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ORIGINAL ARTICLE

Sex-related differences in the effect of exercise on protein profiles of serum in mice

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Abstract

The risk of various diseases could be proven or improved by physical activity. It is known that the expression and/or activation of some proteins were up-/down-regulated by exercise. Whereas several studies suggested that exercise-related factors were expressed sex-dependently. Additionally, exercise decreased androgen levels in woman's plasma, but increased in men. These evidence raise the possibility that there is a sex-related difference in the effect of exercise on several protein expressions and signaling pathways.

In this study, proteome analysis of mice serum was performed to identify the different effects of exercise on protein expression and signaling pathways between male and female mice. The wheel-running exercise was performed, and activity counts were analyzed. After the exercise, cardiac blood samples were collected for proteome analysis. Significant alterations in protein expression levels were identified. However, few protein expression levels were commonly affected by exercise in males and females. The protein with the highest increased expression levels was F13a1 in males and Sepiapterin reductase (Spr) in females. The protein with the most decreased expression levels was thioredoxin-like protein 1 (Txnl1) in males and insulin-like growth factor binding protein 1 (Igfbp1) in females. By the orthogonal partial least square-discriminant analysis, the different proteins between males and females were identified as candidate biomarkers of exercise. In gene ontology analysis, there were dissimilar patterns of up- and down-regulation in some biological processes. These results suggested that there were several differences in exercise-dependent signaling pathways between male and female mice.

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Key words: forced exercise; voluntary exercise; proteomics; sex-related difference.

Introduction

Regular physical activity has proven to be highly beneficial for health, reducing the risk of various diseases like cardiovascular issues, obesity, diabetes, cancer, osteoporosis, autoimmune disease, and asthma[1-5)]. Exercise improves cardiovascular function, modifies fatty acid composition in muscle, liver, and adipose tissue, enhances glucose tolerance and insulin sensitivity, increases bone density, and reduces inflammation[4, 6-12)]. Notably, exercise also has positive effects on mental health, benefiting conditions such as depression, anxiety, schizophrenia, Alzheimer's, and Parkinson's diseases[13-17)].

Several studies have linked exercise to the expression and activation of cytokines, myokines, and neurotrophic factors. For instance, exercise increases proinflammatory cytokines in plasma and enhances immune cell activity[18, 19)]. Myokines and trophic factors like IGF-1 and BDNF are released during exercise and have neuroprotective effects[20, 21)]. These factors can cross the blood-brain barrier and improve hippocampal function by promoting neurogenesis and BDNF

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expression[22-24].

While exercise has beneficial effects, sex-related differences have been observed in the expression of IGF-1 and BDNF. Females tend to have higher concentrations of IGF-1 in serum and BDNF mRNA expression in the hippocampus[25, 26]. Additionally, the response to exercise in skeletal muscle and androgen levels in plasma differs between males and females[27-29]. These findings raise the possibility that exercise may have sex-dependent effects on protein expression and signaling pathway regulation. However, the exact sex-related differences in the effects of physical activity have not been fully studied. In this study, we hypothesize that the exercise-induced alteration in protein expression levels differ between males and females, and that the beneficial effects of exercise are mediated by different pathways in male and female. To investigate the sex-related differences, we performed a proteome assay of 8-weeks exercised male and female mice serum. We found that there were several differences in signaling pathways between male and female mice. These results suggested that the beneficial effects induced by physical activity could appear in the sex-dependent pathway.

Materials and Methods

Animal

Adult male and female C57BL/6J mice were used in this study. Eight-week-old mice were divided into four groups consisting of 5 male mice not exercised (sedentary), five male exercised mice, five female sedentary mice, and five female exercised mice. Animals were group housed at 24°C ± 2°C under a 12-hour light/dark cycle (lights on at 8:00 am), and food and water were available *ad libitum*. All experiments were performed in accordance with the guidelines for animal research issued by the Physiological Society of Japan and the Hirosaki University School of Medicine (approval number M21033), and all efforts were made to minimize the number of animals used and their suffering.

Activity recording and exercise

In this study, both forced running and voluntary running were performed for exercise (Fig. 1A). The exercise group mice were group housed in cages with free access to a running wheel (11 cm in diameter: Able Scientific, WA, Australia) for two weeks before the forced running start. The voluntary activity of group-housed mice was continuously recorded at 5-min bins using a three-axis accelerometer Nano-tag® (Kissei Comtec Co., Ltd. Nagano, Japan). Physical activity values were obtained by a three-axis composite wave of the accelerometer[30]. Nano-tag was implanted into the abdominal cavity under isoflurane anesthesia (0.5~1.5% air) one week before the forced running start. The implanted Nano-tag was set to start recording one week later. The activity of sedentary groups was recorded for 3 days, and the activities of exercise groups were recorded for 8 weeks. Forced running was performed before the dark period onset using a motor-driven running wheel (FWS-1505; Melquest Ltd., Toyama, Japan) for 20–60 minutes/day, 3 days/week for 8 weeks. During the first week, the running speed and time were started at 4 m/minute for 10 minutes, and then increased to 7 m/minute for 10 minutes. The intensity of the exercise was gradually increased for 4 weeks (week 2: 4 m/minute for 10 minutes + increased to 7 m/minute for 10 minutes + 7 m/minute for 10 minutes, week 3: 4 m/minute for 10 minutes + increased to 10 m/minute for 20 minutes + 10 m/minute for 20 minutes, weeks 4–8: Initially 4 m/minute, immediately increased to 10 m/minute for 20 minutes + 10 m/minute for 40 minutes).

Blood sampling

After the exercise protocol, the mice were

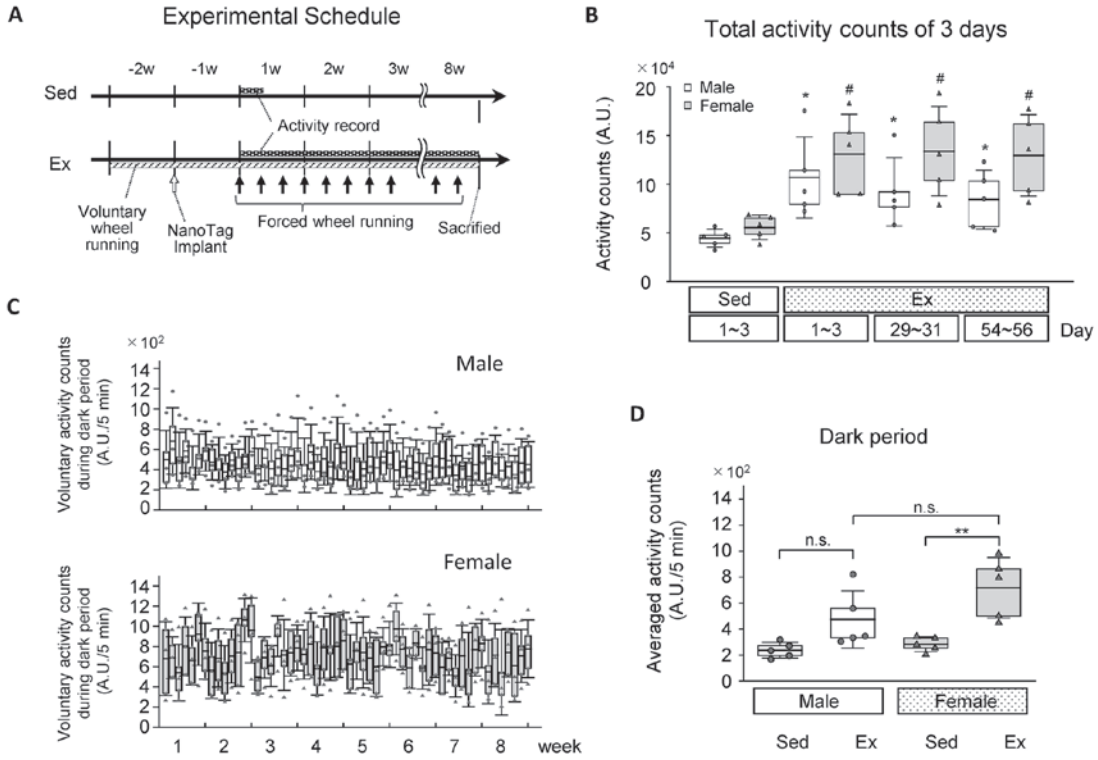

Figure 1 Voluntary running exercise made female mice more active than male mice. A) The experimental schedule of this study. Mice were randomly assigned to the sedentary or exercised groups. Exercise groups were subjected to voluntary running and forced running protocols for 8 weeks each. After the end of exercise, blood samples were collected for the serum proteomics. B) The amount of activity for 3 days of sedentary group in 1 to 3 days and exercised group in 1 to 3, 29 to 31 and 54 to 56 days were box plotted. (*: $P < 0.05$, Student's t-test , vs male sedentary group, #: $P < 0.05$, Student's t-test, vs Female sedentary group). C) Voluntary activity counts for 5 min of male (upper) and female (bottom) mice in the dark period of the light/dark cycle were averaged per a day and box plotted. D) All voluntary activity counts during the dark period of the light/dark cycle were averaged and box plotted (**: $P < 0.01$, one-way ANOVA, Tukey post hoc test). The plot indicate activity count of individual mice. The box indicate interquartile range, and the center line indicate average. Error bars represent standard deviation.

deeply anesthetized by three mixed anesthetics (0.3 mg/kg medetomidine, 4 mg/kg midazolam, and 5 mg/kg butorphanol, i.p.), and blood samples were collected by cardiac puncture. The collected blood was prepared by centrifugation at 1,500 g, 10 min, and 4°C. The serum was immediately aliquoted and stored at −80°C until required for proteome analysis.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) for proteome analysis

Serum samples were diluted 100-fold with 50 mM ammonium bicarbonate. The diluted plasma was precipitated with acetone and resuspended in 50% trifluoroethanol containing 250 mM ammonium bicarbonate. The solvent was reduced with 40 mM dithiothreitol (DTT) at 90°C for 30 min. Free cysteine residues in samples were alkylated with 80 mM iodoacetamide for 60 min at room temperature in the dark, and the remaining iodoacetamide was quenched with DTT. After alkylation, the samples were diluted with 100 mM ammonium bicarbonate and incubated with 5 μg trypsin (TPCK treated, AB Sciex) at 37°C for 18 h. The samples were desalted by MonoSpin C18 (GL Sciences), and desalted samples were rehydrated in 0.1% formic acid and analysed by LC-MS/MS using a

nanoLC Eksigent 400 system coupled online to a TripleTOF 6600 mass spectrometer (AB Sciex). Peptide was separated by using a C18 capillary column (3 μm, 75 μm ID × 125 mm, nano HPLC capillary column, Nikkyo Technos) at 300 nl/min with a 90 min linear gradient of acetonitrile in 0.1% formic acid. The parameters for data-dependent acquisition (DDA, SWATH acquisition) were set as follows: 100 ms TOF MS scan, followed by 200 variable SWATH windows each at 50 ms accumulation time for 400–1250 *m/z*. MS/MS SWATH scans were set at a 5 Da window overlapping by 1 Da for 400 to 1250 *m/z* and varied on each side of the mass range. The total cycle time was 9.6 sec and a rolling collision energy (CE) parameters script was used to automatically control the CE.

Data processing and statistical analysis for proteome analysis

To process all the raw data, DIA-NN (v1.8.1) was used in the default settings of a library-free mode[31]. To identify the protein family, the group column in DIA-NN's report was used. The MaxLFQ column calculated using the MaxLFQ algorithm[32] was used to obtain the normalized quantity. The principal component analysis (PCA) and orthogonal partial least square-discriminant analysis (OPLS-DA) were performed as multivariate statistical analysis using the Simca software program (Infocom Corp., Tokyo, Japan). Pareto scaling was applied to the normalized data prior to the analyses by calculating the relative abundance of each modified peptide using a corresponding non-modified peptide peak area and autoscaling (mean centering and dividing by the standard deviation of each variable). Ingenuity Pathway Analysis (IPA, Qiagen, Hilden, Germany) was used for pathway enrichment analysis of differentially regulated proteins. A p-value reflecting the probability that the association was explained by chance alone was calculated by Fisher's exact test. Proteins altered in the exercised group with $|p1(corr)| > 0.7$ by OPLS-DA were analyzed by IPA.

Gene ontology (GO) analysis

Differentially expressed proteins were subjected to GO analysis using the PANTHER Classification System (http://pantherdb.org/), a part of the Gene Ontology Reference Genome Project. The gene sets that showed significantly different expressions between the control and exercised male and female mice were analyzed, and the number of proteins in each Biological Process was represented by a horizontal bar graph. The gene list input to the PANTHER Classification System was divided according to down- or up-regulation by exercise.

Statistics

All values of activity counts are presented as the mean ± standard deviation. The unpaired Student's t-test and one-way ANOVA followed by Tukey-Kramer's test were performed to compare activity-counted data between groups. Differences were considered statistically significant at $P < 0.05$.

Results

Gender differences in the characteristics of physical activity in forced and voluntary exercise

The physical activities of the exercise groups were evaluated by summarizing the activity counts for three days during the voluntary exercise period (initial phase, 1 to 3 days: middle phase, 29 to 31 days; last phase, 54 to 56 days; Fig. 1B). The activity counts of exercised groups at each time point were significantly higher than those of sedentary groups in both sexes. However, there was no significant difference in the activity counts among each time point of exercise groups. These data confirmed that male and female mice in the exercise groups

performed practical exercises using a voluntary running wheel. Furthermore, they showed regular activities during the dark period throughout the voluntary running period (Fig. 1C). When compared to the sedentary animals, the exercised females showed a significant increase in activity during the dark period, but the exercised males did not (Fig. 1D). There was no significant gender difference between exercise groups. These results suggested that voluntary running made female mice more active than male mice.

Following the voluntary running, we performed the forced running for 8 weeks. Weekly activity changes indicated that mice acclimated to wheel running in the first few weeks and then showed stable running performance (Fig. 2A). In addition, there was no significant gender difference in the mean activity counts (Fig. 2B). These results indicated that the forced running promoted the activity equally in both sexes.

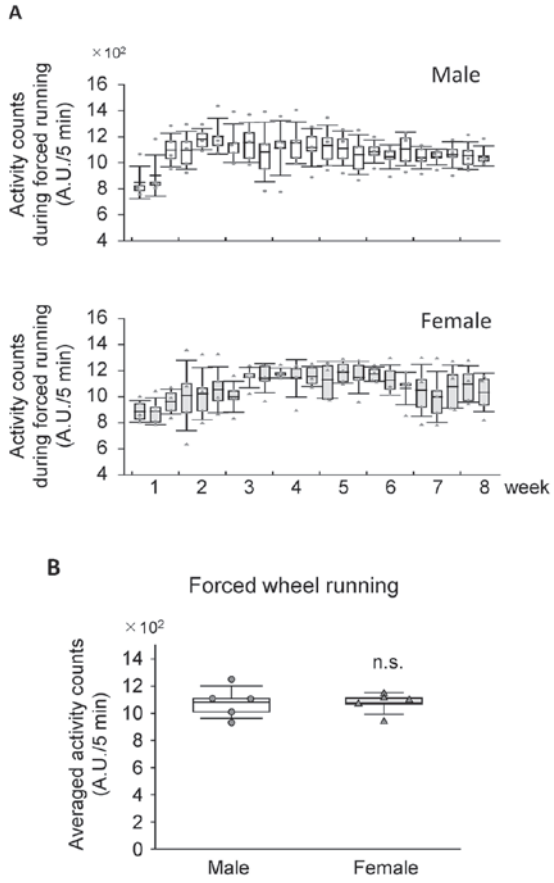

Figure 2 Both male and female mice showed comparable physical activations during the forced running exercise. A) The mean activity counts per a day of male (upper) and female mice (bottom) of exercised group. B) Mean activity counts shown in A were averaged and box plotted. There were no significant differences in the activity between sexes. The plot indicate activity count of individual mice. The box indicate interquartile range, and the center line indicate average. Error bars represent standard deviation.

The effect of exercise on protein expression in serum

To investigate the sex-related difference in protein profiles induced by exercise, proteomic analysis of mice serum was performed. In our condition, a total of 378 proteins in male and 361 proteins in female mice were identified by the proteomic analysis. The 79 proteins in male and 56 proteins in female were found to be differentially expressed with absolute fold change > 1 and ANOVA p-value < 0.05. The volcano plots representing the distribution of the fold changes and p-values of the above proteins were shown in Fig. 3. The exercise induced significant upregulation of 44 proteins and downregulation of 35 proteins expression in male mice serum (Fig. 3Aa). In female mice serum, exercise upregulate 25 and downregulate 31 proteins expression (Fig. 3Ab). The top 5 proteins most changed by exercise were not found to be common between males and females. The p1 (corr) of OPLS-DA shows the correlation of each protein to the discriminant model between the exercise and control groups, and Fig. 3B shows a scatter plot of p1 (corr) in the discriminant model developed for males and females. This figure shows that the commonly 4 proteins altered by exercise in both male and female mice (Fig. 3B, Table 1). The expression of these proteins was inversely altered between males and females; Hrg, Serpina1e and Kng1 were increased in male but decreased in female mice,

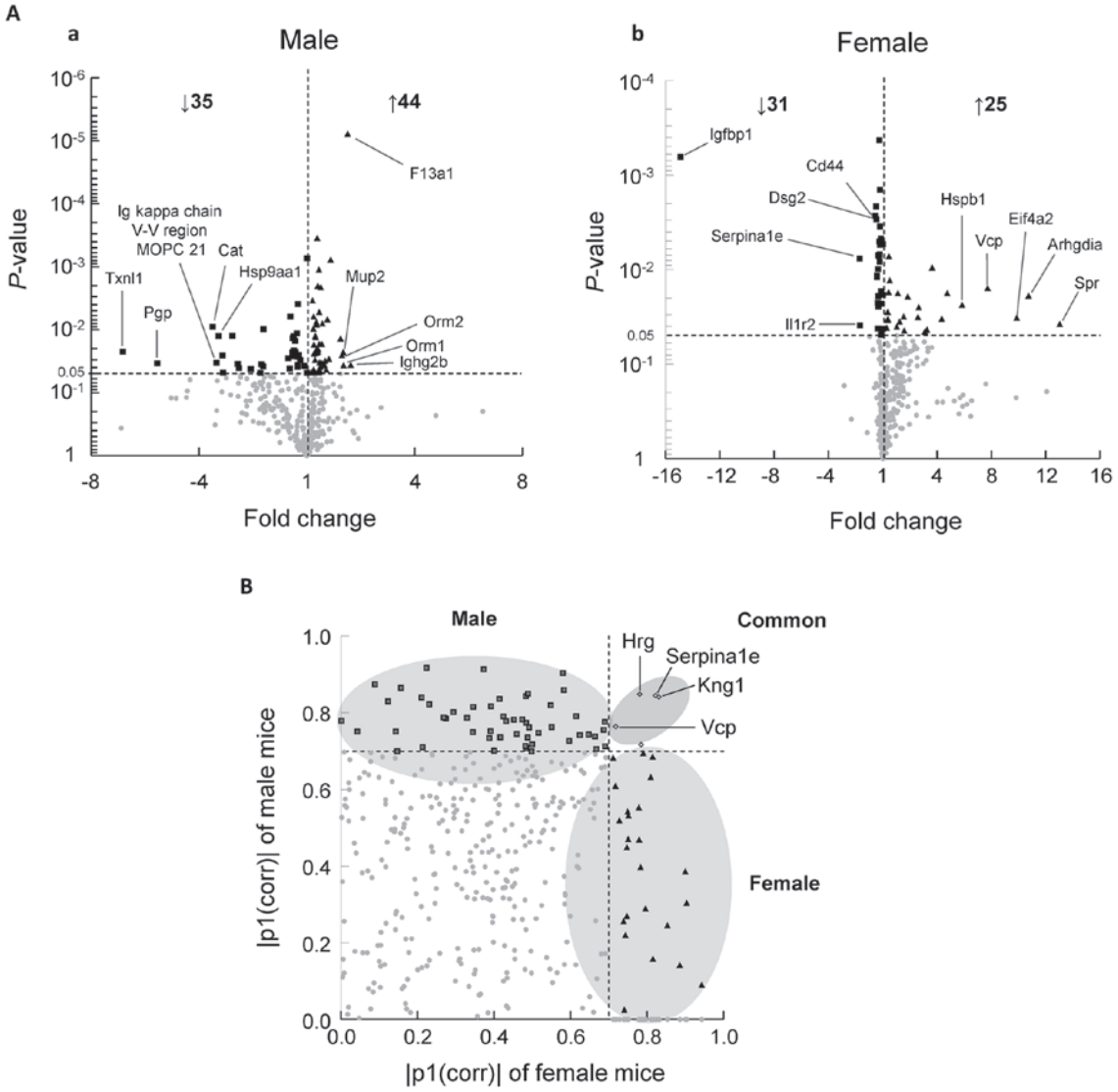

Figure 3 Proteomic analysis results of mice serum. A) A volcano plot of the differentially expressed proteins between sedentary and exercised mice serum in male (a) and female (b) mice. Black dots indicate up (triangular) or down (square)-regulated proteins. The top 5 of most changed proteins were indicated by text label. B) OPLS-DA analysis of the data generated from male vs female. The text labeled proteins were significantly up- or down- regulated by exercise in both sexes.

and Vcp was decreased in male but increased in female mice. These results suggested that exercise induced alterations of serum protein levels were distinctly different between male and female mice.

The difference in GO and pathway profiles between male and female mice

The sex-related difference in the effect of exercise on serum protein levels corresponded to the results of GO analysis. Some processes, such as developmental, multicellular organismal, growth, biological adhesion, and biomineralization process, were mostly facilitated in male but inhibited in female mice (Fig. 4). In addition, there were female-specific changes in some processes. Reproduction and reproductive process related to gametogenesis and meiosis

Table 1. Exploring candidates for exercise-induced markers in serum by proteome analysis and OPLS-DA plot.

Var ID	Genes	p1 (correlation)		Fold change	
		Male	Female	Male	Female
Q9ESB3	Hrg	0.848	0.781	1.21	−1.15
Q00898	Serpina1e	0.841	0.831	1.36	−2.72
O08677	Kng1	0.845	0.823	1.27	−1.21
Q01853	Vcp	0.763	0.719	−2.77	7.73

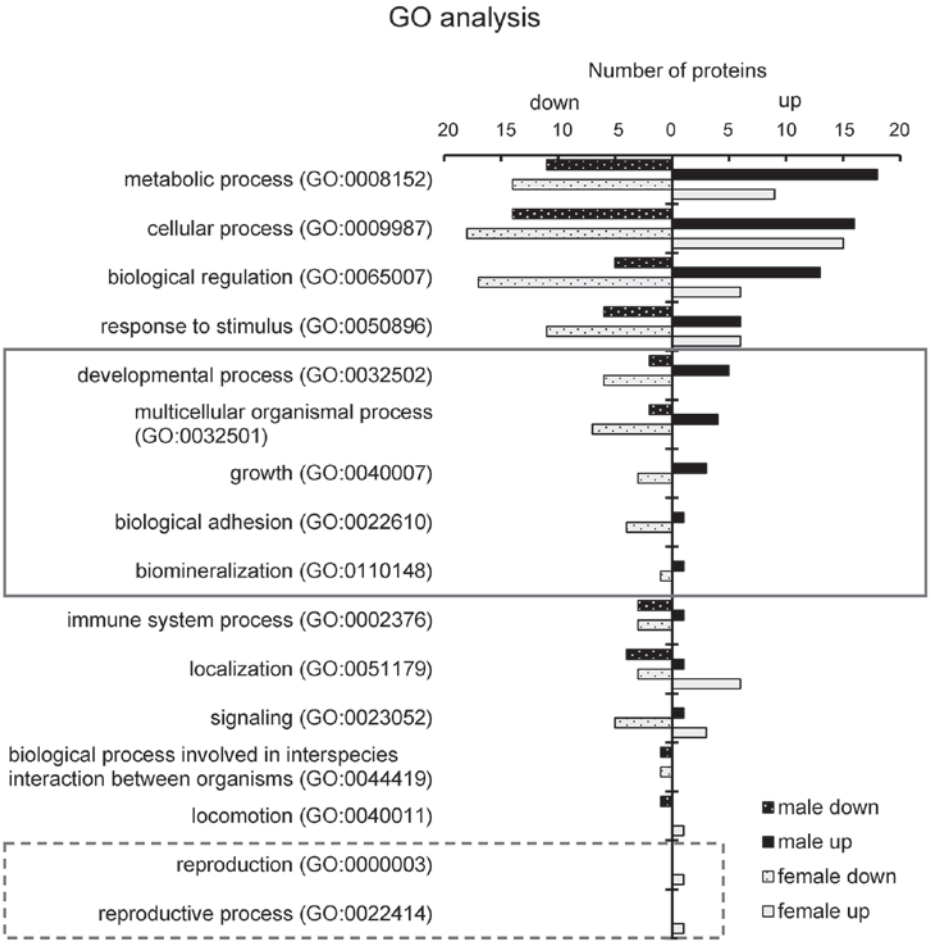

Figure 4 GO analysis of molecular function for up- and down-regulated proteins between sedentary vs exercise groups of both sexes. The up-regulated (solid bar) or down-regulated (dotted bar) biological process in male (black bar) and female (white bar) mice is shown in this figure. Hrizontal axis indicate number of proteins in up- or down-regulated process.

were facilitated only in female mice. In the process of metabolic and cellular process, biological regulation, and response to stimulus, there were both up-regulation and down-regulation in male and female mice. To investigate the detail alteration of biological process, IPA pathway analysis was performed. IPA performs an enrichment analysis considering the regulation of each protein, and the up- and down-regulation of a pathway is calculated as a positive and negative z score, respectively. The top two significant and highly up- or down-regulated (Z-score>2, $P<0.05$) pathways were shown in Table 2. In male mice, the RNA-

Table 2. The results of the IPA pathway analysis. The top two significant and most altered pathways were represented.

		Pathway	-log(p-value)	Z-score	Molecules
Male	Up	Role of PKR in Interferon Induction and Antiviral Response	2.51	2.646	CYCS,HSP90AA1,HSP90AB1,HSP90B1,HSPA5,HSPA8,IFNAR2
		LXR/RXR Activation	35.3	2.535	A1BG,AGT,AHSG,AMBP,APOA1,APOA2,APOA4,APOB,APOC4,APOD,APOE,APOH,APOM,C3,C4A/C4B,C9,CLU,FGA,GC,HPX,IL1R2,IL1RAP,ITIH4,KNG1,LCAT,LYZ,PLTP,PON1,RBP4,SERPINA1,SERPINF1,SERPINF2,TF,TTR,VTN
	Down	PI3K/AKT Signaling	1.64	−2.449	HSP90AA1,HSP90AB1,HSP90B1,IL1R2,YWHAE,YWHAG,YWHAZ
		Epithelial Adherens Junction Signaling	1.63	−2.449	CDH1,EGFR,MST1,YWHAE,YWHAG,YWHAZ
Female	Up	Glycolysis I	8.79	2.828	ALDOA,BPGM,ENO1,GAPDH,GPI,PGK1,PKM,TPI1
		Gluconeogenesis I	8.79	2.828	ALDOA,BPGM,ENO1,GAPDH,GPI,MDH1,MDH2,PGK1
	Down	LPS/IL-1 Mediated Inhibition of RXR Function	2.64	−2.236	APOC4,APOE,CAT,FABP5,GSTM5,GSTP1,IL1R2,IL1RAP,PLTP,SOD3
		SPINK1 Pancreatic Cancer Pathway	10.7	−2.309	CELA1,CELA2A,CELA3B,CPB2,CPN1,CPQ,CTRB2,CTSA,CTSB,KLKB1,PRSS2,SPINK1

dependent protein kinase (PKR)-associated pathway and the liver X receptor-retinoid X receptor (LXR/RXR) pathway were identified as especially and significantly upregulated pathway. Conversely, the PI3K/AKT and the epithelial adherens junction signaling were highly significantly downregulated. Differently to male results, glucose metabolism was significantly upregulated, and RXR inhibition and serine protease inhibitor kazal-type1 (SPINK1) pathway were downregulated (Table. 2). These results indicated that the effect of exercise on pathway regulation was distinctly different between male and female mice.

Discussion

In this study, we investigated the effects of voluntary and forced wheel running exercises on male and female mice and performed proteomic analysis to investigate sex differences in serum protein profiles. We found that voluntary running increased activity levels more in female mice compared to male mice. On the other hand, forced running equally promoted activity in both sexes. Additionally, the results of proteomic analysis suggested the exercise-induced alteration in serum protein levels and pathway regulation differed between male and female mice. Based on these results, we concluded that our findings support our hypothesis that the effects of exercise on the regulation of serum protein expression are mediated by different pathways in male and female mice.

Although the forced exercise protocol induced physical activation equally in both sexes, the voluntary exercise protocol induced more physical activity in female than male mice. The result was consistent with a previous report indicating that voluntary wheel-running activity of young female mice was higher than that of males [33]. Generally, female rodents are more active than male ones [34]. It was reported that sex hormone, estrogen, is a factor that upregulates daily physical activity [35]. Sex differences, including hormones and voluntary activity volume, may affect serum protein profiles.

Proteomic analysis in the current study revealed that changes in serum as a result of physical exercise were distinctly different between male and female mice. Our data identified molecules such as F13a1, Ighg2b, Clu, Serpina1e/3m, and Gpx3 as exercise upregulated molecules in the serum of male mice, and changes in expression of these molecules were consistent with results reported in previous study using similar analyses [36]. These facts imply that our data seems to be correctly obtained and

analyzed.

Recent studies revealed that physical exercise upregulates some molecules in serum such as BDNF, IGF-1, glycosylphosphatidylinositol-specific phospholipase D1 and FNDC5. The PGC-1α/FNDC5 pathway was upregulated by exercise and increase hippocampal BDNF[37]. The fact that these molecules improve hippocampal functions has been reported[24]. However, the pathways including these molecules were not detected as the most significant factors in our condition. The expression of exercise-induced molecules might be affected by the difference in exercise procedure, proteomic analysis tools, and mouse strain.

PKR pathway is involved in inflammation and immune regulation. As exercise increases the amounts of inflammatory cells to maintain physiological homeostasis, the upregulation of PKR signaling pathway in the male exercise group was a well-known response[38]. LXR/RXR activation is involved in lipid metabolism, the inflammatory response, cholesterol degradation, and glucose homeostasis. It was reported that activating LXR regulate neuroinflammation and reduce amyloid-beta peptide accumulation in Alzheimer's disease[39]. It is possible that the beneficial effects of exercise on the CNS are mediated through upregulation of LXR/RXR activation.

In female mice, Spr was the most upregulated molecule in female serum. Sepiapterin is a precursor of the nitric oxide synthase (NOS) cofactor tetrahydrobiopterin (BH4). Spr deficiency is a levodopa-responsive disorder and displays Parkinson's disease-like symptom including cognitive impairment[40]. It is likely that an increase in Spr expression through exercise may be protective against Parkinson's disease and other diseases of the central nervous system. In addition, glycolysis and gluconeogenesis pathways were upregulated by exercise. In general, glycolysis is an important energy supply mechanism for some physiological activities, such as strenuous or prolonged exercise[41]. Gluconeogenesis is the opposite of glycolysis; it consumes energy and produces glucose. It has been reported that exercise induces both glycolysis and gluconeogenesis[42]. The fact that glucose metabolism is modulated by exercise suggests that exercise has a beneficial effects including improvements in the metabolic health of the liver[43].

Meanwhile, there were downregulated expression of proteins and pathways involved in normal biological process. For instance, Txnl1, a member of the thioredoxin superfamily of redox regulators, plays an essential role in the maintenance of cell survival through a variety of antioxidant and anti-apoptotic mechanisms. The Akt/PI3K-Akt pathway is involved in basic cellular processes including protein synthesis, proliferation and survival. Adherens junctions play a role in maintaining tissue homeostasis and regulating epithelial and endothelial permeability. The downregulation of these proteins and pathways is not likely to be a beneficial effect of exercise, suggesting that not all effects of exercise on plasma protein expression are beneficial.

Volcano plot, OPLS-DA and pathway analysis indicated that the molecules regulated by exercise were different in male and female mice. However, it was not clear from the present study why there are differences between the sexes in the effects of exercise on the characteristics of serum protein profiles. Numerous studies have suggested that there are sex differences in the responses of glucose, insulin-like growth factor-1, growth hormone, and various other molecules to exercise[44, 45]. It is possible that sex differences in exercise-regulated serum protein expression may be caused by differences in body fat, muscle mass, and sex hormones[46].

There were some limitations to this study. In the present study, both voluntary and forced

exercise were performed on the same mice using the same protocol. Therefore, further research would be needed to define the effects of exercise intensity and voluntariness to exercise on the sex-specific regulation of serum protein expression. Additionally, the distinct expression levels and function of proteins were not examined. Analysis of the functional properties of each protein would assess the sex difference in exercise-induced beneficial effects. In future studies, the expression of proteins and pathways that confer beneficial effects of exercise would be individually identified in male and female. Thereby it may be possible to prevent a range of diseases that could be ameliorated by exercise, even in patients who are unable to exercise.

Disclosure Statement

All authors have no conflicts of interest directly relevant to the content of this article.

Data Availability Statement

The proteomic data is available online using accession number "PXD041178" for Proteome Xchange and accession number "JPST002106" for jPOST Repositor.

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Author Contribution Statement

T.F. and S.S. are equal contributors to this work and designated as co-first authors. A.K., Y.T. and Y.N. contributed to the interpretation of the results. S.U. supervised the conduct of this study. All authors reviewed the manuscript draft and revised it critically on intellectual content.

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