Lipid Dynamics due to Muscle Atrophy Induced by Immobilization

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Abstract: Muscle atrophy refers to skeletal muscle loss and dysfunction that affects glucose and lipid metabolism. Moreover, muscle atrophy is manifested in cancer, diabetes, and obesity. In this study, we focused on lipid metabolism during muscle atrophy. We observed that the gastrocnemius muscle was associated with significant atrophy with 8 days of immobilization of hind limb joints and that muscle atrophy occurred regardless of the muscle fiber type. Further, we performed lipid analyses using thin layer chromatography, liquid chromatography–mass spectrometry, and mass spectrometry imaging. Total amounts of triacylglycerol, phosphatidylserine, and sphingomyelin were found to be increased in the immobilized muscle. Additionally, we found that specific molecular species of phosphatidylserine, phosphatidylcholine, and sphingomyelin were increased by immobilization. Furthermore, the expression of adipose triglyceride lipase and the activity of cyclooxygenase-2 were significantly reduced by atrophy. From these results, it was revealed that lipid accumulation and metabolic changes in specific fatty acids occur during disuse muscle atrophy. The present study holds implications in validating preventive treatment strategies for muscle atrophy.

Key words: muscle atrophy, immobilization, lipid, fatty acid

1 Introduction

Skeletal muscles comprise approximately 40% of the total body weight and play fundamental roles in exercise, posture control, and energy metabolism¹. These muscles can reconstitute their mass by accelerating protein synthesis and disassembly. However, when this system is distorted to lose its mass, muscle atrophy occurs². Muscle atrophy is associated with the pathogenesis of cancer, diabetes, obesity, and aging. Sarcopenia, which refers to the loss of muscle mass and strength with aging, is one of the most important hindrances in maintaining quality of life. Patients developing sarcopenia show abnormal lipid metabolism such as dyslipidemias; however, the underlying mechanism is not fully understood. Patients with amyotrophic lateral sclerosis (ALS), which causes skeletal muscle decline and muscle atrophy through the progressive loss of motor neurons, are known to have severe muscle atrophy³. Moreover, it is considered that in ALS, mutant Cu/Zn superoxide dismutase is aggregated and mitochondrial function is impaired, thereby causing lipid changes. In other words, lipid metabolism is considered to significantly affect muscle atrophy⁴.

Previously, studies on muscle atrophy models induced by high-fat diet⁵, muscle immobilization⁶, and denervation⁷ clarified that the accumulation of excess lipids is not just associated with muscle atrophy but insulin resistance as well (it has been reported that the accumulation of excess lipids is involved in insulin resistance and muscle atrophy⁸). Of special interest is increased intramyocellular lipids comprising neutral lipids, such as triacylglycerol (TG) and diacylglycerol (DG), which lead to impaired insulin sensitivity and alter lipid metabolic genes⁹,¹⁰ and are associated with muscle atrophy. Reportedly, phosphatidylserine (PS) is increased with muscle atrophy when the gene expressing PS-decarboxylase is knocked out in mice¹¹. However, only a few studies have accurately analyzed the
molecular species of these lipids. In this study, we aimed to
determine the lipid changes caused by muscle atrophy,
thereby demonstrating their roles in muscle atrophy at the
molecular species level.

There are four types of muscle fibers found in skeletal
muscles, namely myosin heavy chain (MyHC) type I, type
IIa (slow-twitch), type IIx, and type IIb (fast-twitch). These
myofibers have different contractile and metabolic charac-
teristics. The gastrocnemius muscle contains all of these
myofiber types and exhibits their characteristic localization.
We developed an experimental model based on 8 days
of immobilization of one limb to pinpoint lipid changes in
the muscles due to muscle atrophy. We compared lipid
content between the immobilized limb (right) and non-im-
obilized limb (left) collected from the same rat.

2 Experimental Procedures

2.1 Animals

Five-week-old male Sprague–Dawley rats (Nihon SLC,
Ltd. Shizuoka, Japan) were housed in stainless steel wire
mesh cages in a temperature-controlled room (24 ± 1°C)
under a 12/12 h light/dark cycle (dark phase: 15:00–3:00).
After 7 days of acclimatization, 12 rats were subjected an-
esthetic immobilization using isoflurane; the right limb was
splinted with a bent plastic plate cast with a wetted plaster
(3M Soft Elastic Tape, width: 5 cm, 3M Japan, Tokyo) and
wrapped with a steel wire mesh to inhibit gnawing. After
the 8-day immobilization period, the rats were sacrificed by
decapitation and the gastrocnemius muscles were removed
and weighed. This study was conducted in accordance with
the ethical guidelines of the Utsunomiya University Animal
Experimentation Committee (Approval No. A20-0016) and
in complete compliance with the National Institutes
of Health Guide for the Care and Use of Laboratory Animals.
All efforts were made to minimize the number of animals
used and limit experimentation sufficient to produce reli-
able scientific information.

2.2 Cryosectioning and hematoxylin and eosin (H&E)
staining

Serial cross-sections of the muscles (10 μm thick) were
produced using a cryostat (CM 1950; Leica Microsystems,
Wetzlar, Germany) and mounted onto Matsunami adhesive
coated slides (Matsunami, Osaka, Japan) for histochemical
staining or indium–tin-oxide-coated glass slides (Bruker
Daltonics, Bremen, Germany) for mass spectrometry (MS)
imaging. For morphological observation, the sections were
stained with hematoxylin and eosin (H&E), followed by
measuring the cross-sectional area (CSA) of each muscle
fiber (total, 600).

2.3 Fluorescence staining and immunoassays

To differentiate between muscle fiber types, the sections
were subjected to immunofluorescence staining using fiber
type-specific antibodies. Briefly, tissue sections were fixed
with 100% acetone and incubated with primary antibodies
against MyHC type I (#BA-F8), MyHC type IIa (#SC-T1),
and MyHC type IIb (#BF-F3), all obtained from the Develop-
ment Studies Hybridoma Bank, University of Iowa
(USA). Further, the sections were incubated with Alexa
Fluor 350 (#A21040), Alexa Fluor 488 (#A21042), and
Alexa Fluor 555 (#A21127)-conjugated secondary antibod-
ies (Thermo Fisher Scientific K.K. Tokyo, Japan). In addi-
tion, unstained black muscle fibers were assigned as MyHC
type IIX. Fluorescence signals were detected with a wide-
field fluorescence microscope (BZ-9000; KEYENCE, Osaka,
Japan).

2.4 Thin-layer chromatography (TLC)

Total lipids were extracted from the gastrocnemius
muscle with chloroform:methanol (2:1, v/v); lipid fractions
were extracted by the Bligh and Dyer method as described
previously. Equal amounts of the extracts were manually
applied to silica gel 60 high-performance TLC plates
(Merck, Darmstadt, Germany). The plates were developed
with the solvent systems methyl acetate/1-propanol/chloro-
form/methanol/0.25% aqueous potassium chloride
(25:25:25:10:9, v/v/v/v/v) and n-hexane/diethyl ether/acetic
acid (80:30:1, v/v/v) for the separation of polar lipids and
neutral lipids, respectively. The chromatograms were
sprayed with primuline reagent, and lipid bands were visu-
alized with a Gel Doc EZ Imager (Bio-Rad Laboratories, CA,
USA) under ultraviolet light. Relative densities of each lipid
were determined quantitatively using ImageJ software
(http://rsbweb.nih.gov/ij/). For TLC imaging, lipid bands
developed on the TLC plates were transferred to a polyvi-
nyl difluoride (PVDF) membrane, as described previously.
TLC-imaging was performed using a TOF/TOF 5800 mass
spectrometer (AB SCIEX, Tokyo, Japan). Briefly, the trans-
ferred PVDF membranes were attached to the MALDI
target plate and deposited matrix for ionization. Positive
ion-mode analyses were applied to obtain the signal, and
m/z values in the range of 400–1000 were measured. To
create two-dimensional ion-density maps, we used Data-
cube Explorer (AMOLF, Amsterdam, Netherlands). Lipids
were identified by referring to a previous report and by
tandem MS analysis.

2.5 Liquid chromatography–mass spectrometry (LC–MS)
analysis

Lipid profiles were acquired with HPLC-electrospray
ionization (ESI)–orbitrap MS (LTQ Orbitrap Discovery,
Thermo Scientific). The stored samples (−80°C) were
thawed at 23 ± 1°C and resolved with 200 μL chloroform:
methanol (1:2, v/v); 5 μL of each sample solution was
applied to the LC-MS system. The samples were separated with a reverse phase C18 column (1.9 μm, 2.1 × 50 mm, JASCO Corporation, Tokyo, Japan). The mobile phase was composed of A = acetonitrile: methanol:water (9:9:2, v/v/v) containing 0.1% formic acid and 0.028% ammonia, and B = isopropanol containing 0.1% formic acid and 0.028% ammonia. The gradient program was as follows: 0 min, 0% B; 5 min, 100% B; 10 min, 100% B; 10.1 min, 0% B; and 13 min, 0% B. The column temperature was maintained at 45°C and the flow rate was set to 0.20 mL/min. To wash the flow channel including the column, 5 μL of mobile phase B was injected after each analysis and the same gradient program was adopted.

MS detection was performed in the positive ion mode. ESI source conditions were set as follows: source voltage and capillary voltage were maintained at 4.0 kV and 20 V, respectively; capillary temperature, sheath gas flow rate, and aux gas flow rate were set at 275°C, 20 arb, and 0 arb, respectively. Data were collected in a data-independent top and capillary voltage were maintained at 4.0 kV and 20 V, respectively. Ion images were constructed using flexImaging software.

2.6 Western blot analysis
Following tissue homogenization in lysis buffer and sonication, the lysate was centrifuged at 14,800 rpm for 40 min at 4°C to pellet the debris. The total protein concentration of the supernatant was determined using a Bradford assay. The supernatant sample was stored at −30°C.

SDS-PAGE analysis was performed as described previously. Samples were diluted in Laemmli sample buffer and boiled for 10 min at 98°C. Protein samples were separated by 10% SDS-PAGE and transferred onto PVDF membranes (Millipore, Bedford, MA, USA). The membranes were blocked for 1 h in tris-buffered saline containing 0.1% Tween-20 and 5% skim milk. Thereafter, membranes were incubated overnight at 4°C with primary antibodies against ATGL (#2138, Cell Signaling Technology, MA, USA) and GAPDH (#2708, Cell Signaling Technology, MA, USA). The next day, the membranes were washed four times with TBST and incubated with horseradish peroxidase (HRP) - conjugated secondary antibodies (anti-rabbit IgG: 1:1000, Cell Signaling Technology, MA, USA) for 1 h at room temperature. The protein bands were detected using enhanced chemiluminescence (Luminata Forte Western HRP Substrate; Merck Millipore, Bedford, MA, USA) with an ImageQuant LAS 500 imaging system (GE Healthcare, Amersham, UK). Quantification was performed using ImageJ software (http://rsbweb.nih.gov/ij/).

2.7 Spectrophotometric analysis of cyclooxygenase (COX) activity
COX activity was determined using a commercial kit (COX Activity Assay Kit, Cayman Chemical, MI, USA). The gastrocnemius muscle was sonicated in 5–10 mL cold buffer (i.e. 0.1 M tris-HCl, pH 7.8, containing 1 mM EDTA) per g of tissue, followed by centrifugation at 10,000 x g for 15 min at 4°C. The supernatant was collected and used for the assay. Briefly, the sample (supernatant), buffer, arachidonic acid (AA), and hemin were placed in a 96-well plate. Samples with inhibitors including 5-bromo-2-(4-fluorophenyl)-3-[4-(methylsulfonyl)phenyl]-thiophene (Dup-697) and 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole(SC-560) were also placed in other wells.

Dup-697 and SC-560 were used as inhibitors of COX-2 and COX-1, respectively. Absorbance was measured at 560 nm (Molecular Devices, CA, USA), and COX activity was calculated from the obtained absorbance.

2.8 MS imaging
MS imaging was performed using the Solarix XR (Bruker Daltonics, Bremen, Germany). Analyses were performed for masses in the range of m/z 400–1200. The matrices for ionization used 40 mg/mL 2,5-dihydroxybenzoic acid in methanol/water (8:2, v/v) for the positive ion mode. Ion images were constructed using flexImaging (Bruker Daltonics, Bremen, Germany). The MS parameters were set to obtain the highest sensitivity. Cryosections as described in section 2.2, for MS imaging, were prepared from three independent rats (n = 3).

2.9 Statistical analysis
All data are expressed as the mean ± standard error of the mean. Statistical analyses were performed using a Student’s t-test with StatView 5.0 (SAS Institute, Tokyo, Japan). The level of significance was set at p < 0.05 (*) or p < 0.01 (**).

3 Results
3.1 Induction of muscle atrophy by immobilization
Previously, it was reported that the weights of the soleus and gastrocnemius muscle decrease after 2 weeks of immobilization of the hind limb joint. In the present study, we tried to analyze the lipid changes in the early stage of muscle atrophy; hence, we immobilized the hind limb joint for 8 days using the same method (Fig. 1A). The weight of the muscle collected from the right limb (immobilized) was reduced as compared to that of the left limb (control; Fig. 1B). Further, immobilized muscle exhibited significantly reduced transverse section areas (Figs. 1C and 1D). We concluded that even an 8-day immobilization procedure can significantly reduce skeletal muscle masses.
3.2 Analyses of muscle atrophy based on each fiber type

We observed muscle atrophic features in the immobilized muscle (Fig. 1). Further, we divided the gastrocnemius muscle into three parts depending on the muscle fiber composition (LGasS: lateral head gastrocnemius muscle of the surface; LGasD: lateral head gastrocnemius muscle of the deep layer; MGasD: medial head gastrocnemius muscle of the deep layer) to determine the level of atrophy in each fiber type (Fig. 2A) and measure the CSA of each muscle fiber. We found that muscle atrophy occurred regardless of the site and muscle fiber type and that all parts and all muscle fibers were significantly atrophied by 0.5 to 0.7 times in the immobilized group as compared to those of the control group (Figs. 2B and 2C). More specifically, the average atrophy rates for each muscle fiber type were 0.53 to 0.59 times for slow-twitch fibers (MyHC types I and IIa, respectively) and 0.60 to 0.61 times for fast-twitch fiber (MyHC types IIx and IIb, respectively).

3.3 Accumulation of neutral lipids by muscle atrophy

We next measured total lipid content, which did not differ between control and immobilized groups (data not shown). To determine lipid changes during skeletal muscle atrophy in detail, we analyzed differences in lipid content between control and immobilized muscles using TLC. Of the neutral lipids, TGs tended to increase in immobilized muscles (Fig. 3A), which was similar to that observed in a previous report. Therefore, we conducted LC–MS analyses and examined the molecular species to understand the quantitative and qualitative changes that occurred with immobilization. We found 24 kinds of TG molecules, wherein signal ratios of the top 10 molecular species are shown in Fig. 3B. As shown in Fig. 3B, all molecular species showed a significant increase in immobilized muscles. To assess the mechanism of TG accumulation, we measured protein expression levels of adipose triglyceride lipase (ATGL), a major lipase that catalyzes TG to DG. The expression of ATGL was significantly reduced in immobilized muscles as compared to that in control muscles (Fig. 3C).

3.4 Accumulation of polar lipids with muscle atrophy

A previous study reported that phosphatidylethanolamine (PE) and PS increased in patients with spinal muscular atrophy. To elucidate if our atrophy model exhibited such a trend, we performed TLC for phospholipid separation. Consequently, we detected PE, phosphatidylinositol (PI), PS, phosphatidylcholine (PC), sphingomyelin (SM), and lysophosphatidylcholine (LPC) (Fig. 4A). Quantitative
analysis revealed that there was an increasing tendency for PS ($p = 0.0755$) and a significant increase in SM ($p = 0.0061$). To clarify the kinds of molecular species of PS that changed, we analyzed PS bands using TLC imaging. As a result, we found that the molecular ion of PS (18:0/18:1) was the main molecular species and tended to increase ($p = 0.0528$) in the immobilized muscle compared to that in the control muscle. Reportedly, PC (18:0/20:4) and SM
3.5 COX activity with muscle atrophy

As seen in Fig. 4B, AA-containing phospholipids, such as PC(18:0/20:4), were significantly increased during muscle atrophy, a finding that is in accordance with our previous report. We found that AA-containing PC(18:0/20:4) and SM(d18:1/24:1) were significantly increased in immobilized muscle as compared to levels in control muscle (Fig. 4B).

3.6 Specific localization of PC

We finally performed MS imaging to visually pinpoint lipid localization. We selected three molecular species of PCs, namely PC(16:0/18:2) at m/z 796.56, PC(16:0/18:1) at m/z 798.56, and PC(18:0/20:4) at m/z 848.58. Consecutive cryosections stained with MyHC isoforms were overlaid to fix the morphological information. The highest intensity was detected at m/z 796.56, which could be assigned as PC(16:0/18:2), since it was detected through the sections...
ubiquitously. We observed that PC(16:0/18:1) was predominately localized to the surface area rather than the deep area of the gastrocnemius muscle. In contrast, PC(18:0/20:4) was mainly localized to the deep area (Fig. 6). These specific localization patterns might be related to lipid dynamics specific to muscle fiber types. Not only quantitative differences in lipid components, but also their differential localization, are essential to elucidate lipid dynamics.

4 Discussion

We previously demonstrated the dynamic lipid changes due to muscle atrophy induced by a high-fat diet, in addition to revealing the characteristic accumulation of lipids. However, these models differed in terms of the intake of lipids, and it was very difficult to reveal lipid dynamics in the muscle. Therefore, we realized that we needed to develop another animal model to efficiently elucidate lipid dynamics in the muscle. Further, in our previous study, we developed a "disuse" muscle atrophy model via 2 weeks of immobilization of the animal’s hind limb joints. We observed a significant reduction (0.6-fold) in the gastrocnemius muscle of this model. In the present study, we reduced the period of immobilization to 8 days to validate lipid changes involved in the early stages of muscle atrophy. Regardless of the short period of immobilization, the muscle weight and CSA of myofibers were significantly reduced in immobilized muscle as compared to those in controls. In various muscle atrophy models, the effect of atrophy has been reported to be muscle fiber-specific; for example, only MyHC type IIx is significantly reduced in quadriceps of patients with chronic obstructive pulmonary disease and MyHC type IIx CSA is significantly reduced in the muscles of spinal cord injury models. To pinpoint which of these muscle fibers exhibited atrophic features, we utilized multiple immunofluorescence studies. Interestingly, all muscle fibers of all types (MyHC type I, IIa, IIx, and IIb) were reduced approximately 0.5–0.7-fold in our model. Furthermore, some reports represented muscle fiber shift from slow to fast and/or fast to slow by muscle atrophy. In our model, the muscle fiber ratio did not change by immobilization (data not shown). We thought that only 8 days were not sufficient to exhibit different protein expression pattern. Therefore, we concluded that the "disuse" atrophy model led to severe muscle atrophy, wherein all myofibers showed atrophic features.

Intramuscular TG accumulation is known to be involved in insulin resistance and obesity; it reportedly occurs in a muscle atrophy model. Furthermore, the amount of intramuscular TG was found to decrease after exercise, indicating its association with increased insulin sensitivity due to exercise.
In the present study, we observed that TG tended to increase with immobilization (Fig. 3A), similar to that in a previous report. Therefore, we suggest that the accumulation of TG is a typical feature of muscle atrophy. We know that lipid bioactivity differs depending upon the type of acylated fatty acid. For example, palmitoleic acid is involved in the activation of phosphatidylinositol-3 kinase signaling and contributes to an improvement in insulin resistance. However, AA inhibits insulin signaling by stimulating the p38 Mitogen-activated Protein Kinase pathway. Hence, we analyzed the lipids that were increased with respect to their molecular species. Our LC-MS analyses detected 24 kinds of TG molecular species, of which almost all were significantly increased, but no molecular specificity in the accumulated TGs was observed. Furthermore, we elucidated the mechanism underlying lipid accumulation by studying the expression level of ATGL. We found that ATGL was significantly reduced with immobilization (Fig. 3C). Previous studies have reported that exercise training increases the expression of ATGL, which promotes the lipolysis of TGs. We conclude that all TG molecular species were accumulated owing to suppressed ATGL expression triggered by inactivity and reduced energy metabolism. We consider that further investigations to determine the regulation of CGI-58 and G0S2, which modulate the expression of ATGL, are necessary.

PS is a membrane-constituting lipid that is known to play an important role during muscle fusion. Previous studies have shown that PS is increased in muscle atrophy models. However, these reports did not distinguish the molecular species that increased. Subsequently, we analyzed molecular species of polar lipids that increased in the present model. Some reports suggested that PS and PS-binding proteins such as Stabilin-2 are very important for muscle fusion, and we will evaluate the increase in PS molecular species that could bind to such proteins in future study.

In the same manner, we detected a specific increase in AA-containing PCs, for which results were consistent with those of the high-fat fed atrophy model, and hence, we suggest that the increase in AA-containing PCs is one of the universal lipid changes that occurs due to muscle atrophy. Previous reports demonstrated that supplementation with AA to C2C12 cells promotes myoblast fusion and is associated with hypertrophic myofibers. In our model, total intake of fatty acids was equal between control (left) and immobilized (right) limbs, and we realized that the increase in AA-containing PCs led to a decrease in free AA, which might be related to the suppression of cell proliferation.

A previous report found that AA supplementation contributes to the activation of COX-2. COX has two isotypes, namely COX-1 and COX-2. COX-1 is ubiquitously present and involved in cell homeostasis, whereas COX-2 is involved in hypertrophy in muscle and is related to chronic exercise. From previous studies, it is known that Prostaglandin E2 levels are increased in muscle atrophy models, such as rheumatoid arthritis. However, it has been confirmed that Prostaglandin F2a (PGF2a) levels increase with the increase in muscle protein synthesis due to exercise. This suggests that PGF2a is involved in muscle hypertrophy, which is catalyzed by the COX-2 pathway. Our model investigating the early stage of immobilization revealed downregulation of the COX-2 pathway. We hypothesized that this phenomenon demonstrated the decrease in free AA in the immobilized limb. Alvarez et al. showed that inflammation induced by atrophy triggers the activation of COX-2 to promote proliferation via interleukin-1β and tumor necrosis factor-α. We postulated that the activity of COX-2 might increase in the later phase of immobilization.

In addition, we observed the specific localization of PCs by MS imaging, since each PC molecule is distributed differently through the muscle, as described in our study previously. We found that AA-containing PC, one of the lipid molecules, is significantly modulated by muscle atrophy, showing specific localization in the deep region that consists of slow-twitch fibers (Fig. 6). Hence, further studies are required to evaluate location-specific lipid changes due to muscle atrophy. Furthermore, muscle fiber shifts might occur in some muscle hypertrophy/atrophy models. However, we acknowledge the limitation that in our model, only 8 days of immobilization was studied, which is not enough time to shift muscle fibers; however, monitoring the phospholipid composition of these myofibers might support the possibility of muscle fiber shifts with our short immobilization model.

5 Conclusion

We demonstrated that muscle atrophy develops with 8 days of immobilization of the limb, which was associated with significant changes in lipid dynamics, with the accumulation of all TGs and specific phospholipids. In summary, we revealed the accumulation of specific lipids and determined their changes at the molecular species level. Further, we suggest that a reduction in ATGL is related to TG accumulation and that the inhibition of COX-2 is related to the accumulation of AA-containing phospholipids and the downregulation of protein synthesis. Our study holds implications in validating preventive treatments for muscle atrophy.
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References
1) Frontera, W.R.; Ochala, J. Skeletal muscle: A brief review of structure and function. Calcif. Tissue Int. 96, 183-195 (2015).
2) Lípina, C.; Hundal, H.S. Lipid modulation of skeletal muscle mass and function. J. Cachexia Sarcopenia Muscle 8, 190-201 (2017).
3) Chaves-Filho, A.B.; Pinto, I.F.D.; Dantas, L.S.; Xavier, A.M.; Inague, A.; Farja, R.L.; Medeiros, M.H.G.; Glezer, I.; Yoshinaga, M.Y.; Miyamoto, S. Alterations in lipid metabolism of spinal cord linked to amyotrophic lateral sclerosis. Sci. Rep. 9, 11642 (2019).
4) Fan, Z.; Xiao, Q. Impaired autophagic flux contributes to muscle atrophy in obesity by affecting muscle degradation and regeneration. Biochem. Biophys. Res. Commun. 525, 462-468 (2020).
5) Vigelsø, A.; Gram, M.; C. Wiufl, C.; Hansen, C.N.; Prats, C.; Dela, F.; Helge, J.W. Effects of immobilization and aerobic training on proteins related to intramuscular substrate storage and metabolism in young and older men. Eur. J. Appl. Physiol. 116, 481-494 (2016).
6) Kumar, R.; Sharma, S. Lipid profile changes in mouse gastrocnemius muscle after denervation and beta-adrenoceptor stimulation. Indian J. Exp. Biol. 47, 314-319 (2009).
7) Goto-Inoue, N.; Yamada, K.; Inagaki, A.; Furuchi, Y.; Ogino, S.; Manabe, Y.; Setou, M.; Fujii, N. Lipidomics analysis revealed the phospholipid compositional changes in muscle by chronic exercise and high-fat diet. Sci. Rep. 3, 3267 (2013).
8) Sitnick, M.T.; Basantani, M.K.; Cai, L.; Schoiswohl, G.; Yazbeck, C.F.; Distefano, G.; Ritov, V.; Delany, J.P.; Schreiber, R.; Stolz, D.B.; Gardner, N.P.; Kienesberger, P.C.; Pulilirikumil, T.; Zechner, R.; Goodpaster, B.H.; Coen, P.; Kershaw, E.E. Skeletal muscle triacylglycerol hydrolysis does not influence metabolic complications of obesity. Diabetes 62, 3350-3361 (2013).
9) Kakehi, S.; Tamura, Y.; Takeo, K.; Sakurai, Y.; Kawaguchi, M.; Watanabe, T.; Funayama, T.; Sato, F.; Ikeda, S.; Kanazawa, A.; Fujitani, Y.; Kawamori, R.; Watada, H. Increased intramyocellular lipid/impaired insulin sensitivity is associated with altered lipid metabolic genes in muscle of high responders to a high-fat diet. Am. J. Physiol. Endocrinol. Metab. 310, E3240 (2016).
10) Selathurai, A.; Kowalski, G.M.; Mason, S.A.; Callahan, D.L.; Poletta, V.C.; Della Gatta, P.A.; Lindsay, A.; Hanley, S.; Kaur, G.; Curtis, A.R.; Burch, M.L.; Ang, T.; McGee, S.L.; Bruce, C.R. Phosphatidylserine decarboxylase is critical for the maintenance of skeletal muscle mitochondrial integrity and muscle mass. Mol. Metab. 27, 33-46 (2019).
11) Goto-Inoue, N.; Hayakawa, T.; Taki, T.; Gonzalez, T.V.; Setou, M. A new lipidomics approach by thin-layer chromatography-blot-matrix-assisted laser desorption/ionization mass spectrometry for analyzing detailed patterns of phospholipid molecular species. J. Chromatogr. A 1216, 7096-7101 (2009).
12) Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685 (1970).
13) Fujitani, M.; Mizushige, T.; Kawabata, F.; Uozumi, K.; Yasui, M.; Hayamizu, K.; Uchida, K.; Okada, S.; Keshab, B.; Kishida, T. Dietary Alaska pollack protein improves skeletal muscle weight recovery after immobilization-induced atrophy in rats. PLoS One 14, e0217917 (2019).
14) Morisasa, M.; Goto-Inoue, N.; Sato, T.; Machida, K.; Fujitani, M.; Kishida, T.; Uchida, K.; Mori, T. Investigation of the lipid changes that occur in hypertrophic muscle due to fish protein-feeding using mass spectrometry imaging. J. Oleo Sci. 68, 141-148 (2019).
15) Borgia, D.; Malena, A.; Spinazzi, M.; Desbats, M.A.; Salvati, L.; Russell, A.P.; Miotto, G.; Tosatto, L.; Pegoraro, E.; Sorarù, G.; Pennuto, M.; Vergani, L. Increased mitophagy in the skeletal muscle of spinal and bulbar muscular atrophy patients. Hum. Mol. Genet. 26, 1087-1103 (2017).
16) Gosker, H.R.; Engelen, M.P.; van Mameren, H.; van Dijk, P.J.; van der Vusse, G.J.; Wouters, E.F.; Schols, A.M. Muscle fiber type IIX atrophy is involved in the loss of fat-free mass in chronic obstructive pulmonary disease. Am. J. Clin. Nutr. 76, 113-119 (2002).
17) Mantilla, C.B.; Greising, S.M.; Zhan, W.Z.; Seven, Y.B.; Sieck, G.C. Prolonged C2 spinal hemisection-induced inactivity reduces diaphragm muscle specific force with modest, selective atrophy of type IIX and/or IIb fibers. J. Appl. Physiol. (1985) 114, 380-386 (2013).
18) Yoshihara, T.; Machida, S.; Kurosa, Y.; Kakigi, R.; Sugiyama, T.; Naito, H. Immobilization induces nuclear accumulation of HDAC4 in rat skeletal muscle. J. Physiol. Sci. 66, 337-343 (2016).
19) Hord, J.M.; Garcia, M.M.; Farris, K.R.; Guzzoni, V.; Lee, Y.; Lawler, M.S.; Lawler, J.M. Nox2 signaling and muscle fiber remodeling are attenuated by losartan administration during skeletal muscle unloading. Physiol. Rep. 9, e14606 (2021).
20) Laurentius, T.; Kob, R.; Fellner, C.; Nourbakhsh, M.;
Bertsch, T.; Sieber, C.C.; Bollheimer, L.C. Long-chain fatty acids and inflammatory markers coaccumulate in the skeletal muscle of sarcopenic old rats. Dis. Markers 2019, 940789 (2019).

21) Turnbull, P.C.; Longo, A.B.; Ramos, S.V.; Roy, B.D.; Ward, W.E.; Peters, S.J. Increases in skeletal muscle ATGL and its inhibitor G0S2 following 8 weeks of endurance training in metabolically different rat skeletal muscles. Am. J. Physiol. Regul. Integr. Comp. Physiol. 310, R125-133 (2016).

22) Talbot, N.A.; Wheeler-Jones, C.P.; Cleasby, M.E. Palmitoleic acid prevents palmitic acid-induced macrophage activation and consequent p38 MAPK-mediated skeletal muscle insulin resistance. Mol. Cell Endocrinol. 393, 129-142 (2014).

23) Talukdar, I.; Szeszel-Fedorowicz, W.; Salati, L.M. Arachidonic acid inhibits the insulin induction of glucose-6-phosphate dehydrogenase via p38 MAP kinase. J. Biol. Chem. 280, 40660-40667 (2005).

24) Park, S.Y.; Y. Yun.; J.S. Lim.; M.J. Kim.; S.Y. Kim.; J.E. Kim and I.S. Kim. Stabilin-2 modulates the efficiency of myoblast fusion during myogenic differentiation and muscle regeneration. Nat Commun 7: 10871 (2016).

25) Markworth, J.F.; Cameron-Smith, D. Arachidonic acid supplementation enhances in vitro skeletal muscle cell growth via a COX-2-dependent pathway. Am. J. Physiol. Cell Physiol. 304, C56-67 (2013).

26) Oak, N.R.; Gumucio, J.P.; Flood, M.D.; Saripalli, A.L.; Davis, M.E.; Harning, J.A.; Lynch, E.B.; Roche, S.M.; Bedi, A.; Mendias, C.L. Inhibition of 5-LOX, COX-1, and COX-2 increases tendon healing and reduces muscle fibrosis and lipid accumulation after rotator cuff repair. Am. J. Sports Med. 42, 2860-2868 (2014).

27) Trappe, T.A.; Liu, S.Z. Effects of prostaglandins and COX-inhibiting drugs on skeletal muscle adaptations to exercise. J. Appl. Physiol. (1985) 115, 909-919 (2013).

28) Alvarez, A.M.; DeOcesano-Pereira, C.; Teixeira, C.; Moreira, V. IL-1β and TNF-α modulation of proliferated and committed myoblasts: IL-6 and COX-2-derived prostaglandins as key actors in the mechanisms involved. Cells 9, 2005 (2020).

29) Goto-Inoue, N.; Morisasa, M.; Machida, K.; Furuichi, Y.; Fujii, N.L.; Miura, S.; Mori, T. Characterization of myofiber-type-specific molecules using mass spectrometry imaging. Rapid Commun. Mass Spectrom. 33, 185-192 (2019).

30) Kosek, D.J.; Kim, J.S.; Petrella, J.K.; Cross, J.M.; Baman, M.M. Efficacy of 3 days/wk resistance training on myofiber hypertrophy and myogenic mechanisms in young vs. older adults. J. Appl. Physiol. (1985) 101, 531-544 (2006).

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