA expression in the adrenal glands
Comparison of renalase mRNA expression in the adrenal glands of rats from the moderate-intensity exercise (MEX) and control (COT) groups. The t-test (*P < 0.05) is used for analysis. No significant differences are observed (P = 0.656).

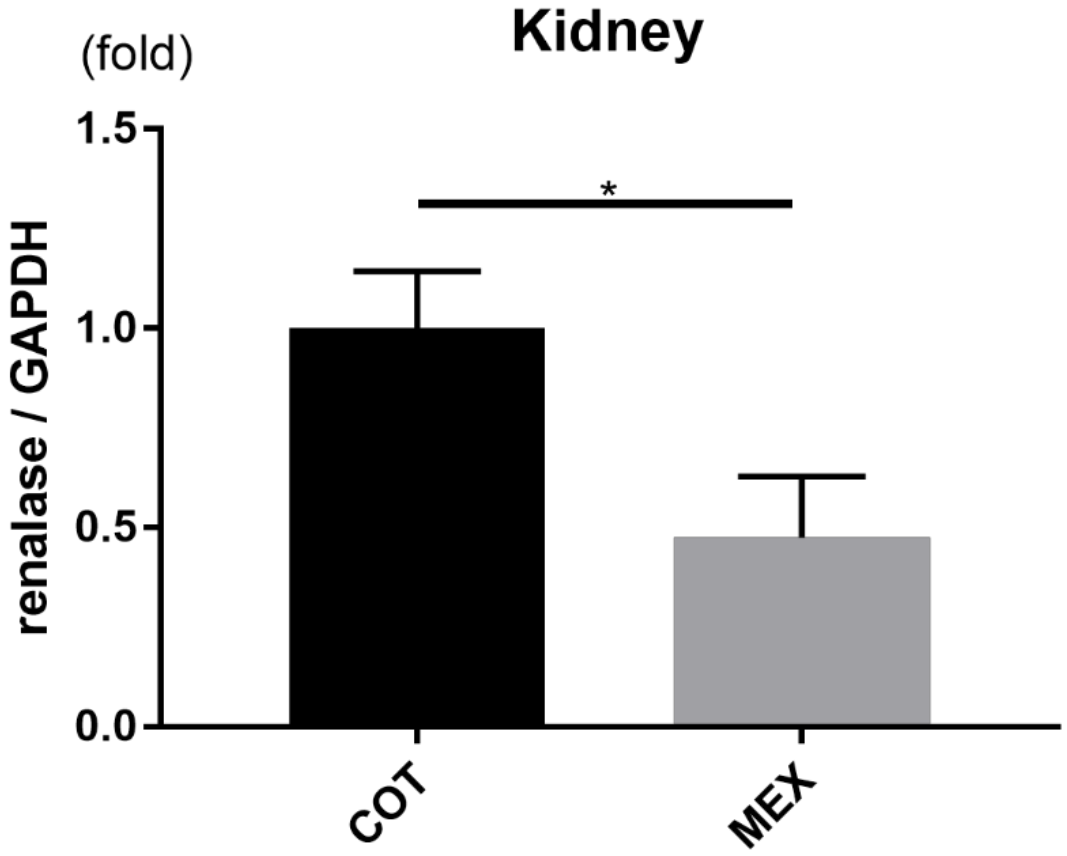


Figure 13. Renalase mRNA expression in the kidney
Comparison of renalase mRNA expression in the kidneys of rats of the moderate-intensity exercise (MEX) and control (COT) groups. The t-test (*P < 0.05) is used for analysis. There was a significant difference in the values (P = 0.032).

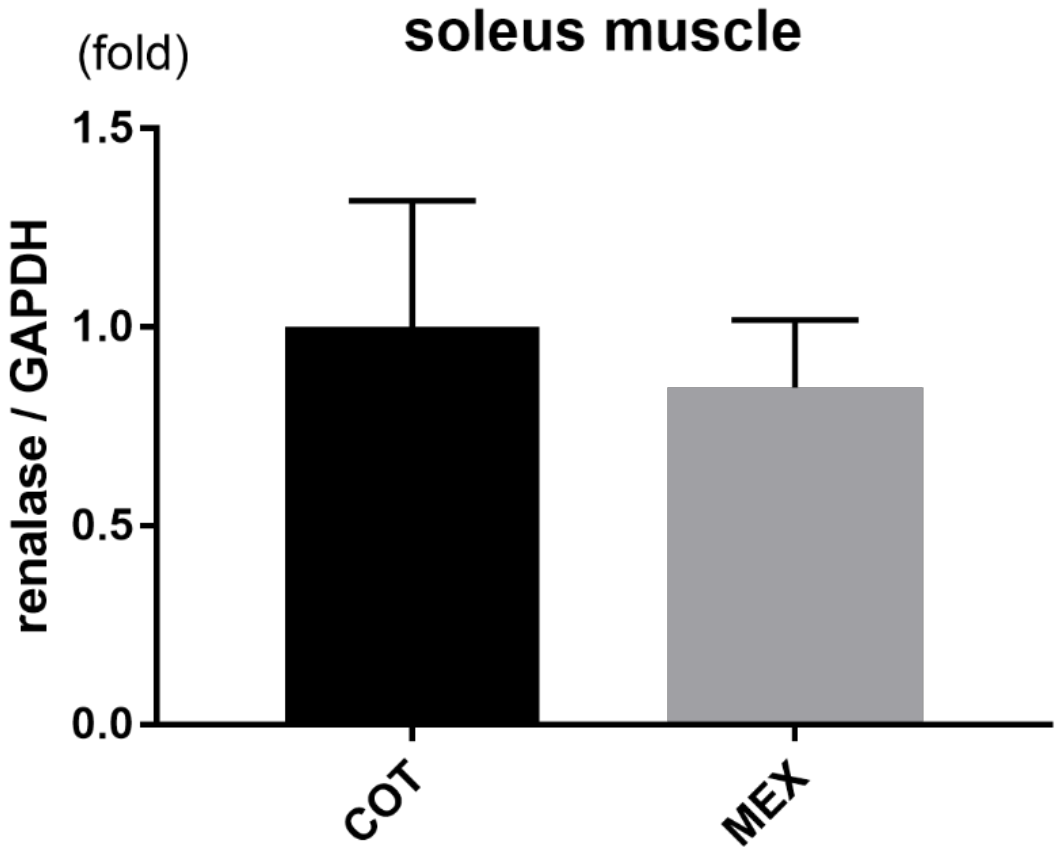


Figure 14. Renalase mRNA expression in the soleus muscle
Comparison of renalase mRNA expression in the soleus muscle of rats of the moderate-intensity exercise (MEX) and control (COT) groups. The t-test (*P < 0.05) is used for analysis. No significant differences were observed (P = 0.683).

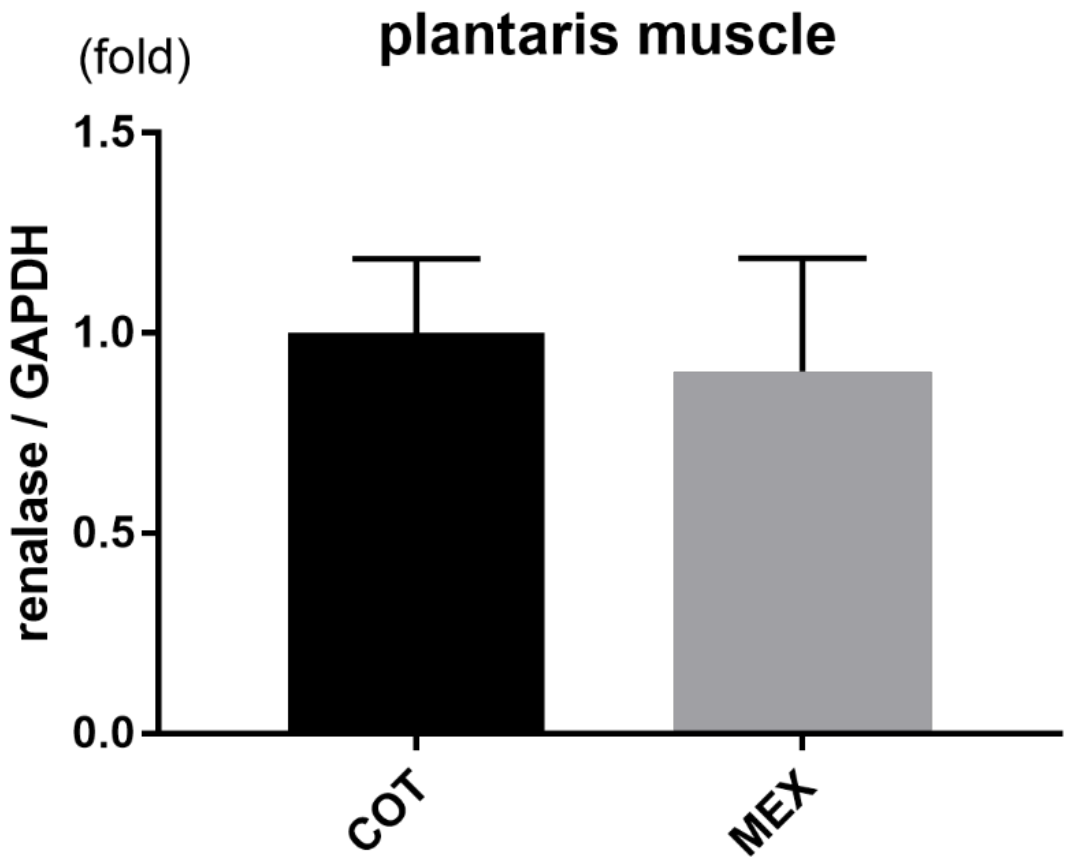


Figure 15. Renalase mRNA expression in the plantaris muscle
Comparison of renalase mRNA expression in the plantaris muscle of rats of the moderate-intensity exercise (MEX) and control (COT) groups. The t-test (*P < 0.05) is used for analysis. No significant differences were observed (P = 0.782).

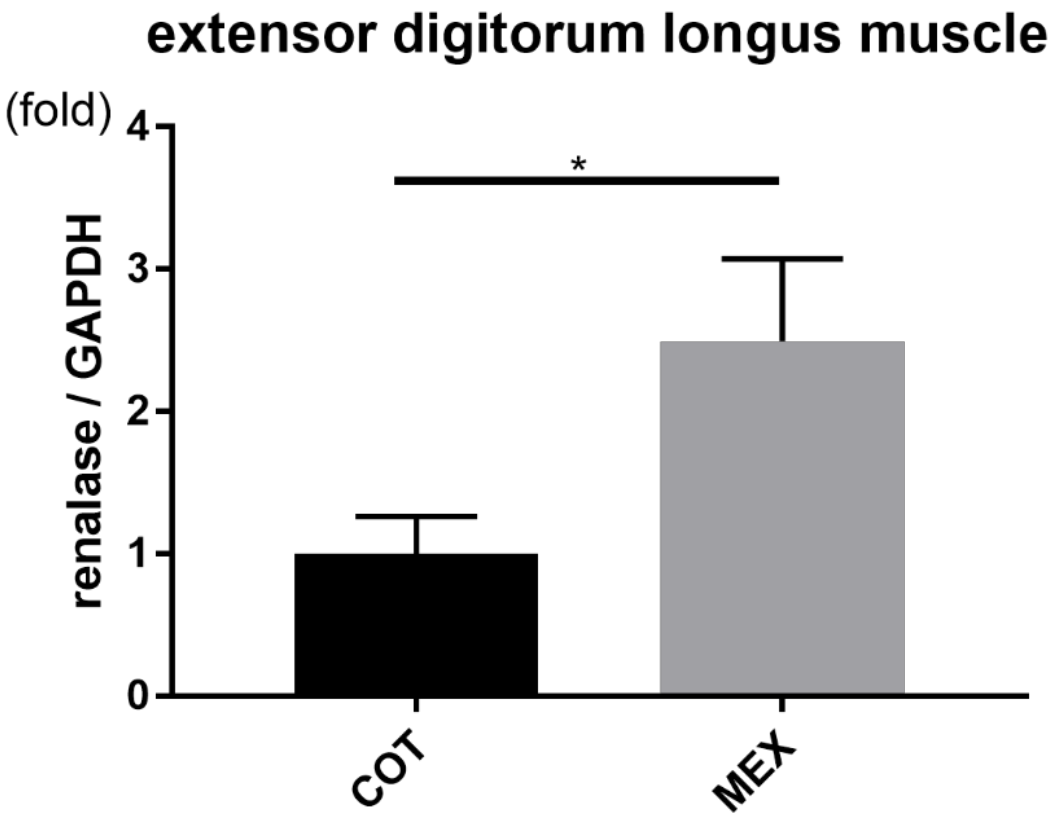


Figure 16. Renalase mRNA expression in the extensor digitorum longus muscle
Comparison of renalase mRNA expression in the extensor digitorum longus muscles of rats from the moderate-intensity exercise (MEX) and control (COT) groups.
The t-test (*P < 0.05) is used for analysis. There was a significant difference in the values (P = 0.041).

Expression of renalase protein

The expression of renalase protein was investigated in and skeletal muscles that had showed fluctuations in the level of renalase mRNA expressed for the running exercise load. The results showed that in the kidneys of rats in the MEX group, the renalase level decreased significantly, as compared to that in the COT group (Figure 17). With regard to the skeletal muscles, there was no significant difference in the renalase levels in the soleus (Figure 18) and extensor digitorum longus muscles (Figure 19). However, the renalase level was more significantly increased in the plantaris muscles of rats from the MEX group, as compared with those of the COT group (Figure 20).

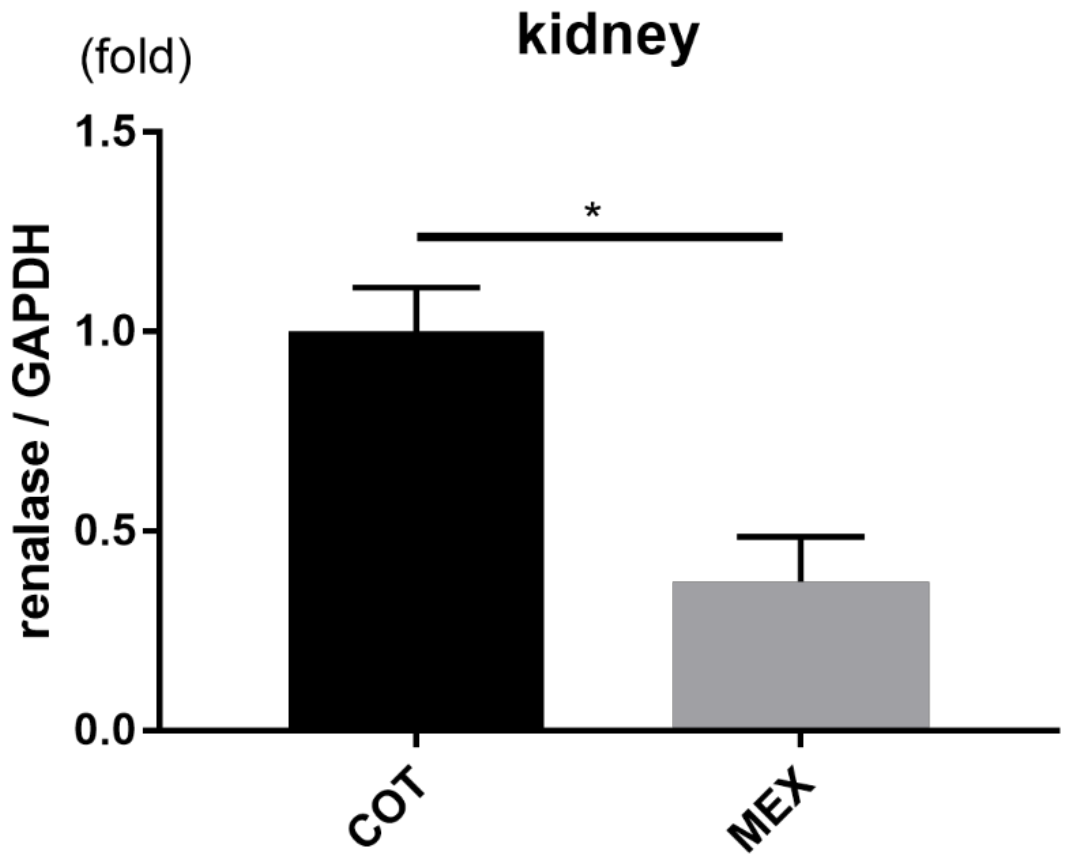


Figure 17. Renalase protein expression in the kidney

Comparisons of renalase protein expression in the kidneys of rats from the moderate-intensity exercise (MEX) and control (COT) groups. The t-test (*P < 0.05) is used for analysis. There was significant difference in values (P = 0.012).

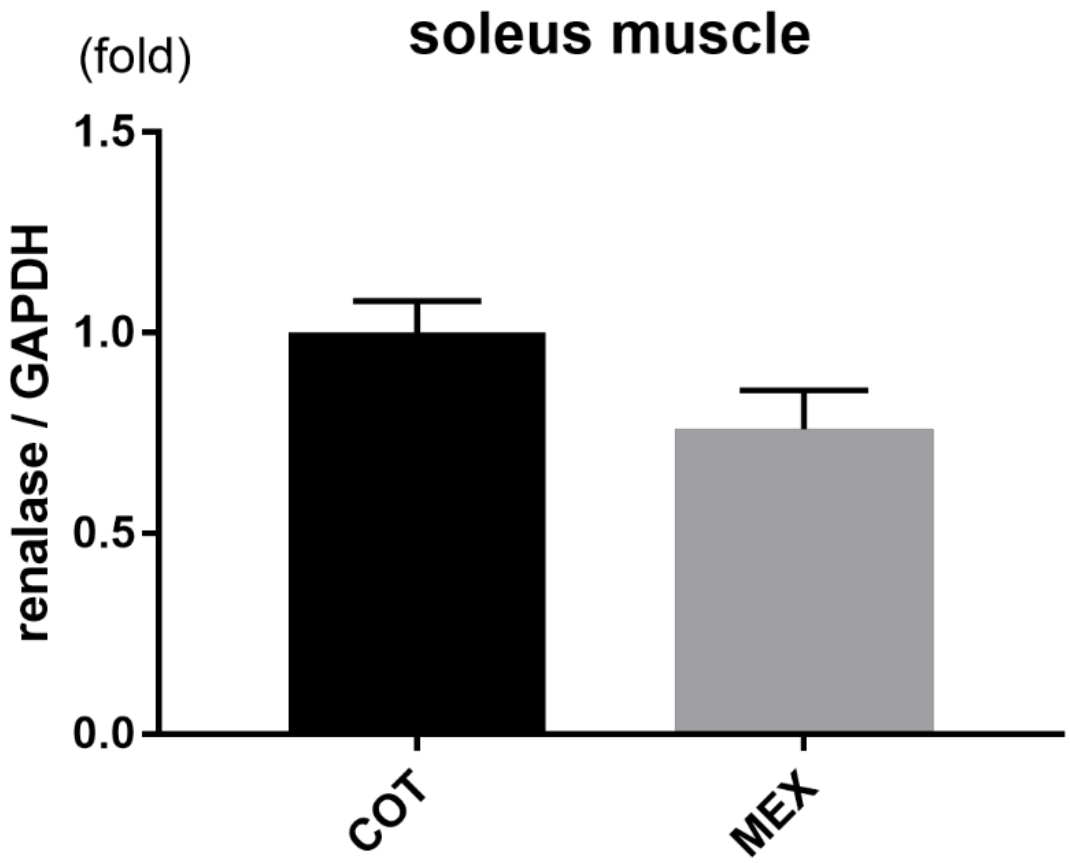


Figure 18. Renalase protein expression in the soleus muscle

Comparison of renalase protein expression in the soleus muscles of rats from the moderate-intensity exercise (MEX) and control (COT) groups. The t-test (*P < 0.05) is used for analysis. No significant differences were observed (P = 0.095).

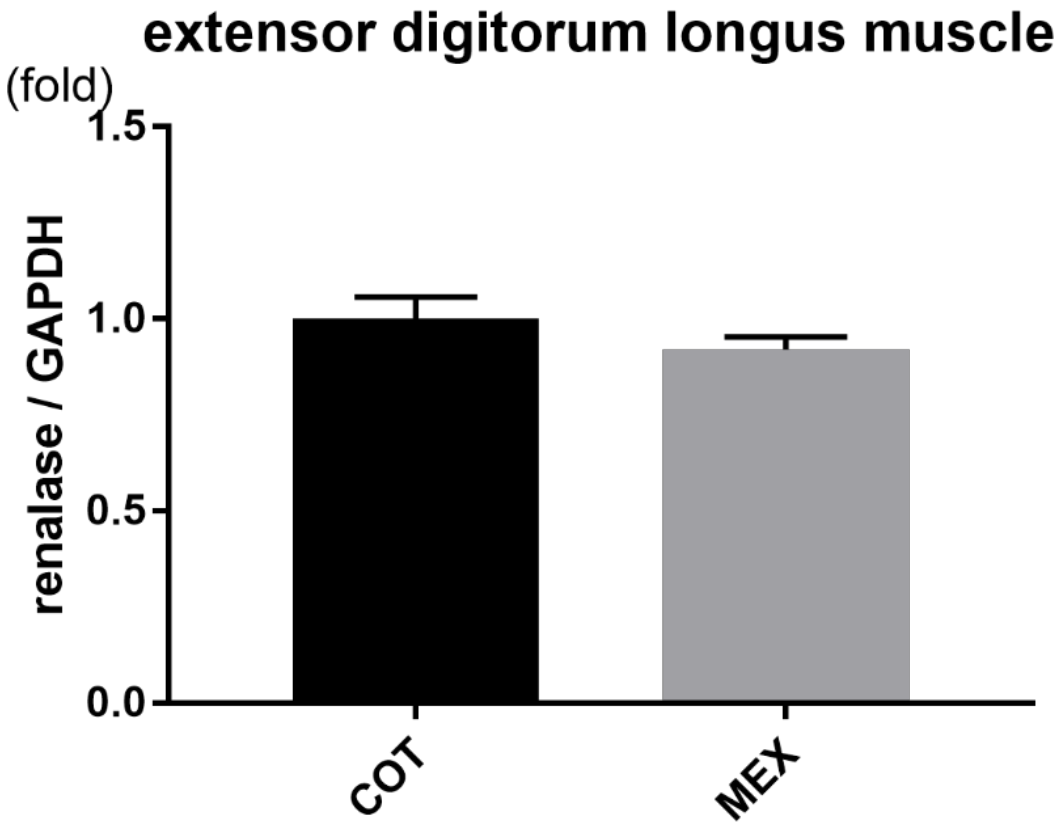


Figure 19. Renalase protein expression in the extensor digitorum longus muscle
Comparisons of renalase protein expression in the extensor digitorum longus muscle between moderate-intensity exercise (MEX) and control (COT). The analysis is t-test (*P < 0.05). No significant differences were observed (P = 0.552).

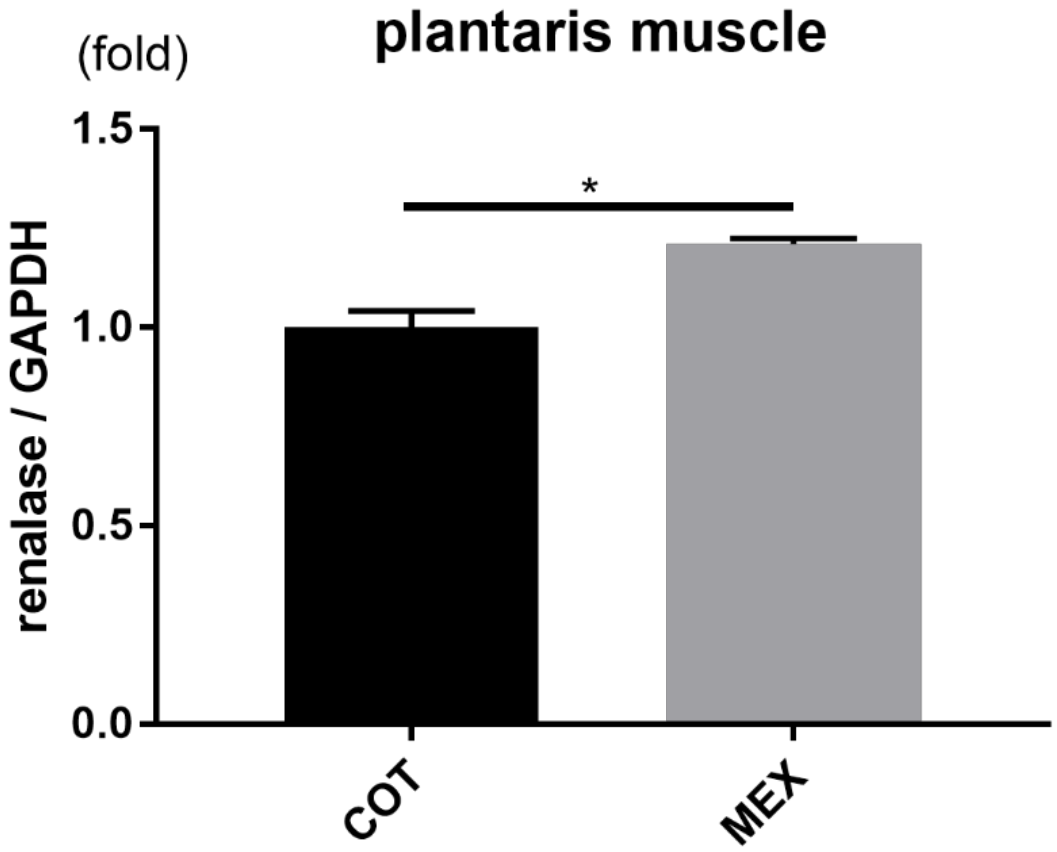


Figure 20. Renalase protein expression in the plantaris muscle

Comparison of renalase protein expression in the plantaris muscles of rats from the moderate-intensity exercise (MEX) and control (COT) groups. The t-test (*$P < 0.05$) is used for analysis. There was a significant difference in the values ($P = 0.014$).

4) Discussion

In this study, the exercise-induced expression of renalase in a rat model, using an exercise load equivalent to that used in a human subject experiment, was investigated. In the study using rat models, as in human subject experiments, the concentration of renalase in the blood was significantly increased by exercise. The purpose of this study is to investigate exogenous renalase expression in visceral and skeletal muscle.

The results showed that the levels of renalase in the blood also increased significantly in the animal models used in this study, which was similar to that observed during human subject research.

In this study, the differences in mRNA expression levels of the renalase gene in visceral and skeletal muscles were investigated. It was confirmed that renalase gene expression was the most abundant in the kidney (Xu J et al, 2005). It is reported that skeletal muscles exhibit renalase gene expression. However, the expression of the renalase gene in each skeletal muscle type has not been reported. Renalase gene expression in each type of skeletal muscle in the soleus, plantaris, and extensor digitorum longus muscles was investigated for the first time in the present study. The results elucidated that renalase gene expression in the soleus muscle, which predominantly consisted of red muscle, was approximately 10 times higher than that in the extensor digitorum longus muscle, which predominantly consisted of white muscle.

Next, the exercise-induced variability of renalase gene expression in visceral and skeletal muscles was examined. The results showed that there was no significant difference in the renalase mRNA expression due to exercise in the heart, liver, lung, and adrenal glands. However, in the kidneys of rats from the MEX group, the renalase level was significantly decreased as compared with that in the COT group. With regard to skeletal muscle, there was no significant difference because of exercise induction in the plantaris and extensor digitorum longus muscles; however, the renalase level was significantly higher in the soleus muscle of rats in the MEX group than that in CON group.

In addition, the expression of renalase protein was examined in the kidney and skeletal muscles of rats in which the renalase mRNA expression was altered. The results showed that the renalase level in the kidney was significantly reduced in the MEX group as compared to that in the COT group, as observed for mRNA expression levels. With regard to skeletal muscles, there was no significant difference in the soleus and extensor digitorum longus muscles of rats in the MEX group, as compared to those in the COT group; however, a significant increase in renalase levels was observed in the plantaris muscle.

The expression of the renalase gene because of exercise was shown to be decreased in the kidney and increased in skeletal muscles. However, the expression in skeletal muscles

differed, depending on the type of muscles. The decrease in expression of the renalase gene in the kidney of rats, observed in this study, is consistent with the reduction in renal function observed in research task 1. These results also support those of a previous study (Zbroch et al, 2012), in which it was revealed that the renalase level decreases with a decrease in renal function, which is similar to the decrease in renalase level observed in renal disease patients with renal impairment. In kidney disease, the concentration of renalase in the blood decreases when renal function decreases. In the present study, the renalase gene expression in the kidney decreased because of exercise. However, the concentration of renalase in the blood increased significantly, which is presumably because of the increased renalase expression in the skeletal muscle. The reasons for this are as follows. In the in vitro study performed by Wang et al, the addition of catecholamines to renal cells caused renalase secretion and mRNA expression to increase (Wang F et al, 2014). In addition, in the in vivo studies conducted by Li et al, the parenteral administration of catecholamines to chronic kidney disease model rats caused the renalase activity and blood renalase levels to increase significantly. In these previous studies, a rise in catecholamine levels increased blood renalase levels because of some physiological effects. In addition, blood flow possibly decreases in visceral organs during exercise and increases in skeletal muscles (Standard physiology, 2000).

V. Research task 3: Cell culture experiment

"Epinephrine upregulates renalase expression in cultured C2C12 muscle cells."

1) Aim

Research tasks 1 and 2 showed that renalase concentration in the blood increased because of exercise. In research task 2, the expression of the renalase gene in was examined in the tissue of an animal model. The results showed a significant decrease in the level of both renalase mRNA and protein expressed in the kidney. In skeletal muscle, there was a significant increase in the levels of renalase mRNA and protein expressed.

There are two possible causes for the increase in the renalase level.

One is to provide enhanced protection to cells. More than 30 reports, including the report by Du et al, have reported about the protective effect of renalase on cells (Du et al, 2014, Wang et al, 2015 etc). In research task 1, the oxidative stress increased significantly, and showed a significant positive correlation with the renalase concentration in the blood. In this study, an increase in the level of renalase is presumed to protect viscera and skeletal muscle cells from exercise-induced oxidative stress.

Another cause pertains to the functioning of renalase as an enzyme. More than 50 papers have reported that renalase might regulate cardiac function and systemic blood pressure by metabolizing catecholamines, which causes the decomposition of catecholamines such as epinephrine and norepinephrine (Desir., 2007, Desir., 2009, etc). In the in vitro study

performed by Wang et al., the addition of catecholamines to renal cells increased the level of renalase secretion and mRNA expression (Wang F et al, 2014). In addition, in the in vivo studies conducted by Li G et al, the parenteral administration of catecholamines to chronic kidney disease model rats significantly increased the activity and levels of renalase I the blood (Li et al, 2008). In these previous studies, a rise in catecholamine levels increased blood renalase levels because of some physiological effects.

Therefore, in this study, the influence of catecholamines is investigated. In research topic 3, it was hypothesized and experimentally proven that catecholamines were involved in increasing the level of expression of the renalase gene in skeletal muscles because of exercise.

2) Materials and methods

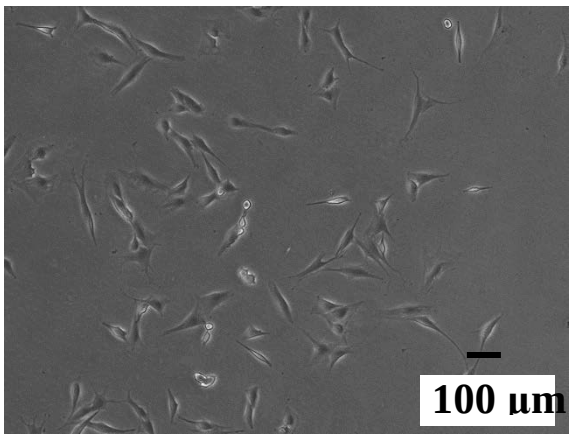
Cell culture

Mouse muscle myoblasts were used for the culture of C2C12 cells (RIKEN BioResource Center, Tsukuba, Japan). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing 10% fetal bovine serum, and maintained at 37 °C under a continuous stream of 5% CO_2 in 6-well plates. After the cultures reached confluence, the medium was replaced with DMEM containing 2% horse serum (differentiation medium) and incubated further for 7

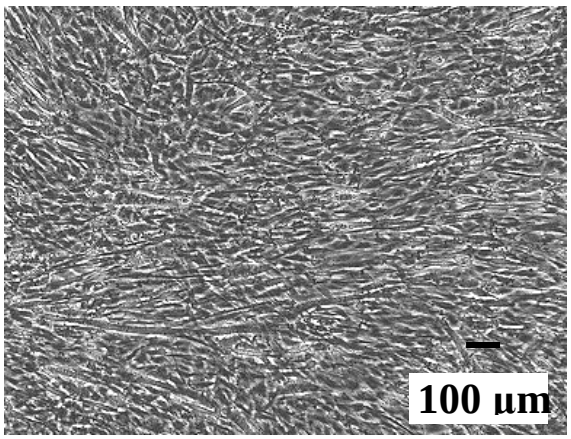
days to stimulate myotube formation (Figure 21).

Figure 21. C2C12 cells

A Myoblasts **B Myotube cell**



40× magnification



40× magnification

Figure 21. C2C12 cells

C2C12 cells of mouse skeletal muscle cells, were cultured (A: Myoblasts), and the cultured C2C12 cells were differentiated (B: Myotube cell).

Addition of epinephrine

The myotubes formed in the serum-free medium were incubated with epinephrine (Sigma-Aldrich Co., St. Louis, MO, USA). In the first study, the epinephrine concentrations were 10^{-6} g/L, 10^{-5} g/L, and 10^{-4} g/L, and the incubation time was 30 min. In the next study, the epinephrine concentration was 10^{-5} g/L and the incubation times were 15 min, 30 min, and 45 min.

Quantitative real-time PCR

Renalase gene expression was measured in C2C12 cells using real-time reverse transcription–polymerase chain reaction (RT–PCR). Total RNA was extracted from samples using Sepasol-RNA I Super G (Nacalai, Kyoto, Japan), according to the manufacturer's instructions. Total RNA was reverse transcribed into cDNA using the PrimeScript RT Master Mix (Perfect Real Time; TAKARA BIO INC., Siga, Japan). To quantify gene expression levels, PCR was carried out using a KAPA SYBR FAST qPCR kit (Kapa Biosystems, Wilmington, USA) and the Applied Biosystems 7500/7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham,USA), according to the manufacturer's instructions. The designed primers are shown below.

Gene (mouse) renalase

Forward: 5'-GGGTGGGGATATAGGGGGAAG-3'

Reverse: 5'-GCTGTGCATCGGGGATTATG-3'

Gene (mouse) GAPDH

Forward: 5'- AGGTCGGTGTGAACGGATTTG -3'

Reverse: 5'- TGTAGACCATGTAGTTGAGGTCA -3'

The cycling program involved preliminary denaturation at 95 °C for 20 seconds, followed by 40 cycles of denaturation at 95 °C for 3 seconds, and annealing and elongation at 60 °C for 3 seconds. Then, it was confirmed by melting curve analysis that non-specific by-products were not contained in the PCR product. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal normalizer control for mRNA. The measured values were analyzed by a comparative CT method (ΔΔCT method).

Statistical analysis

Statistical analysis was conducted using SPSS statistical software (version 24.0; SPSS Inc., Chicago, Illinois, USA). The data were subjected to one-way analysis of variance (ANOVA) and subsequent post-hoc test analyses for comparison between the four groups,

and t-test for the comparison between the two groups. P values that were below 0.05 were considered significant.

3) Results

Epinephrine-induced renalase expression by comparing concentrations.

C2C12 cells were cultured with three different concentrations of epinephrine for 30 min. Only cells incubated with epinephrine at a concentration of 10^{-5} g/L showed a significant increase in renalase mRNA levels ($P < 0.05$, Figure 22), as compared to controls.

Epinephrine-induced renalase expression by time comparison.

C2C12 cells were cultured with epinephrine at a concentration of 10^{-5} g/L over three different time periods. Only cells incubated for 30 min showed a significant increase in the renalase mRNA levels ($P < 0.05$, Figure 23), as compared to controls.

As a result, these experiments demonstrate a significant increase in the epinephrine-stimulated renalase expression in C2C12 cells, when cells were cultured at an epinephrine concentration of 10^{-5} g/L for 30 min.

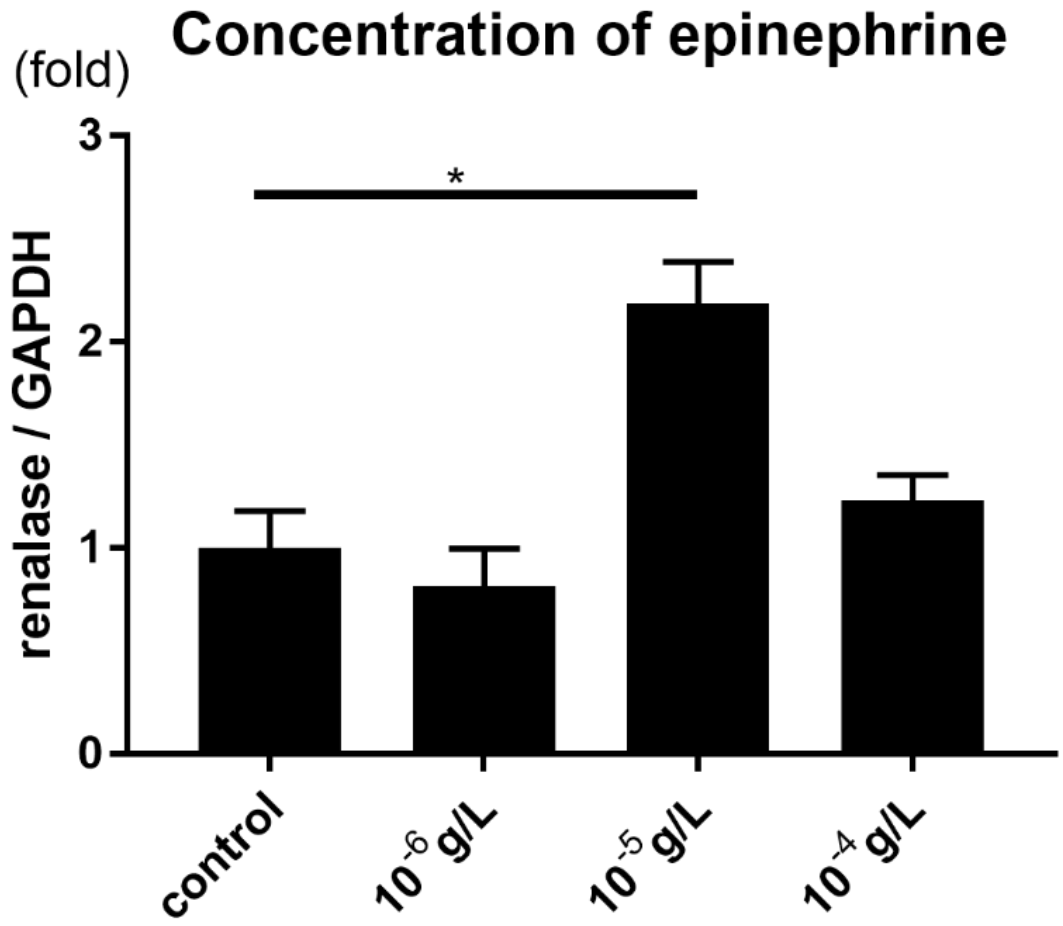
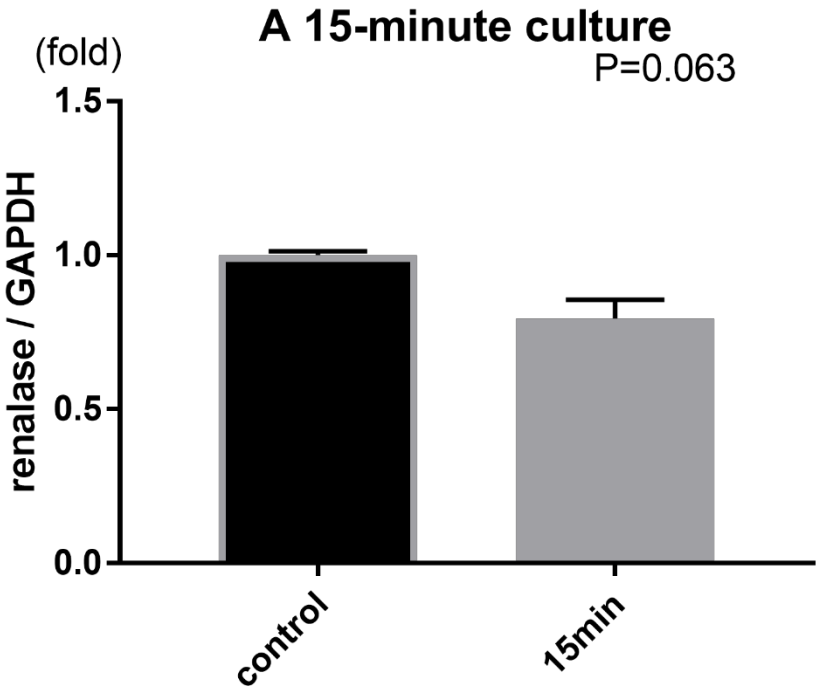
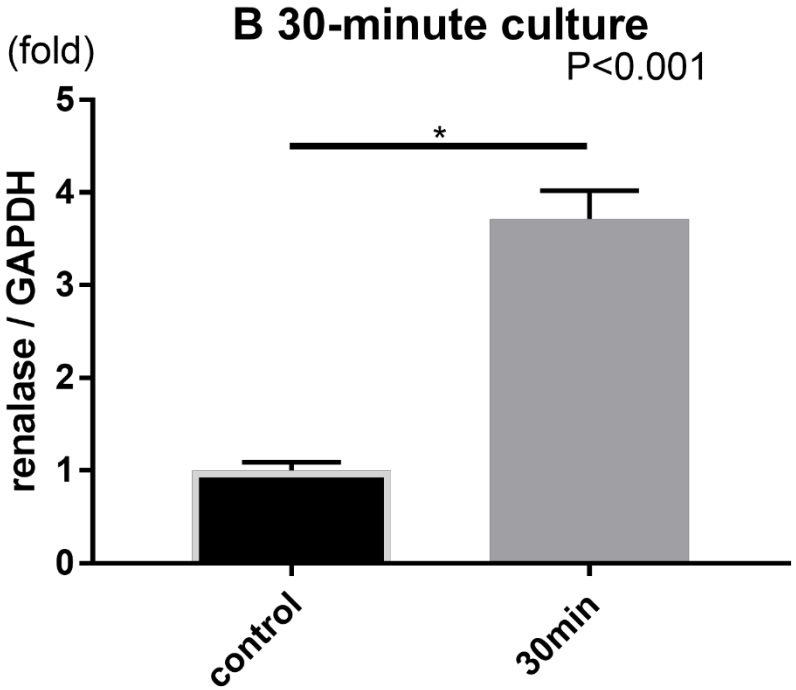


Figure 22. Renalase mRNA expression (Concentration of epinephrine)

C2C12 cells were cultured with three different concentrations of epinephrine for 30 min. Only cells incubated with epinephrine at a concentration of 10^{-5} g/L showed a significant increase in the renalase mRNA levels, as compared to controls.


A 15-minute culture
(fold)
P=0.063
renalase / GAPDH
1.5
1.0
0.5
0.0
control
15min


B 30-minute culture
(fold)
P<0.001
renalase / GAPDH
5
4
3
2
1
0
*
control
30min

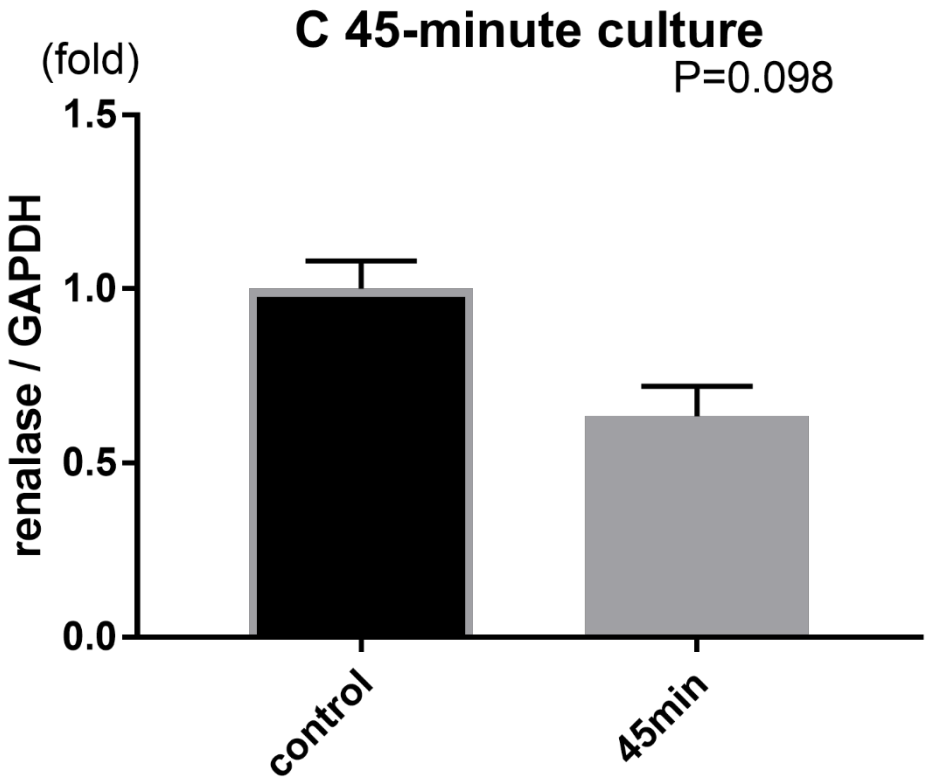


Figure 23. Renalase mRNA expression (Culture time)

C2C12 cells were cultured with three different concentrations of epinephrine for 30 min. Only cells incubated with epinephrine at a concentration of 10^{-5} g/L showed a significant increase in renalase mRNA levels, as compared to controls.

Discussion

This study showed that epinephrine induced renalase expression in differentiated myocytes. This finding has not been previously reported in skeletal muscle cells, and might enable the elucidation of the mechanism of renalase secretion owing to exercise.

This result was also consistent with reports regarding renalase expression in renal cells in vivo and in vitro (Wang et al, 2014; Yin et al, 2016). However, here we noted that there were some differences between the results obtained with kidney cells and myocytes, in terms of the time periods during which epinephrine addition caused a significant increase in the expression of the renalase gene. In addition, there was a difference in the significantly increased epinephrine concentrations. Renalase expression was significantly increased in cultured renal cells 6–24 h after the addition of epinephrine. However, when experiments involving long-term epinephrine addition were conducted, no significant differences in renalase levels were observed in muscle cells. The time required for epinephrine to upregulate renalase expression might be shorter in muscle cells than in renal cells. Further, the concentrations of epinephrine were significantly increased in the renal cells at doses of 10^{-6}, 10^{-7}, and 10^{-8} g / L; however, these concentrations were insignificant in muscle cells. During vigorous exercise, the rate of blood flow to skeletal muscles is 80% that of the total cardiac output; on the contrary, the rate of blood flow to the kidney and internal organs is greatly reduced (Standard physiology, 2000). The muscle

cells are thought to change in response to a high concentration of epinephrine in a short time, to cope with such a drastic change.

The muscle and renal cell types differ with regard to the adrenergic receptors involved in renalase expression. A previous paper has shown that the adrenaline α1 receptor is involved in renal cell functioning, while it is possible that adrenergic β2 receptors are involved in myocyte functioning; however, this has not yet been investigated and needs to be studied further. Furthermore, the pathways mediating renalase expression in each cell type are still unknown (Wang et al, 2014).

Renalase is a monoamine oxidase that decomposes catecholamines, and is involved in the regulation of blood pressure (Zbroch et al, 2012; Wang et al, 2014; Yin et al, 2016; Malyszko et al, 2013; Fedchenko et al, 2013). In recent years, the association between renalase and various diseases has also been studied (Ficek et al, 2015; Elcioglu et al, 2015; Wang et al, 2014; Malyszko et al, 2012 a; Malyszko et al, 2012 b; Desir et al, 2012); however there is almost no report regarding its relationship with skeletal muscles.

VI. Research task 4: Expression factor of renalase

“Expression factor of catecholamine-induced renalase secretion in skeletal muscle tissue in an animal model of transient medium intensity exercise.”

1) Aim

Research tasks 1 and 2 showed that the renalase concentration in the blood increased with exercise. In research task 2, the expression of the renalase gene in the tissue was examined in an animal model. The results showed that in skeletal muscle, a significant increase in both renalase mRNA and protein expression was observed. Therefore, it was hypothesized that catecholamines might be involved, as indicated by Wang et al (2014) in kidney cells. Research task 3 was to conduct experiments with skeletal muscle cells. The results showed that the addition of epinephrine, a catecholamine, significantly increased renalase expression in skeletal muscle cells. Therefore, it was speculated that catecholamines might have been involved in increasing the renalase expression level in skeletal muscles of the animal model in research task 2. SP1 (specificity protein 1), STAT3 (signal transducer and activator of transcription 3), and ZBP89 (zinc binding protein 89) might be involved in the increase of renalase expression by catecholamines. In addition, these proteins are crucial transcription factors required for renalase gene expression (Sonawane et al, 2014; Shih JC et al, 2011; He G et al, 2011; Merchant JL et al, 2003).

Therefore, in research task 4, it was hypothesized that STAT 3, Sp 1, and ZBP 89

increased the renalase gene expression levels in skeletal muscle; experiments were conducted to investigate this hypothesis.

2) Materials and methods

Animals

The animals used were the same as those used in research task 2.

Moderate Exercise

The moderate exercises were the same as those for research task 2.

Real-time PCR

Isolation of mRNA was carried out using the following method. The skeletal muscle tissues were homogenized in a Tissue Lyser bead mixer (Qiagen, Germany) at a frequency of 25 Hz for 2 – 5 min. Total mRNA isolation was performed using a Sepasol-RNA ISuper G (Nacalai, Kyoto, Japan), according to the manufacturer's instructions. Total RNA concentrations were measured at 260 nm using the ND-1000 Spectrophotometer (NanoDrop Thermo Fisher Scientific Inc., Massachusetts, USA). Samples were then frozen and stored at -80 °C for further analyses.

Reverse transcription was performed using the following method. Total RNA was reverse transcribed into cDNA using PrimeScript RT Master Mix (Perfect Real Time; TAKARA BIO INC., Siga, Japan). To quantify gene expression levels, PCR was carried out using a KAPA SYBR FAST qPCR kit (Kapa Biosystems, Wilmington, USA) and the

Applied Biosystems 7500/7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, USA), according to the manufacturer's instructions. The designed primers are shown in Table 8. The cycling program involved preliminary denaturation at 95 °C for 20 seconds, followed by 40 cycles of denaturation at 95 °C for 3 seconds, and annealing and elongation at 60 °C for 3 seconds. Then, it was confirmed by melting curve analysis that non-specific by-products were not contained in the PCR product. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal normalizer control for mRNA. The measured values were analyzed by a comparative CT method (ΔΔCT method).

Table 8. Primer sequences for each gene used in real-time quantitative PCR

Gene (rat)	***STAT3***	Forward:	5'-CCTTGGATTGAGAGCCAAGAT-3'
		Reverse:	5'-ACCAGAGTGGCGTGTGACT -3'
Gene (rat)	***Sp1***	Forward:	5'-GCTATAGCAAACACCCCAGGT-3'
		Reverse:	5'-CAGGGCTGTTCTCTCCTTCTT-3'
Gene (rat)	***Zep89***	Forward:	5'-GGGTGGGGATATAGGGGGAAG-3'
		Reverse:	5'-GGGTGGGGATATAGGGGGAAG-3'
Gene (rat)	***GAPDH***	Forward:	5'-GGAAACCCATCACCATCTTC-3'
		Reverse:	5'-GTGGTTCACACCCATCACAA -3'

Statistical analysis

Statistical analysis was conducted using SPSS statistical software (version 24.0; SPSS Inc., Chicago, Illinois, USA). The data were subjected to the t-test for comparison between the two groups. P values that were below 0.05 were considered to be statistically significant.

3) Results

STAT3

STAT3 mRNA expression in the skeletal, soleus, plantaris, and extensor digitorum longus muscles of rats in the control group (COT group) and the medium intensity exercise group (MEX group) were compared. The results showed that STAT3 mRNA expression increased significantly only in the soleus muscle ($P < 0.05$, Figure 24, 25, 26).

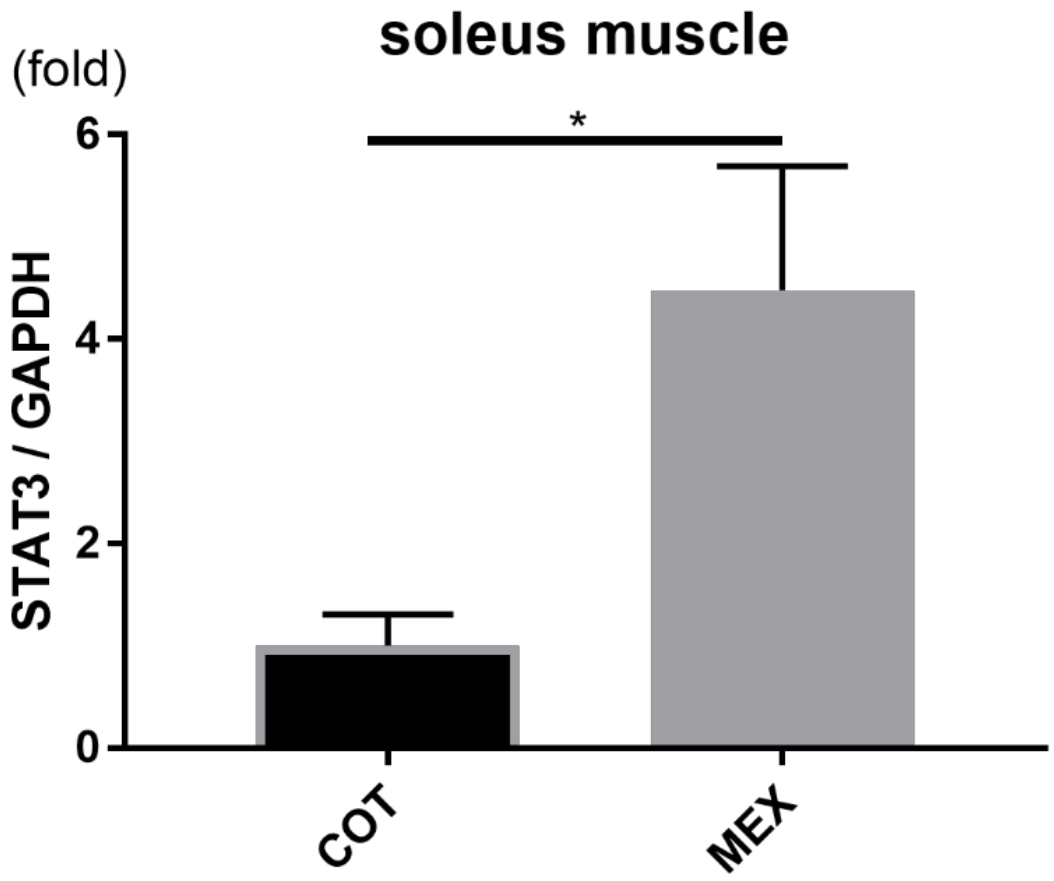


Figure 24. STAT3 mRNA expression in the soleus muscle

Comparison of renalase mRNA expression in the soleus muscles of rats in the moderate-intensity exercise (MEX) and control (COT) groups. The t-test (*P < 0.05) is used for analysis. There was a significant difference in the values (P = 0.034).

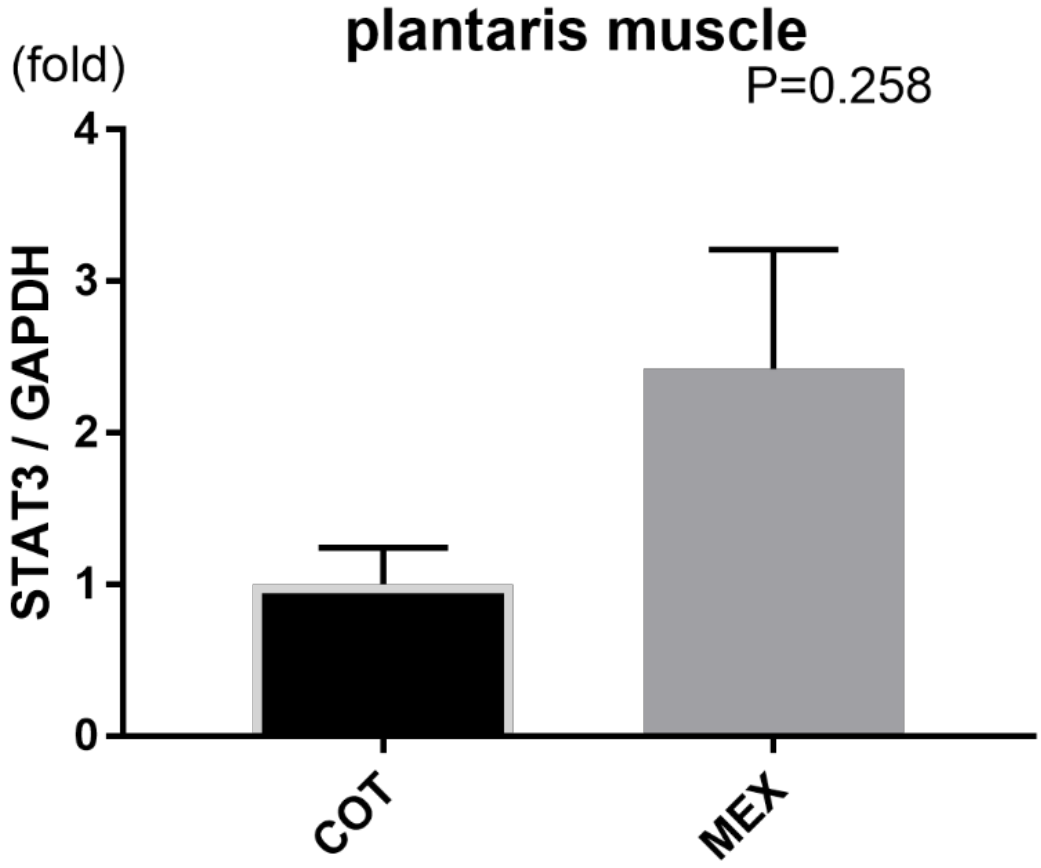


Figure 25. STAT3 mRNA expression in the plantaris muscle
Comparison of renalase mRNA expression in the plantaris muscles of rats in the moderate-intensity exercise (MEX) and control (COT) groups. The t-test (*P < 0.05) is used for analysis. No significant differences were observed.

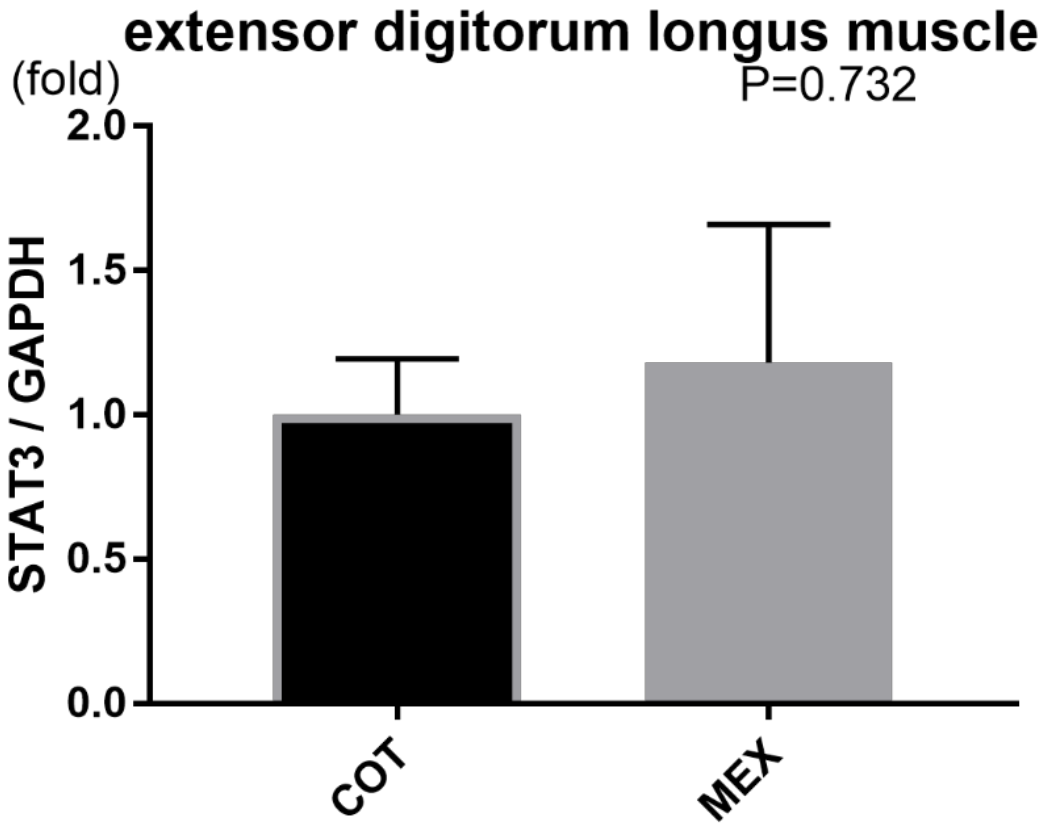


Figure 26. STAT3 mRNA expression in the extensor digitorum longus muscle
Comparison of renalase mRNA expression in the extensor digitorum longus muscles of rats in the moderate-intensity exercise (MEX) and control (COT) groups. The t-test (*P < 0.05) is used for analysis. No significant differences were observed.

Sp1

Sp1 mRNA expression in the skeletal, soleus, plantaris, and extensor digitorum longus muscles of the COT and MEX groups were compared. The results showed that Sp1 expression increased significantly only in soleus muscles ($P < 0.05$, Figure 27, 28, 29).

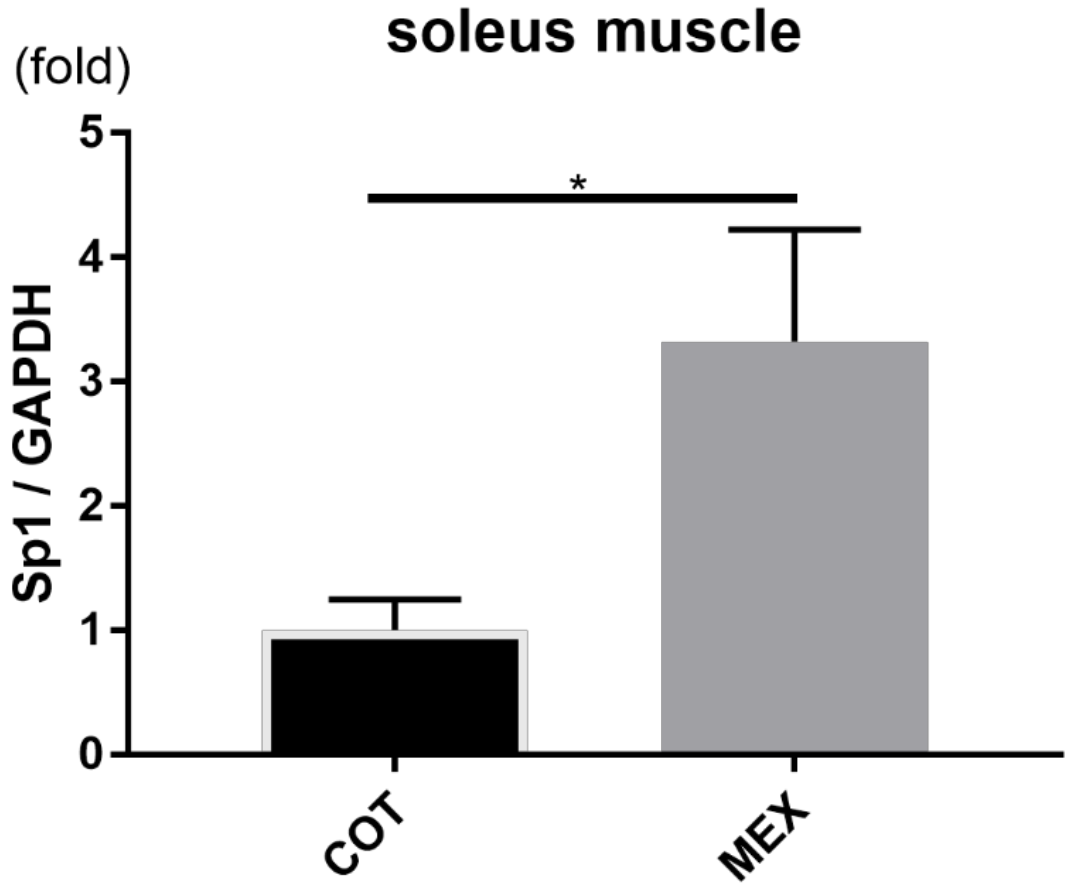


Figure 27. Sp1 mRNA expression in the soleus muscle

Comparison of renalase mRNA expression in the soleus muscles of rats in the moderate-intensity exercise (MEX) and control (COT) groups. The t-test (*P < 0.05) is used for analysis. There was a significant difference in values (P = 0.049).

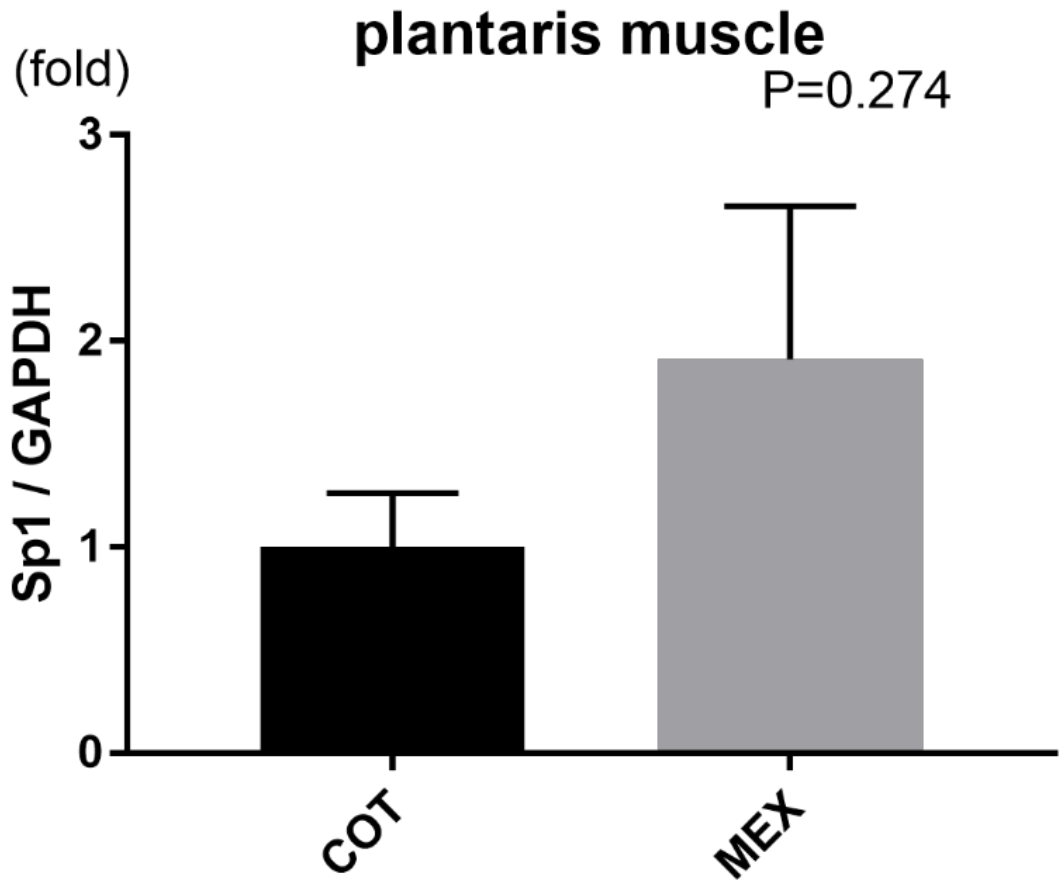


Figure 28. Sp1 mRNA expression in the plantar muscle

Comparisons of renalase mRNA expression in **the plantar muscles of rats in** the moderate-intensity exercise (MEX) and control (COT) groups. The t-test (*P < 0.05) is used for analysis. No significant differences were observed.

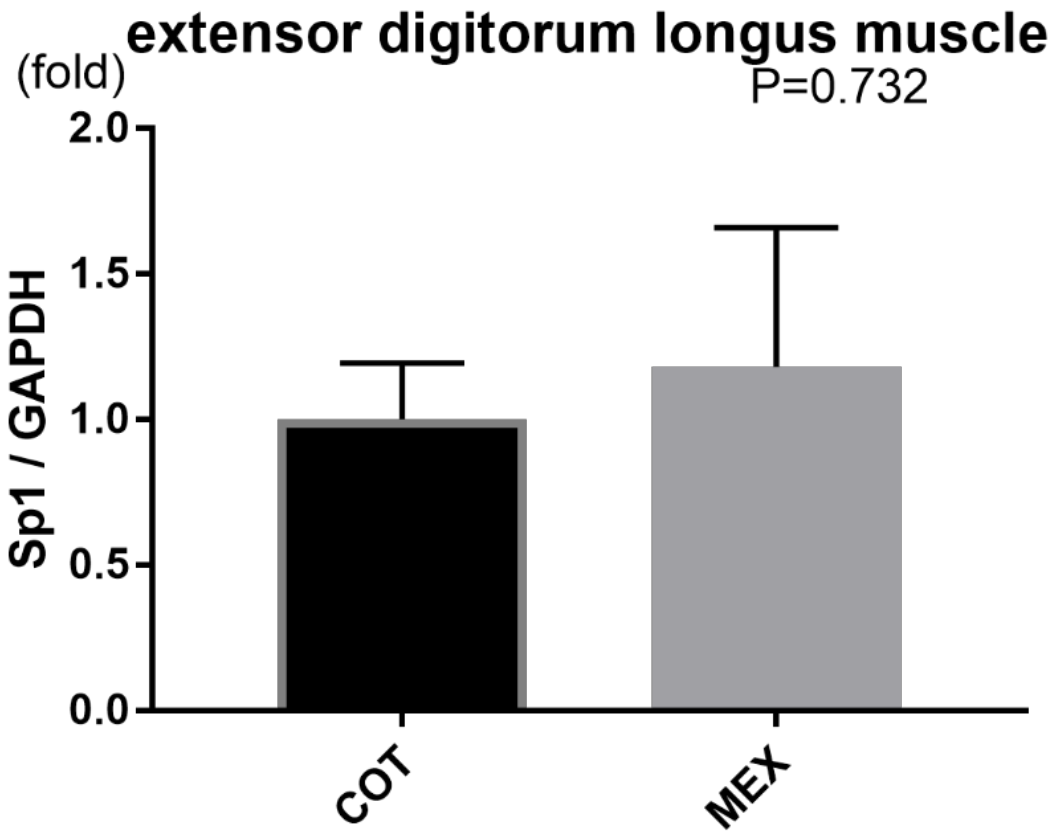


Figure 29. Sp1 mRNA expression in the extensor digitorum longus muscle
Comparison of renalase mRNA expression in extensor digitorum longus muscles of rats in the moderate-intensity exercise (MEX) and control (COT) groups. The t-test (*P < 0.05) is used for analysis. No significant differences were observed.

ZBP89

ZBP89 mRNA expression in the skeletal, soleus, plantaris, and extensor digitorum longus muscles of rats in the COT and MEX groups was compared. The results showed that ZBP89 mRNA expression levels increased significantly ($P < 0.05$, Figure 30, 31, 32) only in the soleus muscle.

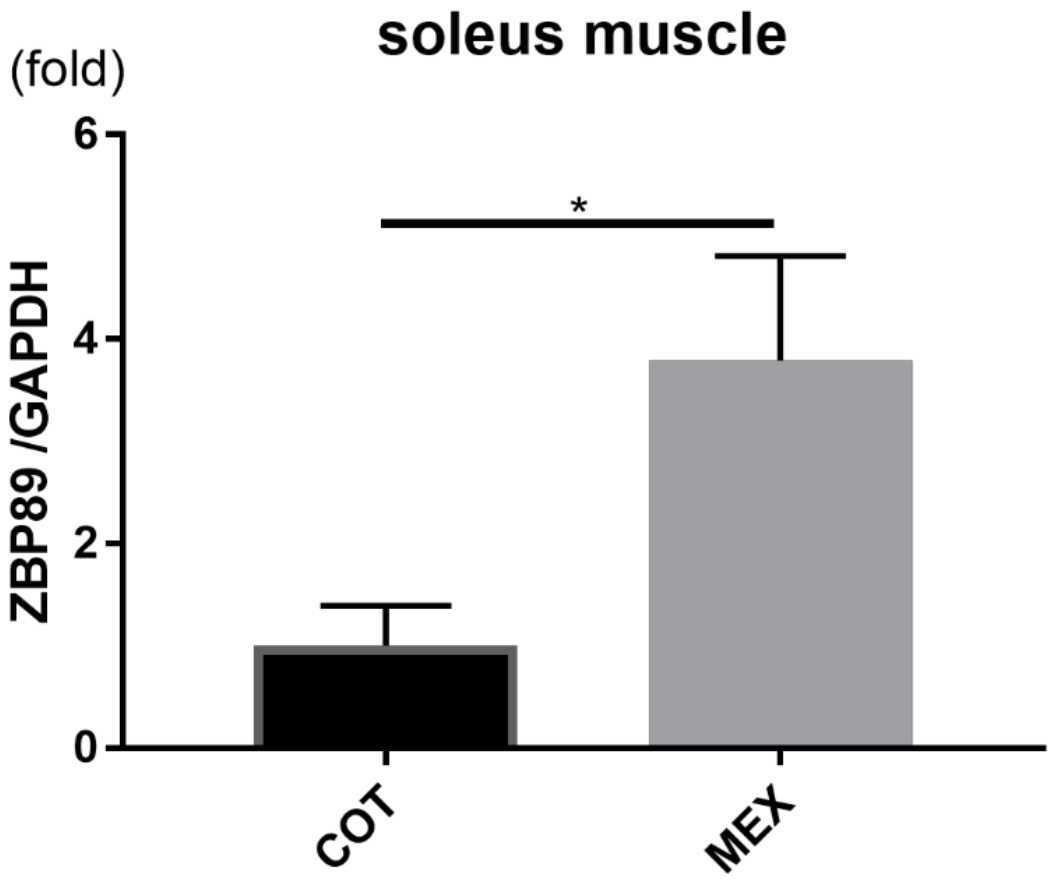


Figure 30. ZBP89 mRNA expression in the soleus muscle

Comparison of renalase mRNA expression in the soleus muscles of rats in the moderate-intensity exercise (MEX) and control (COT) groups. The t-test (*P < 0.05) is used for analysis. There was a significant difference (P = 0.040) in the values.

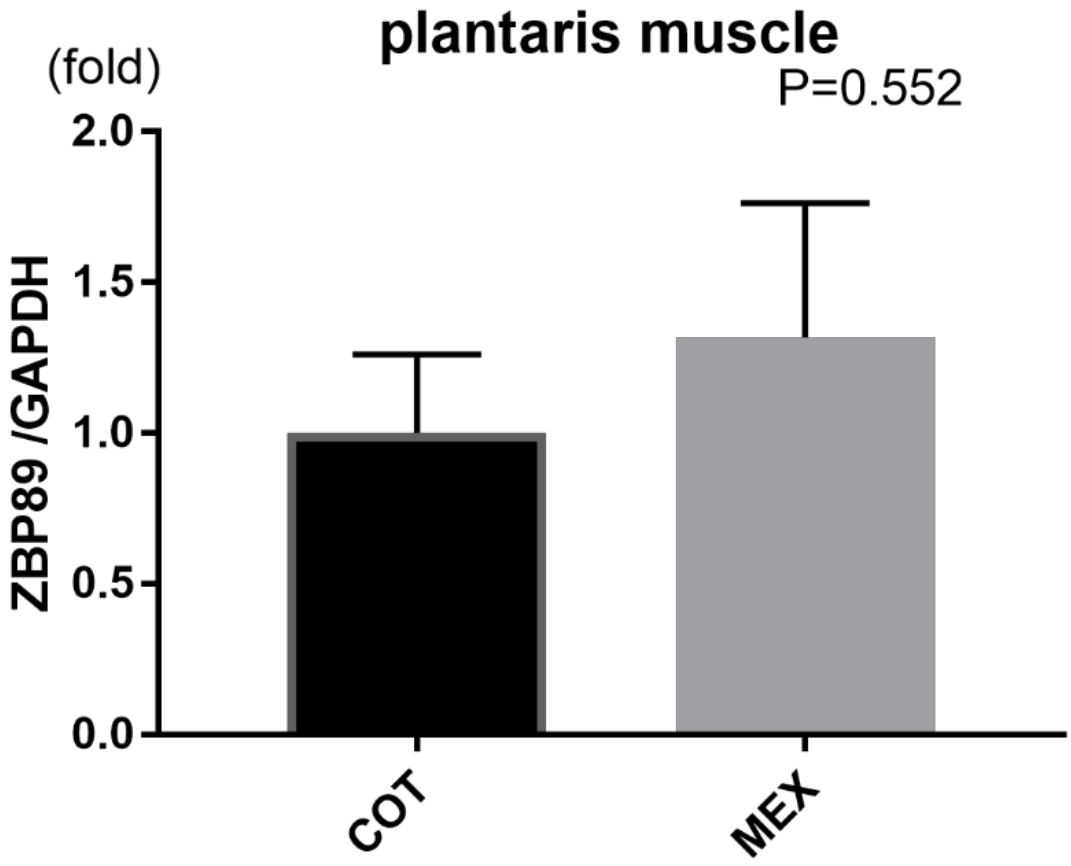


Figure 31. ZBP89 mRNA expression in the plantar muscle

Comparisons of renalase mRNA expression in the plantar muscles of rats in the moderate-intensity exercise (MEX) and control (COT) groups. The t-test (*P < 0.05) is used for analysis. No significant differences were observed.

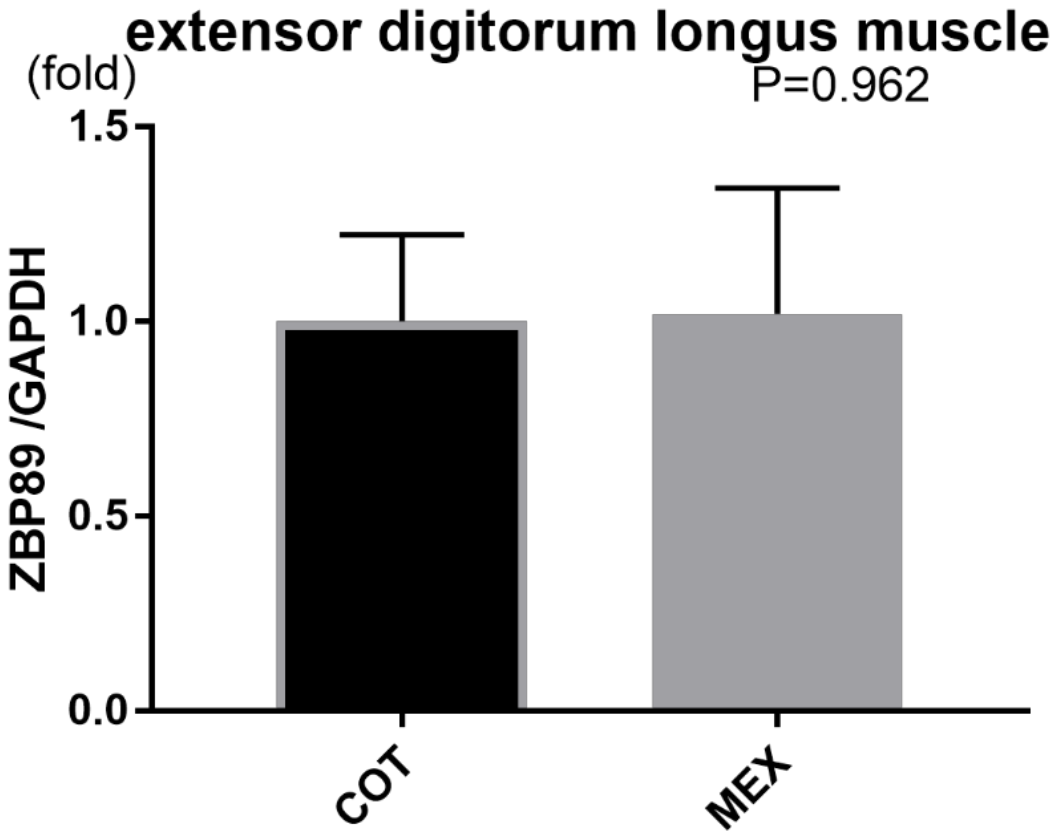


Figure 32. ZBP89 mRNA expression in the extensor digitorum longus muscle
Comparison of renalase mRNA expression in the extensor digitorum longus muscles of rats in the moderate-intensity exercise (MEX) and control (COT) groups. The t-test (*P < 0.05) is used for analysis. No significant differences were observed.

Discussion

Research tasks 1 and 2 showed that exercise causes the renalase concentration in the blood to increase. Research task 2 showed that the skeletal muscle expresses renalase, and is thus involved in causing the increase in renalase levels in the blood. Research task 3 showed that catecholamines increased renalase gene expression in cultured skeletal muscle cells. In research task 4, experiments were conducted to show that catecholamines were involved in exercise-induced renalase gene expression in skeletal muscle.

The skeletal muscle tissues used in research task 2 were used in this study. Then, the genetic expression of STAT 3, Sp 1, and ZBP 89 were measured. These transcription factors, which are very important for renalase gene expression, might be catecholamine regulators (Sonawane et al, 2014). The results showed that levels of STAT 3, Sp 1, and ZBP 89 were significantly increased in the soleus muscle of the skeletal muscle. However, similarly measured levels of transcription factors in the plantaris and extensor digitorum longus muscles did not change significantly. The exercise-induced expression of the renalase gene in skeletal muscle varied, depending on the type of skeletal muscle. In research task 2, the extensor digitorum longus muscle, which was predominantly composed of white muscle, showed a significantly increased level of mRNA expression and plantaris muscle showed a significantly increased level of protein expression.

However, in this study, the gene expression of the catecholamine regulators STAT 3, Sp 1, and ZBP 89 were significantly increased in the soleus muscle, in which red muscles are dominant. In a study regarding acute exercise that was conducted by Czarkowska-Paczek et al, the renalase mRNA expression was measured. The Gastrocnemius muscle was divided into white and red muscles and measured. The results showed that the the renalase levels in white muscles significantly increased immediately after exercise, as compared with that at rest, but the levels in red muscles were not significantly different (Czarkowska-Paczek et al, 2013). These results are consistent with those of research task 2. In research task 4, it was observed that levels of important expression factors for the renalase gene increased significantly only in red muscles. The expression of renalase gene is different in the muscles in which the white and red muscles are dominant. However, it became clear that both white muscle and red muscle showed exercise-induced renalase expression. The difference between the red muscle and the white streak is the difference in the expression level of the renalase gene inducer. There are two functions of renalase. One is to metabolize catecholamines as an enzyme. The other one is to have a protective effect on cells as a growth factor. In this study, the factor for catecholamine regulation was significantly increased in the soleus muscle, which is predominantly composed of red muscles, i.e., it the red muscle is considered to have induced the expression of the

renalase gene via the catecholamines. In addition, in the previous paper, the β-adrenergic receptor density of the red muscle is more than twice that of the white muscle (Wade H et al, 1989, Alex J et al, 2017). Thus, the red muscle is considered to have induced the expression of the renalase gene via catecholamines. On the other hand, the white muscle is presumed to be caused because of oxidative stress and similar factors, such as the cell protective function.

VII. Conclusions

The function of renalase is to regulate blood pressure by metabolizing catecholamines. Another function is to protect cells from damage. Although the renalase protein was discovered in 2005, there was no report of an investigation into the relationship between renalase and exercise in human subjects.

Therefore, in this study, I focused on the fluctuation in renalase levels during exercise, and examined the organs and skeletal muscle.

●Research task 1: Human subject research

The results of research task 1 showed that the running exercise load significantly increased the concentration of renalase in the blood.

In the in vitro study performed by Wang et al., the addition of catecholamines to renal cells increased renalase secretion and mRNA expression (Wang et al, 2014). In addition, in the in vivo studies conducted by Li G, the parenteral administration of catecholamines to chronic kidney disease model rats significantly increased the renalase activity and blood renalase levels (Li G et al, 2008). In the present study, it was presumed that the catecholamine level increased due to exercise, from which it was inferred that the serum renalase concentration was increased.

In this study, the levels of serum renalase and eGFR-CysC showed a significant

negative correlation, i.e., as the serum renalase concentration increased, the concentration of eGFR-CysC, which is an indicator of kidney function, decreased.

A significant positive correlation was found between the rate of increase of TBARS and serum renalase concentration in the period before running 10 km, after running, and the period before running 20 km, and the period after running this distance.

Although the antioxidant effect could not be measured in this study, it could be a possible cause of the decrease in the TBARS level (Kayatekin et al, 2002; Tong et al, 2016). The study by Li et al showed a significant increase in renalase expression with increasing oxidative stress in a mice model of ischemia–reperfusion injury; renalase expression was suppressed by antioxidants (Li et al, 2016). In addition, it has been reported that the renalase level increases during ischemia–reperfusion, and has a protective effect on organs (Lee et al, 2016; Yin et al, 2016; Wu et al, 2011). There was also a significant increase in oxidative stress in this study, which is presumed to be because of ischemia of an organ or organ damage due to exercise (Niemelä et al, 2016). Therefore, it is speculated that an increase in serum renalase concentration due to exercise might also contribute to protection from organ damage due to exercise.

●Research task 2: Animal model research

The results of research task 2 showed that the levels of renalase in the blood increased

significantly in the animal models, which was similar to that observed during human subject research. In this study, the differences in mRNA expression levels of the renalase gene in visceral and skeletal muscles were investigated. Renalase gene expression in each type of skeletal muscle in the soleus, plantaris, and extensor digitorum longus muscles was investigated for the first time in the present study. The results elucidated that renalase gene expression in the soleus muscle, which predominantly consisted of red muscle, was approximately 10 times higher than that in the extensor digitorum longus muscle, which predominantly consisted of white muscle.

Next, the exercise-induced variability of renalase gene expression in visceral and skeletal muscles was examined.The results showed that there was no significant difference in the renalase mRNA expression due to exercise in the heart, liver, lung, and adrenal glands. However, in the kidneys of rats from the MEX group, the renalase level was significantly decreased as compared with that in the COT group. With regard to skeletal muscle, the renalase level was significantly higher in the soleus muscle of rats in the MEX group than that in COT group.

In addition, the expression of renalase protein was examined.The results showed that the renalase level in the kidney was significantly reduced in the MEX group as compared to that in the COT group. With regard to skeletal muscles, there was a significant increase

in renalase levels was observed in the plantaris muscle.

The expression of the renalase gene because of exercise was shown to be decreased in the kidney and increased in skeletal muscles. However, the expression in skeletal muscles differed, depending on the type of muscles.

●Research task 3: Cell culture experiment

The results of research task 3 showed that epinephrine induced renalase expression in differentiated myocytes. This finding has not been previously reported in skeletal muscle cells, and might enable the elucidation of the mechanism of renalase secretion owing to exercise.

This result was also consistent with reports regarding renalase expression in renal cells in vivo and in vitro (Wang et al, 2014; Yin et al, 2016). However, here we noted that there were some differences between the results obtained with kidney cells and myocytes, in terms of the time periods during which epinephrine addition caused a significant increase in the expression of the renalase gene.

The muscle and renal cell types differ with regard to the adrenergic receptors involved in renalase expression. A previous paper has shown that the adrenaline α1 receptor is involved in renal cell functioning, while it is possible that adrenergic β2 receptors are involved in myocyte functioning; however, this has not yet been investigated and needs

to be studied further. Furthermore, the pathways mediating renalase expression in each cell type are still unknown (Wang et al, 2014).

●Research task 4: Expression factor of renalase

The results of research task 4 showed catecholamines were involved in exercise-induced renalase gene expression in skeletal muscle.

The skeletal muscle tissues used in research task 2 were used in this study. Then, the genetic expression of STAT 3, Sp 1, and ZBP 89 were measured. These transcription factors, which are very important for renalase gene expression, might be catecholamine regulators (Sonawane et al, 2014). The results showed that levels of STAT 3, Sp 1, and ZBP 89 were significantly increased in the soleus muscle of the skeletal muscle.

There are two functions of renalase. One is to metabolize catecholamines as an enzyme. The other one is to have a protective effect on cells as a growth factor. In this study, the factor for catecholamine regulation was significantly increased in the soleus muscle, which is predominantly composed of red muscles, i.e., it the red muscle is considered to have induced the expression of the renalase gene via the catecholamines. In addition, in the previous paper, the β-adrenergic receptor density of the red muscle is more than twice that of the white muscle (Wade H et al, 1989, Alex J et al, 2017). Thus, the red muscle is considered to have induced the expression of the renalase gene via catecholamines. On

the other hand, the white muscle is presumed to be caused because of oxidative stress and similar factors, such as the cell protective function.

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