博士論文

The effect of aldehyde dehydrogenase 2 deficiency on the adaptation of skeletal muscle

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Chapter 1

General Introduction

1-1. Skeletal muscle

1-1-1 physiological and clinical significances of skeletal muscle health

Skeletal muscle is the biggest tissue in the human body and comprises approximately 40% of body weight. The roles of skeletal muscle in health and diseases are underappreciated. First of all, the main role of skeletal muscle is to convert chemical energy into mechanical energy to make force and power, maintain posture, and produce movement that is the most important for independent life and furthermore the quality of life. Furthermore, from the metabolic perspective, skeletal muscle stores important substrates such as amino acids and carbohydrates and produce heat for maintaining temperatures, and regulates insulin balance (Wolfe et al., 2006, Frontera and Ochala, 2015). Recent studies have well reported the role of skeletal muscle as a secretory organ of cytokines and other peptides (Pratesi et al., 2013).

Muscle loss is a severe disorder that worsens with age and accelerates with inactivity (bed rest) and other systematic conditions such as cancer, cardiovascular disorders, etc.

Primarily, muscle loss occurs from an acceleration of protein degradation, and is often combined with decreased protein synthesis (Cohen et al., 2015). The balance of protein synthesis and breakdown determines muscle mass, and both are affected by factors such as diet, hormones, physical activity/inactivity, injury, and chronic diseases, etc. In both directions, health and muscle mass are deeply related. Furthermore, it has been reported that the survival probability (mortality rate) is significantly determined by muscle mass (Srikanthan and Karlamangla, 2014, Figure 1-1). The paper strongly suggests that maintaining skeletal muscle affects directly to mortality of human and to fully understand the metabolism of muscle atrophy is unavoidable challenge for healthy life span.

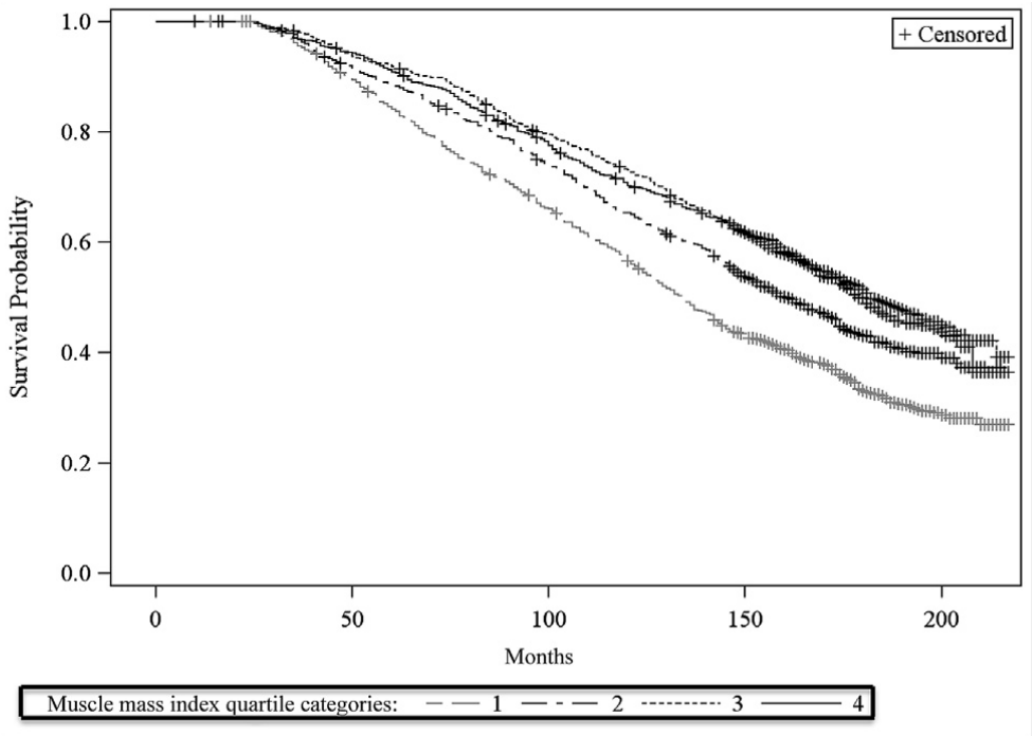

Figure 1-1. survival probability depending on muscle mass

This figure represents the difference in mortality between four groups divided by muscle mass. These 4 groups show notable differences.

(Srikanthan, P., & Karlamangla, A. S. 2014)

1-1-2 muscle plasticity

Repeated, episodic bouts of muscular contraction are powerful stimuli for physiological adaptation. Skeletal muscle exhibits extraordinary malleability in functional adaptation and remodeling in response to contractile action over time. Moreover, muscle contraction-induced adaptation occurring in muscle hypertrophy alters contractile protein and function, mitochondrial function, metabolic regulation, intracellular signaling, and transcriptional responses (Flück and Hoppeler, 2003). More specifically, protein accretion is driven by the regulation of protein translation and synthesis during muscle hypertrophy. Furthermore, satellite cell activation and integration promote the addition of freshly produced myofibrils to the contractile machinery (Tidball et al., 2005). The most important condition of muscle hypertrophy is that muscle protein synthesis has to exceed muscle protein breakdown by muscle contraction (Millward et al., 1976). It is strongly associated with the degree of p70S6K phosphorylation, which is downstream of mTORC1 signaling. The Insulin/IGF1-AKT-mTOR signaling (Figure 1-3) regulates cell growth and proliferation by integrating nutritional and metabolic stimuli (Goodman et al., 2011). Its pathway promotes translational processes,

increases the rate of muscle protein synthesis, and mediates muscle hypertrophy via protein accretion (Terzis et al., 2008).

As well as muscle hypertrophy, muscle atrophy occurs in the circumstance of unbalance of muscle protein synthesis and muscle protein breakdown. Skeletal muscle atrophy is characterized by a reduction in protein content, fiber diameter, force generation, and fatigue resistance, regardless of the triggering event. The many circumstances (aging, diseases, denervation, inactivity, etc.) inducing muscle atrophy indicate various biological triggers and signaling pathways for muscle loss (Jackman and Kandarian et al., 2003). The most common regulators of protein degradation are the ubiquitin-proteasome system (UPS) (Figure 1-4) and autophagy (Figure 1-5), as well as through suppression of anabolic pathways. UPS mostly attacks myofibrillar components and autophagy degrades mitochondria and soluble proteins. (Hoppeler, 2016). Furthermore, IGF1-Akt-FoxO signaling (Figure 1-3) upregulates Murf1 and Atrogin-1 and several autophagy related genes (Sandri et al., 2004).

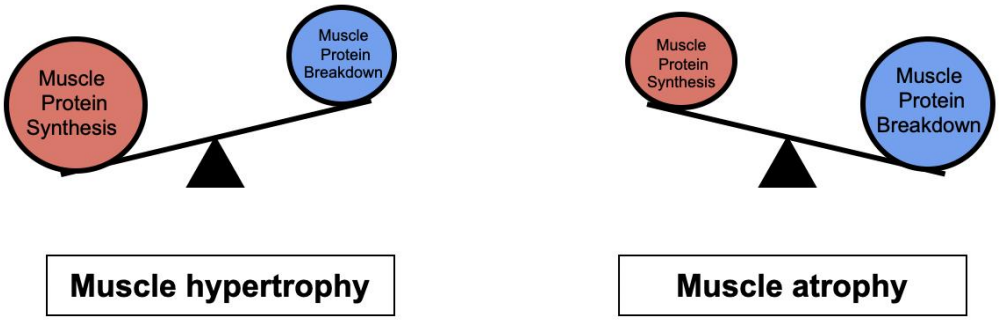

Figure 1-2. Muscle plasticity

Muscle mass is determined by the balance of muscle protein synthesis and muscle protein breakdown.

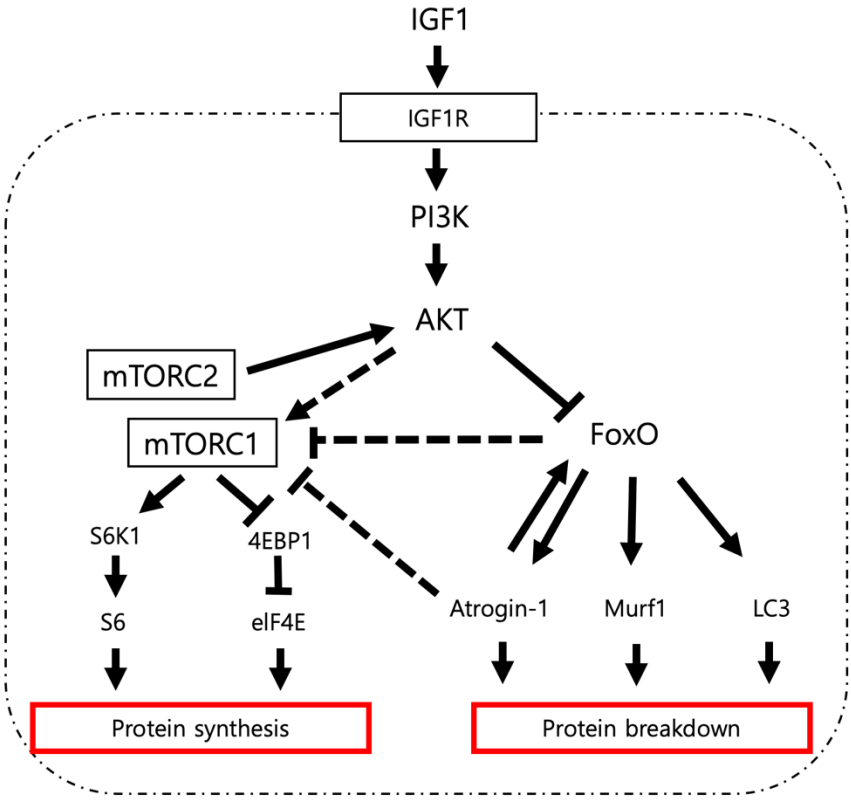

Figure 1-3. IGF-AKT-mTOR and IGF-AKT-FoxO signaling pathways

IGF-AKT-mTOR and IGF-AKT-FoxO signaling pathways are the most common pathways to regulate muscle protein metabolism.

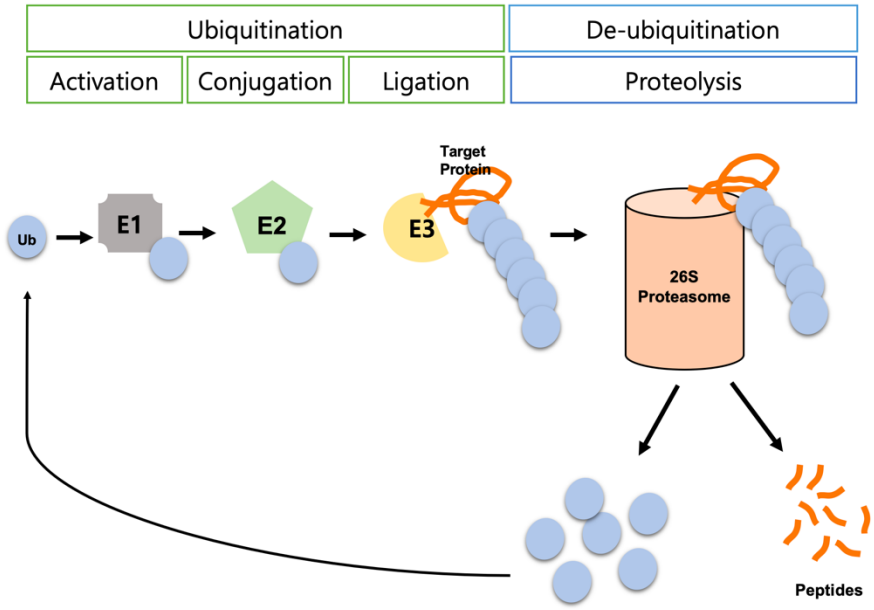

Figure 1-4. The Ubiquitin Proteasome System (UPS)

Proteasomes are a crucial process in degrading misfolded proteins which are unneeded and damaged.

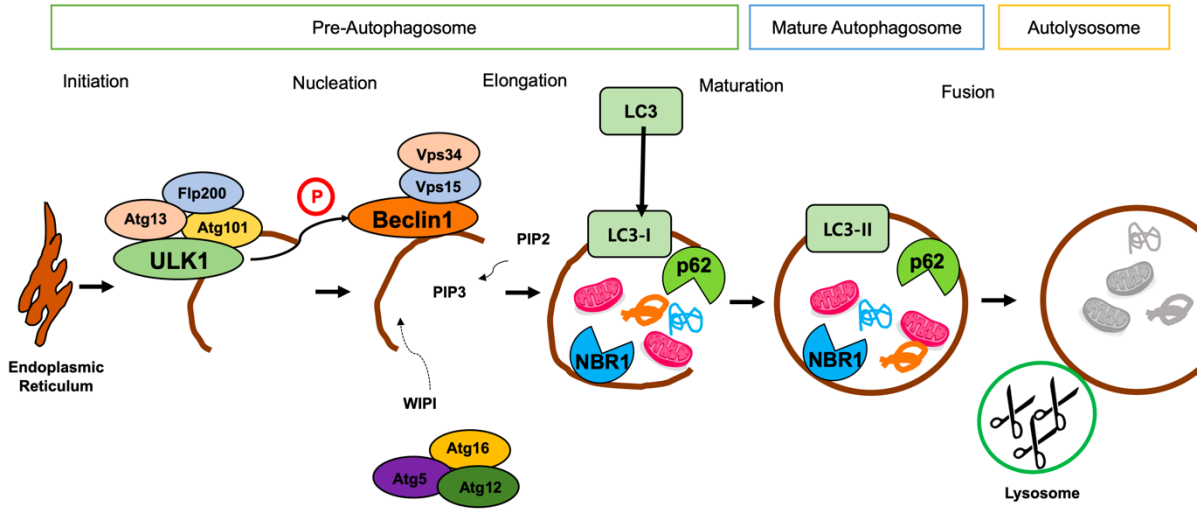

Figure 1-5. The process of autophagy

Autophagy is the lysosome-dependent degradation of cytosolic contents which are unnecessary or dysfunctional.

1-1-3 Mitochondrial dysfunction and muscle health

Mitochondria control numerous important biological processes in skeletal muscle metabolism such as energy supply, ROS production/signaling, calcium homeostasis and apoptosis (Brookes et al., 2004). It is not unexpected that many harmful disorders affecting the health of skeletal muscles have been linked to mitochondrial dysfunction. For instance, there are the sarcopenia (aging-induced muscle atrophy), disuse, inactivity, Duchenne muscular dystrophies, cachexia, chemotherapy-induced muscle dysfunction (Gouspillou and Hepple, 2016).

Mitochondria are the primary source of ROS in cells. ROS are produced as byproducts of aerobic respiration, particularly, in the electron transport chain of mitochondria (Figure 1-6) (Kirkinezos and Moraes, 2001). ROS, the highly reactive molecules, consists of a number of diverse chemical species including superoxide anion, hydroxyl radical, and hydrogen peroxide (Lee et al., 2004). Furthermore, it negatively affects DNA, protein, and lipid by causing oxidative deterioration. Thus, ROS is implicated as a cause of diseases because of its effect on not only mitochondria but also the health of whole cells and furthermore tissues (Cui

et al., 2012).

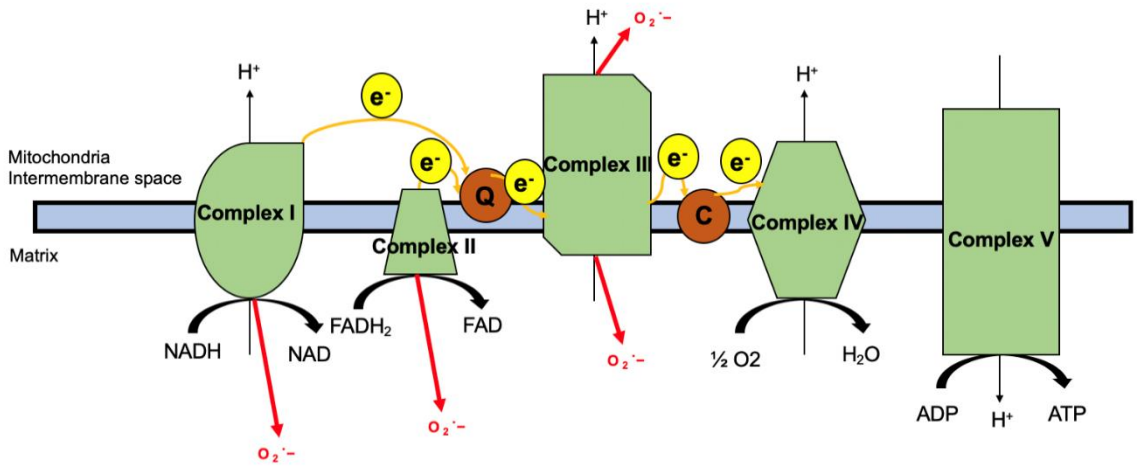

Figure 1-6. Mitochondrial respiration and ROS production

Respiration chain complexes generate superoxide from molecular oxygen within the mitochondrial intermembrane space.

From four decades ago, it has been identified that ROS plays a pathogenic role in muscular dystrophies and various muscular diseases (Murphy and Kehrer et al., 1986, Rochard et al., 2000, Tidball et al., 2005). There is a couple of evidence of the molecular mechanisms of how ROS alters muscle health. For instance, it is well known that oxidative signals affect mitochondrial biogenesis, morphology, and function in skeletal muscle cells (Irrcher et al.,2009). Furthermore, ROS may trigger either autophagy or apoptosis, or both. However, the cell context and availability of specific modulators of ROS activity determine these two pathways (Scherz-Shouval et al., 2007). Furthermore, there is some evidence that mitochondrial dysfunction contributes to muscle atrophy during inactivity. However, direct evidence of the relationship between mitochondrial dysfunction and muscle atrophy is lacking until recently. As the first evidence, it has been reported that the Knockout of proteins involved in mitochondrial fragmentation and degradation partially rescue muscle atrophy due to denervation (Romanello et al., 2010). However, the pathways of mechanisms that can fully understand it are still elusive (Hyatt et al., 2019). Moreover, it has also been demonstrated that ROS emission negatively impact muscle atrophy by regulating UPS and autophagy system

and the level of ROS in skeletal muscle can negatively impact the initiation of mRNA translation (Shenton et al., 2006).

1-1-4 summary

Skeletal muscle plays important roles as locomotor, metabolic, and/or endocrine organs. Maintaining muscle mass is an important key to maximizing a healthy life span. Furthermore, skeletal muscle has unique characteristics of plasticity that alters muscle mass and function through stimuli such as contractile stimulation, loading, nutrition, and environmental factors (aging and chronic diseases). The balance of muscle protein synthesis and breakdown determines muscle mass through several molecular mechanisms in the change of environments of skeletal muscle. To deeper understand muscle dynamics and plasticity, it is necessary to examine the change of skeletal muscle in a variety of circumstances such as muscle hypertrophy and atrophy. In addition, since mitochondrial regulate a lot of biological pathways in muscle metabolism, ROS which is the most important factor of mitochondrial dysfunction should be also well considered to understand muscle metabolism.

1-2. Aldehyde dehydrogenase 2 (ALDH2)

1-2-1 Molecular function of ALDH2

Aldehyde dehydrogenase (ALDH) has 19 ALDH isoforms in the Human genome. The ALDH superfamily is found in all three taxonomic domains (Archaea, Eubacteria, and Eukarya) indicating an important role in the evolution. Members of this superfamily, as the name indicates, metabolize both physiologically and pathophysiologically relevant aldehydes Its function prevents cells from exposure to toxic aldehydes and maintains cellular homeostasis and organismal functions (Singh et al., 2013). Moreover, ALDH superfamily functions extend beyond detoxification (Table 1). For instance, ALDH is also required for the synthesis of vital biomolecules through the metabolism of aldehyde intermediates such as retinoic acid, folate and, betaine (Jackson et al., 2011).

ALDH2 is one of the superfamily members and a 517-amino acid polypeptide encoded by a nuclear gene located at chromosome 12q24 (Raghunathan et al., 1988). The 17-amino acid mitochondrial targeting region of the protein, which is cleaved as part of the enzyme's complete folding and maturation inside the mitochondria, is required for the process of

delivering the protein to the mitochondrial matrix (Braun et al., 1987).

Table 1. the locations and catalytic functions of 19 ALDH family members

ALDH superfamily has 19 isoforms in human genome. The location of 19 isoforms are all different and its each function extends beyond aldehyde detoxification as well.

Gene	location	Catalytic function and phenotype
ALDH1A1	9q21.13(216)	Drug resistance, Alcohol sensitivity, Alcohol-induced flushing Parkinson disease and schizophrenia
ALDH1A2	15q21.3(8854)	Tumors, Early embryonic death
ALDH1A3	15q26.3(220)	Perinatal lethality
ALDH1B1	9q11.1(219)	Development, hemorrhagic shock
ALDH1L1	3q21.3(10840)	Tumors, Methanol toxicity, neural tube defect
ALDH1L2	12q23.3(160428)	Tumors
ALDH2	12q24.2(217)	Alcohol sensitivity, Cancer, Cardiovascular disorders
ALDH3A1	17q11.2(218)	Tumors, Sensitivity to UV light, Lens Opacification, and cataract Drug resistance
ALDH3A2	17q11.2(224)	Sjögren-Larsson syndrome
ALDH3B1	11q13(221)	Paranoid schizopherenia
ALDH3B2	11q13(222)	Unknown
ALDH4A1	1p36(8659)	Type II hyperprolinemia
ALDH5A1	6p22(7915)	Neurological disorder; 4-hydroxybutyric aciduria
ALDH6A1	14q24.3(4329)	Developmental delay
ALDH7A1	5q31(501)	Hyperosmotic stress, Pyridoxine-dependent epilepsy
ALDH8A1	6q23.2(64577)	Unknown
ALDH9A1	1q23.1(223)	Role in GABA and dopamine pathways Nonalcoholic steatohepatitis
ALDH16A1	19q13.33(126133)	Gout, Mast syndrome
ALDH18A1	10q24.3(5832)	Hypoprolinemia, hypoornithinemia, hypocitrulinemia, hypoargininemia, hyperammonemia and cataract formation, neurodegeneration, connective tissue anomalies

ALDH2, like the bulk of the ALDH family members, is a tetrameric enzyme with identical subunits of around 56 kDa. Since only two of each enzyme complex's catalytic sites are still active, the tetramer is referred to as a dimer of dimers. Each subunit is made up of three core domains: the catalytic domain, the coenzyme- or NAD+-binding domain, and the oligomerization domain. ALDH2 has reductase and esterase activities in addition to dehydrogenase activity (Mukerjee and Pietruszko, 1992). ALDH2 is present in all tissues but is especially numerous in the liver and organs that need strong mitochondrial oxidative phosphorylation, such as the heart, brain, and skeletal muscle (Oyama et al., 2005).

1-2-2 polymorphisms of ALDH2

The most relevant human ALDH2 variant is the ALDH2*2 (rs671) allele. ALDH2*2 carriers exhibit the well-known Asian flushing syndrome which is the symptom of face flushing, nausea, dizziness, and elevated blood pressure after consuming alcohol (Wall et al., 1992). ALDH2*2 polymorphism has been observed in 40% of East Asians and 8% of the global population. As a result, ALDH2*2 is one of the most frequent human enzyme deficiencies (Eng et al., 2007). Hence, ALDH2 biochemical and molecular characterizations have been intensively explored during the last few decades. Furthermore, it has been sufficiently reported that ALDH2 deficiency is highly related to chronic diseases such as Alzheimer's disease, cancer, cardiovascular diseases, etc (Chen et al., 2022). For instance, aldh2 mRNA expression increased in Alzheimer's disease patients of the Japanese population. The mutation of ALDH2 showed a significantly higher odd ratio in late-onset Alzheimer's diseases in the meta-analysis of the Asian population (Ueno et al., 2022). In addition, Epidemiological studies associate ALDH2*2 with tumorigenesis and progression (Zhang et al., 2021). Furthermore, an epidemiological study revealed that polymorphisms of ALDH2 rs671 are associated with

cardiovascular risk factors, especially hypertension and diabetes in males of the Chinese population (Wang et al., 2020).

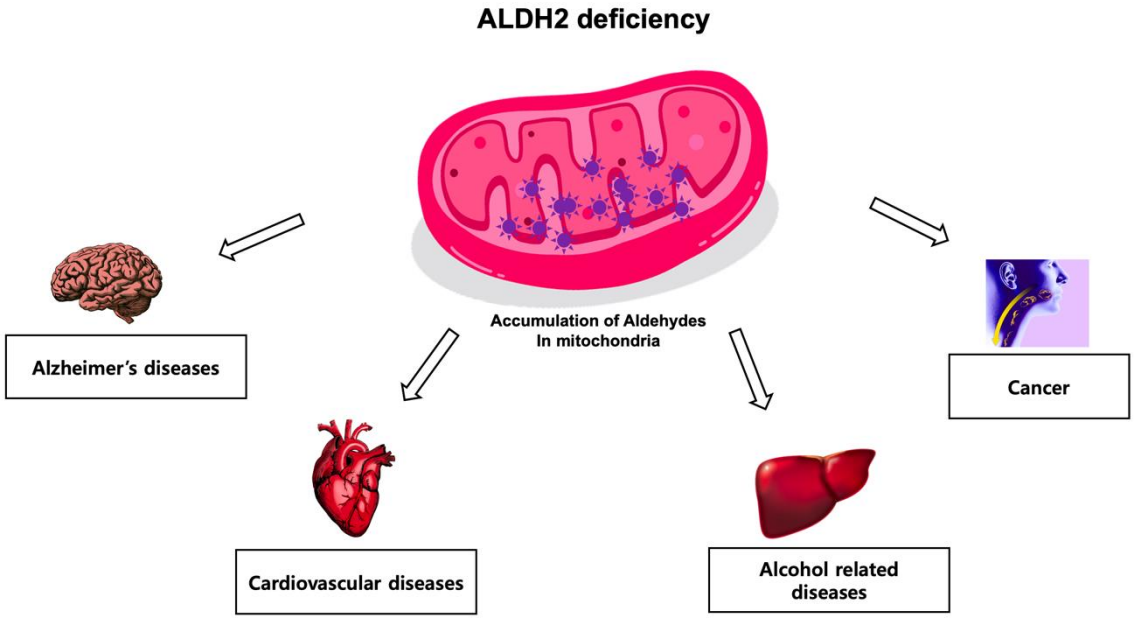

Figure 1-7. ALDH2 deficiency are highly related to a variety of chronic diseases

ALDH2 is located in tissues having sufficient mitochondria. The loss of ALDH2 function induces the accumulation of aldehydes in mitochondria and increases the risks for chronic diseases.

1-2-3 Previous studies about ALDH2 and skeletal muscle

While the association between ALDH2 deficiency and chronic diseases is sufficiently addressed, the effect of ALDH2 deficiency on skeletal muscle is still elusive. There are a few studies about ALDH2 polymorphism and skeletal muscle in human studies. A recent previous research clarified the relationship between the ALDH2 rs671 polymorphism and performance in power/strength athletes. As results, there was no difference in allele frequencies of ALDH2 polymorphism compared to controls. However, total record of powerlifters with GG genotype (WT) was significantly higher than GA + AA genotype (mutation allele) (Saito et al., 2022). Furthermore, there is another research about ALDH2 and athletic capacity subjecting people who are not elite athletes. GG + GA carriers (WT allele) have better muscle strength than those who carry the AA genotype (mutation) (Kikuchi et al., 2022). This research was replicated with the subjects over 55 years, therefore, the results showed same tendency indicating lower grip strength of AA carriers (De Almeida et al., 2022). The above three research suggest that the loss of ALDH2 function affect negatively the muscle strength.

There is not only research on human polymorphism but also research using rodent

models to examine the relationship between ALDH2 and skeletal muscle. The first research about ALDH2 deficiency and skeletal muscle was that anti-oxidant substrates called Chlorella have the potential to prevent age-related muscle atrophy using ALDH2*2 transgenic mice (Nakashima et al., 2014). After this research, it has been also reported that ALDH2 may reverse mitochondrial dysfunction in skeletal muscle due to exhaustive exercise using ALDH2-expressing transgenic mice (Zhang et al., 2017). Furthermore, it has been recently reported that ALDH2*2 transgenic mice have a smaller body and muscle size and lowered turnover of muscle protein metabolism (Kobayashi et al., 2021). These three previous studies suggested the possibility that ALDH2 is closely related to muscle metabolism. A previous study using KO mice showed that ALDH2 is sufficiently expressed in skeletal muscle and is located in the mitochondria of skeletal muscle. Moreover, ALDH2 deficiency increased the accumulation of ROS and damaged mitochondrial networking (Wakabayashi et al., 2020). In the KO aging model, it has been also reported that ALDH2 deficiency showed an increased tendency of ROS production and enhances aging-induced muscle atrophy, especially in the oxidative muscle (Kasai et al., 2022). Both previous studies indicate that ALDH2 deficiency negatively affects

muscle mass and function through decreased mitochondrial function.

1-2-4 summary

Mitochondrial ALDH2 is one of the ALDH superfamily members and metabolizes the toxic aldehydes to non-toxic acetic acid. ALDH2 express in the tissues having sufficient mitochondria such as the brain, liver, heart, and skeletal muscle. While ALDH2 mutation is well addressed as a risk factor for a variety of chronic diseases, the function of ALDH2 in skeletal muscle is barely known. There are some reports that ALDH2 mutation carriers have a lower capacity for strength in both athletes and non-athletes. Furthermore, ALDH2 deficiency increased ROS production and decreased mitochondrial function in the rodent model. Both human- and rodent model suggest that the loss of ALDH2 function negatively affect skeletal muscle.

1-3. Objectives

1-3-1 limitations of previous studies and necessities of the present study

The epidemiological findings of the above previous studies indicate important evidence that the loss of ALDH2 function might negatively affect muscle strength. However, there is little biological evidence to support these findings. Moreover, the research using a rodent model supports these epidemiological studies by showing decreased mitochondrial function and increased ROS production in ALDH2 KO mice. However, as mentioned above, muscle is a highly plastic tissue. Thus, a deeper understanding of the role of ALDH2 during muscle hypertrophy and atrophy is necessary for the comprehension of the physiological characteristics of people with functional ALDH2 deficiency. For instance, understanding how ALDH2 deficiency affects muscle metabolism during muscle atrophy might help to establish of personalized therapeutic approach for people with functional ALDH2 deficiency. Moreover, the effect of ALDH2 deficiency during muscle hypertrophy might explain the different training effects between ALDH2 WT and mutation. Furthermore, metabolic abnormalities of muscle affect tissues other than just the skeletal muscle, thus understanding the role of ALDH2 in

muscle mitochondria seems necessary from the pathological perspective.

1-3-2 purpose of the study

Based on the background in chapter 1, I found that ALDH2 deficiency affects negatively maintaining muscle homeostasis because of the alteration of mitochondrial function. Furthermore, its alteration might impact muscle mass. Therefore, I speculate that ALDH2 deficiency might adversely alter the adaptation of skeletal muscle, especially during muscle atrophy. In this thesis, I will focus on the adaptation of skeletal muscle using the muscle atrophy and hypertrophy model.

Chapter 2: To measure the adaptabilities of gene and protein expression of ALDH2 in different conditions of muscle activities such as muscle atrophy and muscle hypertrophy.

It is necessary to confirm whether there are possibilities that ALDH2 physiologically intervenes in muscle metabolism during muscle atrophy and hypertrophy. Thus, I aimed to examine whether denervation-induced muscle atrophy changes the gene and protein expression

of ALDH2 and then whether mechanical overload-induced muscle hypertrophy alters the gene and protein expression of ALDH2.

Chapter 3: To examine whether ALDH2 deficiency alters denervation-induced muscle protein breakdown and muscle atrophy using ALDH2 KO mice.

Then, I focused on the adaptation of skeletal muscle during muscle atrophy. Referencing the results of chapter 2, I replicated the experimental protocol and analyzed muscle mass, markers of muscle protein breakdown and synthesis, and mitochondrial function.

Chapter 4: To investigate whether ALDH2 deficiency changes mechanical overload-induced muscle protein synthesis and muscle hypertrophy using ALDH2 KO mice.

Finally, I examined the adaptation of skeletal muscle during muscle hypertrophy. As in chapter 3, the experiment of synergist ablation was replicated with the same condition. In this chapter, I focused on analyzing markers of muscle protein synthesis and the rate of muscle protein synthesis.

Chapter 2

Title

Effect of muscle atrophy and hypertrophy on the gene and protein expression of ALDH2 in C57BL/6J mouse skeletal muscle

2-1. Abstract

Aldehyde dehydrogenase 2 (ALDH2) is an enzyme that detoxifies aldehydes and is primarily involved in alcohol metabolism. Recently, it has been reported that ALDH2 also plays an important role in skeletal muscle homeostasis. To better understand the role of ALDH2 in skeletal muscle, it is necessary to clarify the adaptability of ALDH2 during muscle activity and inactivity. In this study, I examined the effects of denervation-induced atrophy and compensatory hypertrophy by synergist ablation (SA) on gene and protein levels of ALDH2 in skeletal muscle. Ten-week-old C57BL/6J mice were subjected to each intervention and the plantaris muscle was collected. Denervation increased both the Aldh2 gene and ALDH2

protein levels. Furthermore, the gene expression of Aldh2 was decreased after SA, but ALDH2 protein levels remained unchanged. Taken together, both interventions of muscle hypertrophy and muscle atrophy altered ALDH2 activities in skeletal muscle. It indicates that ALDH2 might be involved in muscle metabolism.

2-2. Introduction

Skeletal muscle is a highly plastic tissue, and this plasticity is strongly dependent on the mode of contraction (Hoppeler et al. 2016). For example, resistance training induces skeletal muscle hypertrophy and physical inactivity induce muscle atrophy. The contractile activities produced by exercise and training are useful for improving the mass and function of skeletal muscle (Holm et al. 2008). However, decreased contractile activity of skeletal muscle caused by nerve injury, aging, or disease may decrease muscle mass and function (Degens and Alway 2006). The molecular mechanisms underlying skeletal muscle plasticity are not fully understood. To better understand the pathogenesis of muscle disorders and develop effective exercise therapy, it is necessary to elucidate the direct responses and adaptations that regulate skeletal muscle plasticity. Furthermore, the responses and adaptations of skeletal muscle at the cellular and molecular levels require investigation from a broader perspective.

ALDH2 is a key factor in endogenous and exogenous aldehyde detoxification and has been thought to be mainly involved in alcohol metabolism in the liver (Jackson et al. 2011). It has been recently reported that skeletal muscle from mice lacking ALDH2 has elevated levels

of reactive oxygen species (ROS), which are associated with mitochondria oxygen consumption and increased expression of the mitochondrial serine protease Omi/HtrA2 (Wakabayashi et al. 2020). Recent reports from other investigators have also revealed that ALDH-positive cells are increased in skeletal muscle from Duchenne muscular dystrophy patients, and the expression pattern of ALDH2 may be related to the ability of the skeletal muscle to differentiate (Etienne et al. 2020). These findings suggest that ALDH2 and ALDH2-mediated metabolic processes in skeletal muscle may be novel factors that regulate skeletal muscle homeostasis. However, there have been few studies on ALDH2 in skeletal muscle.

In the chapter 2, the aim of this study is to clarify the adaptability of ALDH2 in skeletal muscle. To achieve this objective, denervation-induced muscle atrophy and compensatory hypertrophy were used as experimental models. Since it is possible to use these surgical methods to induce unilateral atrophy and hypertrophy and compare the left leg and right leg in one mouse, thus, I have chosen these two methods. By examining these two models, new insights can be obtained regarding the adaptation of ALDH2 to muscle atrophy and hypertrophy and changes in skeletal muscle size.

2-3. Materials and Methods

Ethical Approval

All animal experiments were approved by the Animal Experimental Committee of Nippon Sport Science University (no. 018-A03 and no. 020-A05). All authors have read, and all experiments complied with the regulations of the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions published by the Ministry of Education, Culture, Sports, Science and Technology, Japan (Notice no. 71, 2006).

Animals

Ten-week-old male C57BL/6J mice (CLEA Japan; Tokyo, Japan) were used for the analyses of denervation (muscle atrophy model) and synergist ablation (muscle hypertrophy model). Mice were housed in ventilated cages at 22 °C (5–6 mice/cage; cage size, 19.7 cm × 37.5 cm × 13.3 cm) and exposed to a 12 h light–dark cycle (dark from 18:00 to 06:00). All mice had access to standard chow (CE-2; CLEA Japan) and water ad libitum. All mice were

sacrificed by cerebral dislocation throughout this study.

Muscle atrophy protocol for denervation (DEN)

Twelve mice (3-day and 7-day recovery groups: n=6/group) were used for the DEN model of muscle atrophy. The mice were anesthetized with isoflurane (2.5%, 300 mL/min) during the surgery. The DEN surgery involved removing approximately 0.8 cm of the sciatic nerve proximal to the bifurcation of the tibial and peroneal nerves. The sham surgery was performed on the left leg and comprised making an incision at the same location as that in the right leg, finding the sciatic nerve, and then closing the incision. After a 3- or 7-day recovery period, the plantaris muscles of both legs were isolated (Fig. 2-1). Collected muscle tissues were immediately frozen in liquid nitrogen until further analyses.

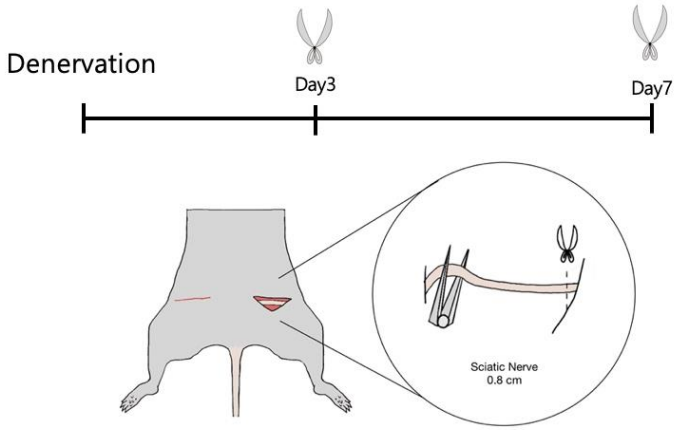

Figure 2-1 Experimental protocol and surgical method of denervation

Muscle hypertrophy protocol for synergist ablation (SA)

Fourteen mice (3-day and 7-day recovery groups: n=7/group) were used for the SA model to study muscle hypertrophy. The mice were anesthetized with isoflurane (2.5%, 300 mL/min) during the surgery. The SA surgery involved the unilateral removal of the soleus muscle and distal half of the gastrocnemius muscle in the right leg of each mouse (Goodman et al. 2011). The sham surgery consisted of opening and then closing an incision on the lower left leg. After the surgical procedures, the incisions were closed with surgical glue. The mice were allowed 3 or 7 days to recover, and then the plantaris muscles of both legs were collected (Fig. 2-2). Collected muscle tissues were immediately frozen in liquid nitrogen until further analyses.

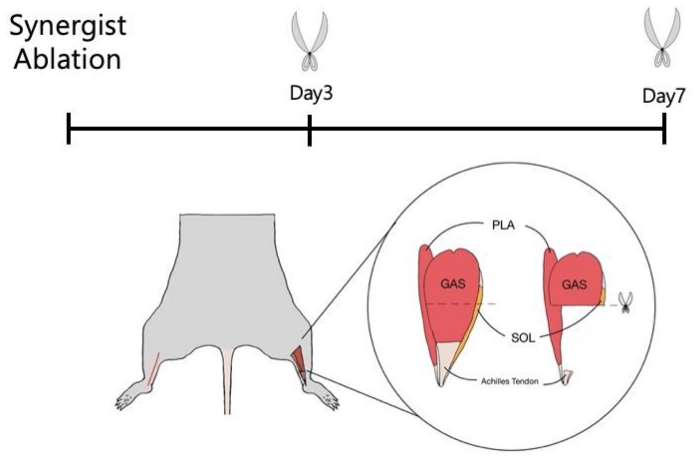

Figure 2-2 Experimental protocol and the surgical method of synergist ablation

RNA extraction, cDNA preparation, and real-time quantitative polymerase chain reaction (PCR)

RNA was isolated from the plantaris muscle as previously described (Kitaoka et al., 2016). Briefly, the plantaris muscle was homogenized in Trizol reagent (Thermo Fisher Scientific; Waltham, MA, USA) on ice and then separated into organic and aqueous phases using chloroform. Total RNA was isolated from the aqueous phase following precipitation with ethanol using a commercial kit (RNA Basic Kit; Cat no. FG-80050; Nippon Genetics Co., Ltd; Japan). RNA concentrations were measured by spectrophotometry (Nanodrop One, Thermo Fisher Scientific).

First-strand complementary DNA (cDNA) synthesis from 1 μg of total RNA was performed with random hexamer primers using a high-capacity cDNA reverse transcription kit (ReverTra Ace®; Toyobo Corp.; Osaka, Japan). The gene expression of aldehyde dehydrogenase 2 (Aldh2), F-box protein 32 (Fbxo32; also known as muscle atrophy F-box gene or atrogin-1), and the E3 ubiquitin-protein ligase gene Trim63 (also known as muscle-specific ring finger 1 or MuRF1) was quantified using the Thermal Cycler Dice Real-Time

System (Takara Bio, Inc.; Shiga, Japan). The TATA-binding protein gene (Tbp) was used as a control housekeeping gene. The primer sequences used in the PCR analyses were: Aldh2 (Forward : 5′ GCTGGGCTGACAAGTACCAT 3′, Reverse: 5′ TTGATCAAGTTGGCCACGTA 3′), Fbxo32 (Forward: 5′ AAGGAGCGCCATGGATACTG 3′, Reverse: 5′ AGCTCCAACAGCCTTACTACG 3′), Trim63 (Forward: 5′ GACATCTTCCAGGCTGCCAA 3′, Reverse: 5′ TGCCGGTCCATGATCACTTC 3′), Tbp (Forward: 5′ CTGCCACACCAGCTTCTGA 3′, Reverse: 5′ TGCAGCAAATCGCTTGGG 3′).

Western Blot Analysis

Western blot analysis was performed as previously described (Tomiya et al. 2019). Briefly, muscle samples were homogenized in radioimmunoprecipitation buffer (188-02453; Fujifilm Wako Pure Chemical; Osaka, Japan) containing a protease and phosphatase inhibitor cocktail (169-26063/167-24381; Fujifilm Wako Pure Chemical). The BCA method (295-78401, Fujifilm Wako Pure Chemical) was used to measure protein concentrations. Equal amounts (10 μg) of protein were separated using standard SDS-PAGE with 10% and 12% (wt/vol) TGX

polyacrylamide gels (161– 0173/161–0175; Bio-Rad; Hercules, CA, USA) and transferred to polyvinylidene difluoride (PVDF) membranes (IPVH00010; Merck Millipore; Burlington, MA, USA). The ponceau S reagent (33427.01; SERVA Electrophoresis; Heidelberg, Germany) was used to confirm complete transfer of proteins to the PVDF membrane. Membranes were blocked with blocking reagent (NYPBR01; Toyobo Corp.) for 1 h and incubated for 1 h with primary antibodies diluted in Can Get Signal Reagent 1 (NKB-101, Toyobo Corp.). The antibodies used in this study are listed in Table 1. Membranes were washed with Tris-buffered saline containing 0.01% Tween 20 (TBST; T9142; Takara Bio, Inc.), incubated for 1 h with secondary antibody diluted in Can Get Signal Reagent 2 (Toyobo Corp.), and washed 3 times with TBST. Proteins were detected using Chemiluminescent reagents (SuperSignal West Pico Chemiluminescent Substrate, Thermo Fisher Scientific). Blots were scanned and quantified using ChemiDoc XRS (170-8071, Bio-Rad) and Quantity One software (170-9600, version 4.5.2 for Windows, Bio-Rad). As a loading control, the Ponceau S signal intensity (25–150 kDa) was used.

Statistical Analysis

Data are presented as means ± SE. Paired (synergist ablation and denervation studies: Sham vs SA or DEN) Student's t-tests were conducted to compare two groups. Statistical significance was defined as $p < 0.05$. GraphPad Prism (v.8.4.3 for MacIntosh) was used for all statistical analyses and preparation of figures.

2-4. Results

Denervation-induced muscle atrophy increases ALDH2 in skeletal muscle

The denervation model was used to examine whether adaptation of ALDH2 was induced during skeletal muscle atrophy. I examined the 3- and 7-day time points to evaluate the early gene responses and later protein adaptations. While I found a downward trend in plantaris muscle mass on day 3 (Fig. 2-3A, 10.29 ± 4.9 % reduction, $p = 0.09$), a significant decrease was observed only on day 7 (Fig. 2-3B, 18.03 ± 5.4 % reduction, $p = 0.02$). The gene expression levels of the E3 ubiquitin ligase Trim63 (MuRF1) and Fbxo32 (Atrogin-1), which are involved in skeletal muscle atrophy, were unchanged on day 3 (Fig. 2-4A, $p = 0.2$ and Fig. 2-4B $p = 0.3$, respectively) but were found to be markedly increased on day 7 (Fig. 2-4C, +112.6 ± 15.4 %, $p = 0.0007$ and Fig. 2-4D, +64.7 ± 12.5 %, $p = 0.004$).

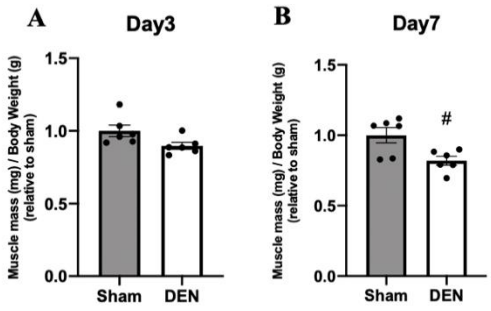

Figure 2-3 Muscle atrophy was successfully induced on day 7 by DEN. (A) The plantaris muscle weight did not decrease on day 3 after DEN. (B) The plantaris muscle weight significantly decreased by day 7 after DEN compared with that for the sham surgery. Data are presented as means ± SE. Paired t tests were performed to compare the Sham and DEN groups. #, $p < 0.05$.

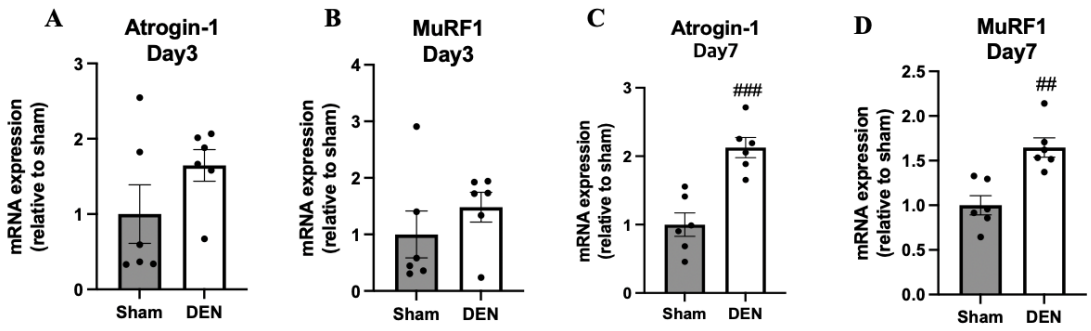

Figure 2-4 7 days of denervation increased mRNA expression of Atrogin-1 and MuRF1. (A-D) While 3 days of DEN did not change Atrogin-1 and MuRF1, 7 days of DEN significantly increased Atrogin-1 and MuRF1. Data are presented as means ± SE. Paired t tests were performed to compare the Sham and DEN groups. ##, $p < 0.01$; ###, $p<0.001$.

Compared with that in the sham group, both gene and protein expression of ALDH2 was significantly increased on day 7 after denervation (Fig. 2-5C, 58.8 ± 16.5 % increase, p = 0.02 and Fig. 2-5 D, 23.9 ± 6.9 % increase, p = 0.02, respectively), but not day 3 (Fig. 2-5A and 2-5B).

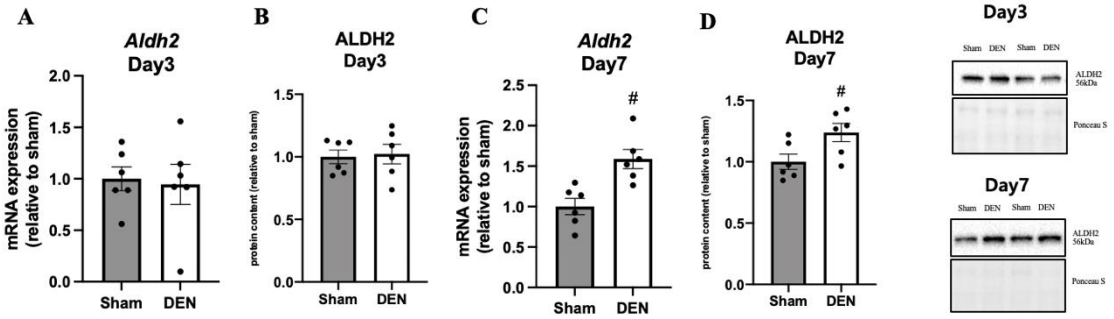

Figure 2-5 The gene and protein expressions of ALDH2 on the day3 and the day 7 after DEN. (A-B) DEN did not change gene and protein expression of ALDH2 on day 3. (C-D) DEN significantly increased gene and protein expression of ALDH2 on day 7. Data are presented as means ± SE. Paired t tests were performed to compare the Sham and DEN groups. #, $p < 0.05$.

Synergist Ablation induced muscle hypertrophy does not change gene and protein expression of ALDH2

I next investigated the adaptability of ALDH2 during the development of skeletal muscle hypertrophy after SA. Two time points after surgery (3 and 7 days) were examined to evaluate the early effects of SA on gene expression and the later adaptation of the protein. A previous study demonstrated that SA induced an increase in muscle mass and muscle protein synthesis (Roberts et al. 2020). Additionally, skeletal muscle overload after SA induced alterations in the protein expression of mammalian target of rapamycin complex 1 (mTORC1) (Chalé-Rush et al. 2009). Compared with that in the sham group, I observed increases in skeletal muscle weights on day 3 (Fig. 2-6A, +29.7 ± 5.5 %, p = 0.003) and day 7 (Fig. 2-6B, +44.7 ± 11.1 %, p = 0.009) after SA. Activation of mTORC1 signaling, which is involved in skeletal muscle hypertrophy, was demonstrated on days 3 and 7 by increases in phosphorylated p70S6K (Fig. 2-7A, 3343 ± 940.3 % , p = 0.01 and Fig. 2-7D, +378 ± 167.3 %, p=0.06, respectively), rpS6 (Fig. 2-7B, +907.5 ± 167.5 %, p = 0.001 and Fig. 2-7E, +57 ± 23.1 %, p = 0.05, respectively), and 4EBP1 (Fig. 2-7C, +42.1 ± 11.4 %, p = 0.01 and Fig. 2-7F, +130.3 ± 43.2 %, p = 0.02,

respectively). These data indicated that the expected responses and adaptations were observed.

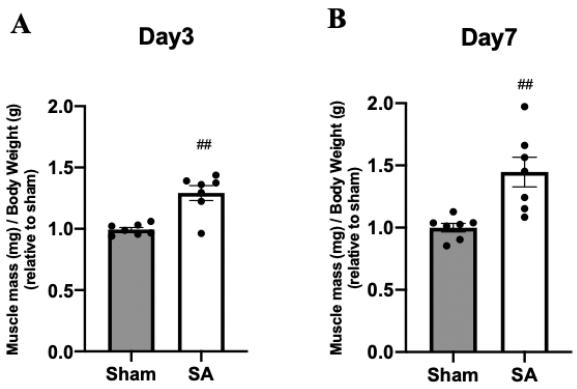

Figure 2-6 Muscle hypertrophy after SA was successfully induced. (A-B) SA increased plantaris muscle mass on day3 and day 7. Data are presented as means ± SE. Paired t tests were performed to compare the Sham and SA groups. ##, $p < 0.01$.

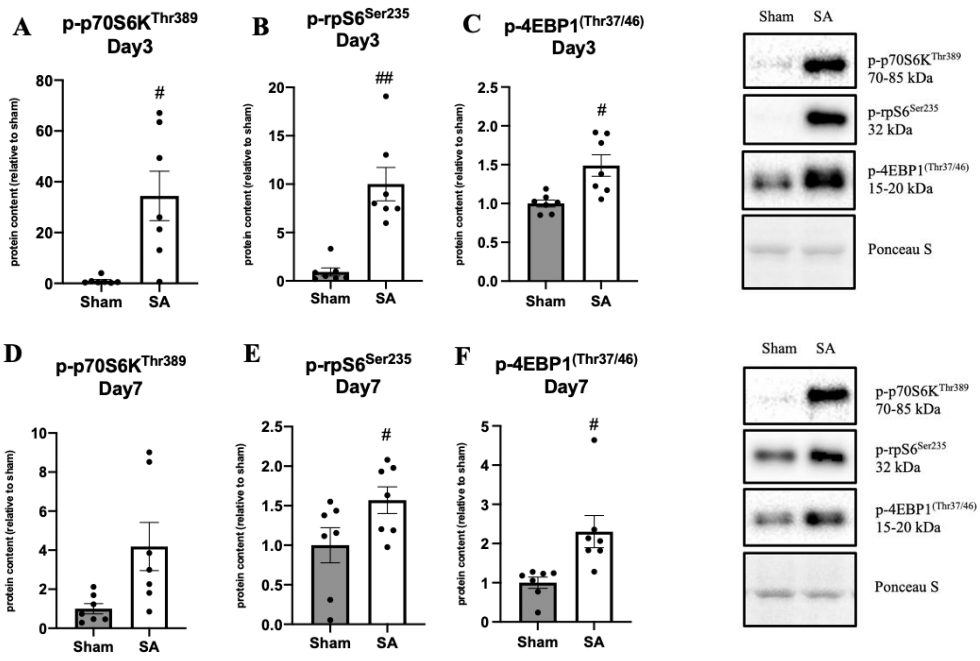

Figure 2-7 Anabolic markers increased after synergist ablation. Expression of proteins involved in the mammalian target of rapamycin complex 1 (mTORC1) signaling pathway on day 3 and day 7 after SA. Data are presented as means ± SE. Paired t tests were performed to compare the Sham and SA groups. #, $p < 0.05$; ##, $p < 0.01$.

On day 3, *Aldh2* gene expression decreased significantly (Fig 2-8A). However, gene expression on day 7 remained unchanged compared with that of the sham group (Fig 2-8C). At the protein level, no changes in the protein levels of ALDH2 were detected at either time point (Fig. 2-8B, p = 0.45; and 2-8D, p = 0.75).

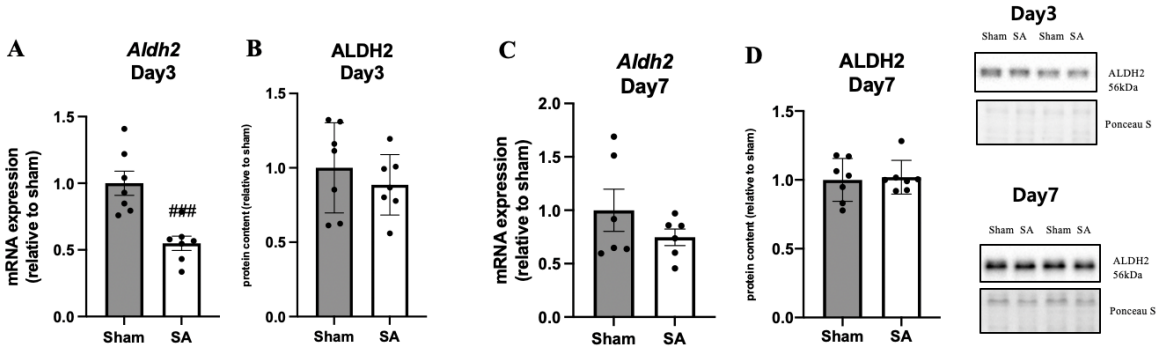

Figure 2-8 Gene and protein expressions of ALDH2 on the day3 and the day7 of SA. (A-B) 3 days of SA significantly decreased gene expression of *Aldh2,* but not protein expression. (C-D) 7 days SA did not alter the gene and protein expression of ALDH2. Data are presented as means ± SE. Paired t tests were performed to compare the Sham and SA groups. ###, $p < 0.001$.

2-5. Discussion

This is the first study that examined changes in gene and protein expression levels of ALDH2 during alterations in skeletal muscle size resulting from denervation and compensatory hypertrophy. The results demonstrated that ALDH2 exhibits various adaptations that are common or specific to experimental models. First of all, denervation changed the gene and protein expression of ALDH2 in skeletal muscle. Secondly, SA changed the early adaptation of *Aldh2* gene expression, however, it was not maintained up to protein expression and chronic adaptation. The findings of this study indicate that 1) ALDH2 might regulate denervation-induced muscle atrophy and 2) ALDH2 might not change SA-induced muscle hypertrophy. Comparisons among the experimental models of adaptation for ALDH2 and the potential mechanisms underlying adaptations are discussed below.

In denervated muscles, increases in both gene and protein expression of ALDH2 were observed. NRF2 is a possible transcription factor candidate for ALDH2. In mice, pharmacological activation of NRF2 induced the expression of ALDH2, and silencing of Nrf2 expression by RNA interference decreased ALDH2 levels (Ishida et al. 2021). Furthermore,

denervation was a sufficient stimulus to activate NRF2 in skeletal muscle, which would also support the possibility that NRF2 contributed to ALDH2 induction in denervated muscle (Kitaoka et al. 2016).

In contrast, under the conditions of skeletal muscle hypertrophy after SA, I observed a decrease in the *Aldh2* gene expression but not in the protein expression in early adaptation. The discrepancies between mRNA and protein levels happens often in experiments. Gene expression is regulated by multiple processes such as histone modification, DNA methylation, RNA modification, and micro RNA regulations after transcription. Its process is necessary to maintain cellular homeostasis or adapt to the environment (Wang et al., 2008). It indicates that the decreased level of *Aldh2* gene expression in early adaptation might interrupt the maintenance of cell homeostasis and its level is quickly modified by multiple processes, especially post-transcriptional and translational regulation.

2-6. Conclusion

In chapter 2, I investigated whether the change in muscle mass alters the gene and protein expression of ALDH2. As result, I found that muscle atrophy increased gene and protein expression of ALDH2 in chronic adaptation and muscle hypertrophy decreased gene expression of *Aldh2* in early adaptation but not protein expression. Herein, I suggest that ALDH2 is closely involved in muscle metabolism during muscle atrophy. Thus, further researches are necessary to clarify how the loss of ALDH2 function affects muscle metabolism during muscle atrophy and also muscle hypertrophy in the following chapters.

Chapter 3

Title

ALDH2 deficiency exacerbates denervation-induced muscle atrophy and decreased function of mitochondrial respiration

4-1. Abstract

A prolonged period of muscle disuse such as aging, chronic diseases, and injuries leads to a loss of muscle mass and decrease muscle function. Aldehyde dehydrogenase 2 is a key enzyme to detoxify the aldehyde in Mitochondria. Recently, it has been reported that ALDH2 deficiency negatively affects muscle homeostasis by increasing ROS production. Furthermore, denervation-induced muscle atrophy increased gene and protein expression of ALDH2. Thus, I aimed to investigate whether ALDH2 deficiency affects denervation-induced muscle atrophy and mitochondrial function in skeletal muscle. Ten-week-old male ALDH2 knockout mice (background strain: C57BL/6J) and wild-type (WT) littermates were used for this study.

Unilateral denervation (DEN) and sham surgery were performed and gastrocnemius, plantaris, and soleus muscles were collected after seven days of recovery time. As result, ALDH2 deficiency enhanced denervation-induced muscle atrophy in plantaris muscle. Furthermore, plantaris and gastrocnemius muscles of KO mice were smaller than WT with and without DEN. In mitochondria function, ALDH2 deficiency showed a tendency to decrease mitochondrial respiration after DEN. Moreover, ROS increased in ALDH-deficient mice with and without DEN. In conclusion, I suggest ALDH2 deficiency negatively affects muscle metabolism during muscle atrophy by decreasing muscle mass and damaging mitochondrial function.

4-2. Introduction

Maintaining healthy skeletal muscle is a crucial key to increasing quality of life through independent daily activities. A lot of health-related conditions such as aging, chronic diseases, and disuse caused by injuries, coma, etc. induce severe muscle loss. Moreover, muscle loss can result in increased morbidity of a variety of diseases and further mortality (Roubenoff, 2007). Since atrophy of skeletal muscle is required changes in extensive gene expressions and signaling pathways, the complete understanding of muscle atrophy on a molecular basis remains a challenge (Bonaldo and Sandri, 2013).

Aldehyde dehydrogenase 2 (ALDH2) has been identified as the key enzyme of catalyzes aldehyde detoxification in mitochondria (Jackson et al., 2011). Accumulation of toxic aldehyde induces cellular homeostasis impairment, enzyme inactivation, damage of DNA, and ultimately cell death (Fritz and Petersen, 2013). More than 40% of Asians and 8% of the world's population have the mutation of ALDH2 and the people with the mutation do not break down alcohol in the liver, which is well known as “Asian Flushing”. Moreover, the mutation of ALDH2 is reported as a risk factor for several chronic diseases such as cancer, Alzheimer,

etc. (Eng et al., 2007). The function of ALDH2 in other tissues and the relation between ALDH2 mutation and diseases have been sufficiently researched, while the function of ALDH2 in the skeletal muscle is still elusive. Recently, it has been reported that ALDH2 deficiency highly expresses in skeletal muscle and its deficiency induces the accumulation of ROS and mitochondrial depolarization (Wakabayashi et al., 2020).

Mitochondria mediate energy metabolism, calcium homeostasis, apoptosis in skeletal muscle (Figueiredo et al., 2008). Thus, maintaining the function and homeostasis of mitochondria is crucial for healthy skeletal muscle cells. Chronic muscle inactivity (aging, disuse, and denervation) induces mitochondrial dysfunction. Especially, denervation is well known as an effective model for muscle atrophy. It has been reported that alteration of mitochondrial function during denervation is related to mediating muscle atrophy (Bhattacharya et al., 2009), and the damaged muscle contraction after denervation induces loss of muscle mass and mitochondrial function (Wicks and Hood 1991). Furthermore, denervation increases mitochondrial ROS production and lipid peroxide (O'Leary and Hood 2008; Abruzzo et al. 2010). Hence, it is necessary to observe both muscle mass and mitochondrial function in

the muscle atrophy model by denervation.

In chapter 2, it has been revealed that denervation-induced muscle atrophy significantly increased ALDH2 protein and gene expression suggesting that ALDH2 deficiency might also change muscle adaptations during muscle atrophy (Jee et al.,2022). Thus, the purpose of this chapter is to investigate whether ALDH2 deficiency affects muscle adaptation to denervation-induced muscle atrophy using ALDH2 KO mice.

4-3. Methods

Ethical Approval

As shown in chapter 2.

Animals

Ten-week-old ALDH2 Knockout (KO) mice (n=13) on a C57BL/6J background and their Wild type (WT) littermates (n=13) were obtained from the RIKEN BioResource Center (Ibaraki, Japan). The mice were housed in cages (5-6 mice/cage, cage size: 19.7 cm x 37.5 cm x 13.3 cm) with a 12-h light/dark cycle (light: 06:00-18:00 h / dark: 18:00-06:00 h). A Standard rodent solid chow (CE-2: CLEA Japan) and water were provided *ad libitum*. The Cages were well ventilated and the room temperature was maintained at 23°C.

Genotyping

Genotyping was performed as previously described (Kitagawa et al., 1999). Briefly, an ear punch biopsy was incubated in 90μl of 50mM NaOH in the heat box at 95°C for an hour.

10μl of 1M Tris pH 6.8 (neutralization buffer) was added. Extracted DNA was amplified by PCR using EmeraldAMP MAX PCR Master Mix (Takara Bio, Shiga, Japan). The primer sequences were forward primer (common) 5′-CTTCCTCGGAAGCACGGAAA-3′; reverse primer 1 (amplified in wild-type mice) 5′-CCATCAGGGTGGCCAAGGTA-3′, and reverse primer 2 (amplified in KO mice) 5′-GGTGGATGTGGAATGTGTGC-3′. By electrophoresis using 2.0% agarose gels, the amplified PCR products were separated. After that, separated bands were visualized using fluorescent DNA-binding dyes (Midori Green Advance, Nippon Genetics, Tokyo, Japan).

Denervation (DEN) surgery

As shown in chapter 2. The mice's sciatic nerve was denervated for 7 days and the mice had a recovery time of 7 days. Both sides of the Gastrocnemius, Plantaris, and Soleus muscle were collected and immediately frozen in liquid nitrogen and saved in -80°C freezers until further analysis.

Western Blotting

As shown in chapter 2.

Muscle Cross-sectional Area

Muscle cross-sectional area was measured as previously described (Bloemberg and Quadrilatero, 2012). Briefly, muscle samples were sectioned at 8μm using a Cryostat (Leica CM 1860, Leica biosystems, Wetzlar, Germany) and fixed with 4% paraformaldehyde for 10 min and then incubated with blocking reagent (NYPBR01; Toyobo Corp.) for 1 h at room temperature. Sections were then incubated for 2 h with a rabbit anti-laminin primary antibody (L9393, Sigma-Aldrich, Missouri, US) diluted in Can Gen Signal Reagent 1 (NKB-101, Toyobo Corp.). Secondary antibody (Alexa Flour 555, Thermo Fisher Scientific) diluted in Can Get Signal Reagent 2 was then applied for 1 h at room temperature. Muscle cross-sections were imaged using a fluorescent microscope with a 20x objective. Approximately 400 randomly selected myofibers per muscle were measured using ImageJ software (National Institutes of Health Bethesda, MD, USA).

Muscle Protein Synthesis Rate

The rate of protein synthesis in the skeletal muscle was conducted using the SUnSET method as previously described (Goodman and Hornberger, 2013). Mice were given an intraperitoneal injection of 0.04 μmol puromycin/g body dissolved in sterile saline. At exactly 15 min after injection, muscle tissues were removed and homogenized as described for western blotting.

RNA Extraction, Reverse Transcription, and Real-time Quantitative PCR

As shown in chapter 2.

Library preparation, RNA-seq, and Bioinformatics Analyses

RNA was extracted as described above and an RNA sequence library was conducted using the QuantSeq 3 mRNA-Seq Library Prep Kit (Lexogen, Vienna, Austria) following the manufacturer's recommendation. The prepared library was sequenced using the MiniSeq system (Illumina, San Diego, CA, USA). Bioinformatics analyses were performed with

integrated differential expression and pathway (iDEP, v0.95). K-Means was used to cluster 2000 variable genes into four clusters using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Differential gene expression analyses were conducted with DEseq2 [false discovery rate (FDR) cutoff: 0.05, minimal fold change: 2].

Statistical Analysis

Data are presented as means ± standard error of mean (SEM). For comparisons of body weight, muscle weight change, and ALDH2 expression, the unpaired (WT vs KO) student's *t*-test was performed to compare two groups. Two-way ANOVA (KO x SA) was conducted to compare four groups. If an interaction was observed, the Tukey-Kramer multiple-comparison test was performed to determine the comparison between each group. Statistical significance was defined as $p < 0.05$ and a trend was defined as $p < 0.10$. All statistical analyses were performed using GraphPad Prism (ver. Version 9.4.1, Macintosh, GraphPad Software, La Jolla, CA, USA).

4-4. Results

Protein level of ALDH2 and ALDH family members

In chapter 2, I found that 7 days of denervation significantly increased ALDH2 gene and protein expression (Jee et al., 2022). I measured protein expression of ALDH2 to confirm that ALDH2 WT and KO mice successfully separated and protein expression of ALDH2 shows the same tendency as chapter 2. ALDH2 expression showed an upward tendency after denervation ($p=0.0879$, Fig. 3-1A). I then examined the protein level of ALDH1A1 and ALDH1B1 which are ALDH family members and most highly expressed in skeletal muscle (Etienne et al., 2020). Denervation decreased the level of ALDH1A1 ($p<0.0005$) and ALDH2 deficiency increased the level of ALDH1A1 ($p<0.05$) However, there was no interaction between denervation and ALDH2 deficiency (Fig. 3-1B). In contrast, the protein expression of ALDH1B1 increased after denervation ($p < 0.0005$) and in KO mice ($p < 0.05$). However, I did not find the interaction between denervation and ALDH2 deficiency (Fig. 3-1C).

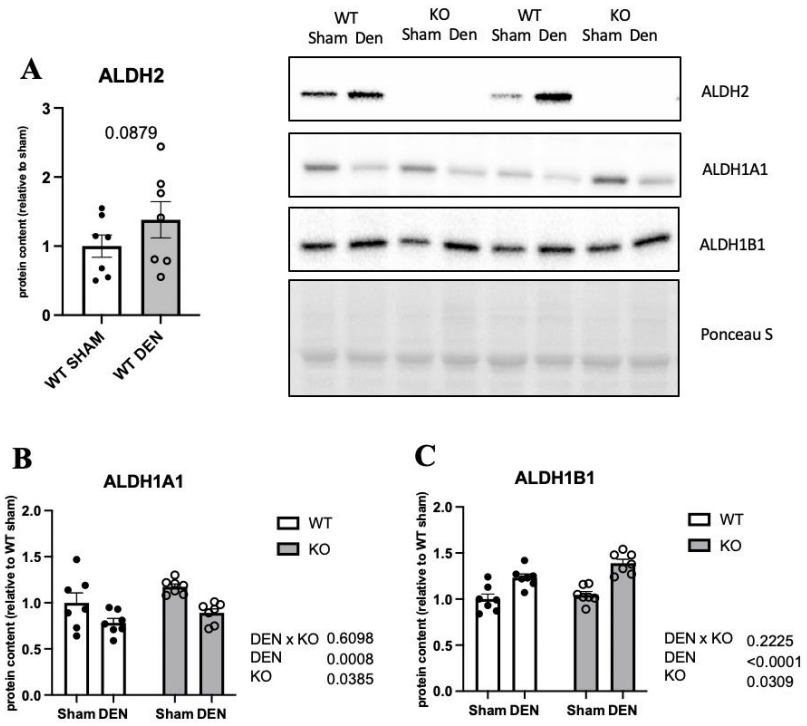

Figure 3-1. The protein expression of ALDH2, ALDH1A1, and ALDH1B1. (A) ALDH2 protein expression showed a tendency to an increase after DEN. (B) ALDH1A1 decreased by DEN and increased by KO. However, there was no interaction between DEN and KO. (C) ALDH1B1 increased by DEN and KO, but there was no interaction between DEN and KO. Data are presented as means ± SE. Paired t tests were performed to compare the WT Sham and WT DEN groups. Two-way ANOVA was conducted to compare the 4 groups (the statistical results are shown on the right side of each figure).

ALDH2 deficiency decreased muscle weight of gastrocnemius and plantaris muscle with and without denervation

First of all, I compared body weight and muscle weight (gastrocnemius, soleus, and plantaris) and then measured the percentage of muscle weight change between WT and KO mice. There was no difference in body weight between WT and KO mice (Fig. 3-2A). In all muscle, denervation significantly decreased muscle weight (Fig. 3-2B~D, $p < 0.0001$). It indicates that denervation surgery has successfully induced muscle atrophy. ALDH2 deficiency decreased muscle weight with and without denervation in gastrocnemius (Fig. 3-2B, $p=0.0005$) and plantaris muscle (Fig. 3-2D, $p<0.0001$). However, I did not find interactions between denervation and ALDH2 deficiency. I then measured the magnitude of muscle weight change in each muscle. I found that plantaris muscle weight decreased significantly in KO mice compared with WT mice (Fig. 3-2G, $p < 0.05$, WT; -10.95%, KO; -16.55%, -5.6±2.6% in KO), while gastrocnemius and plantaris did not (Fig. 3-2E and Fig.3-2F).

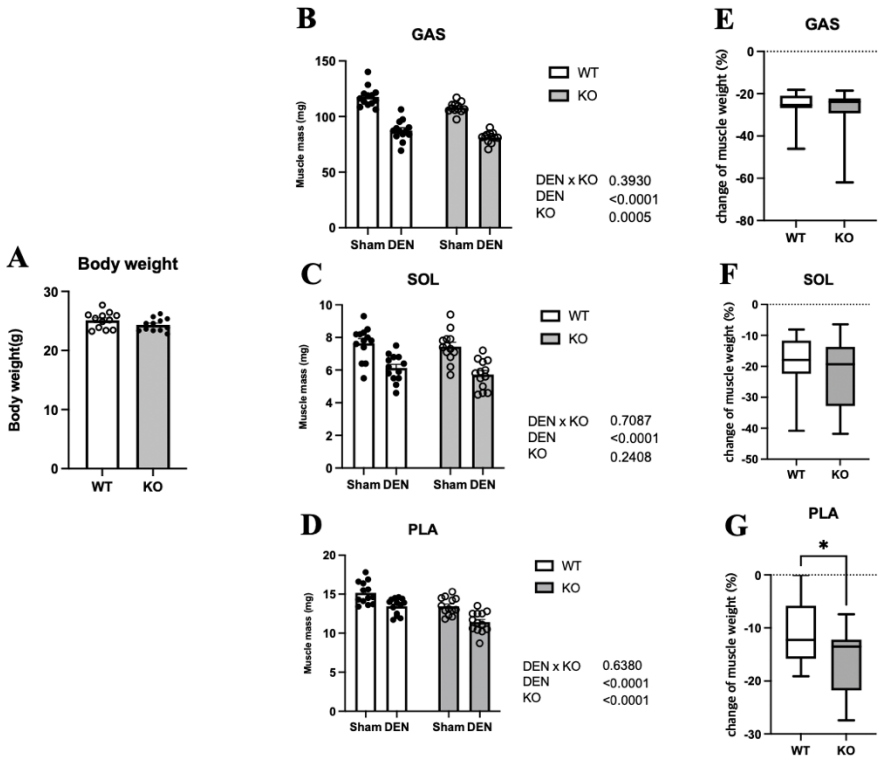

Figure 3-2 ALDH2 deficiency exacerbates DEN-induced muscle atrophy in PLA. (A) There was no difference between WT and KO in body weight. (B-D) GAS, SOL, and PLA muscles decreased after DEN. The muscle weight of GAS and PLA in the KO group significantly decreased with and without DEN. (E-G) the change of muscle weight in PLA showed significant difference between WT and KO, but not in GAS and SOL. Data are presented as means ± SE. Unpaired t tests were performed to compare the WT and KO groups. Two-way ANOVA was conducted to compare the 4 groups (the statistical results are shown on the right side of each figure).

To access whether the change of muscle weight coincide with muscle fiber size, I examined cross section of plantaris muscle fiber. Denervation significantly decreased muscle fiber size and ALDH2 deficiency decreased muscle fiber size with and without denervation. It shows the same tendency to the result of muscle weight above (Fig. 3-3A). I then examined the distribution of cross-sectional area in fiber size from 0 to 5000μm^2. Both the lines of WT DEN and KO DEN groups moved left side compared to WT sham and KO sham groups. However, there was no notable differences between genotypes (Fig. 3-3B). In the visualized Images of CSA, I did not find any remarkable changes between WT and KO groups (Fig. 3-3C).

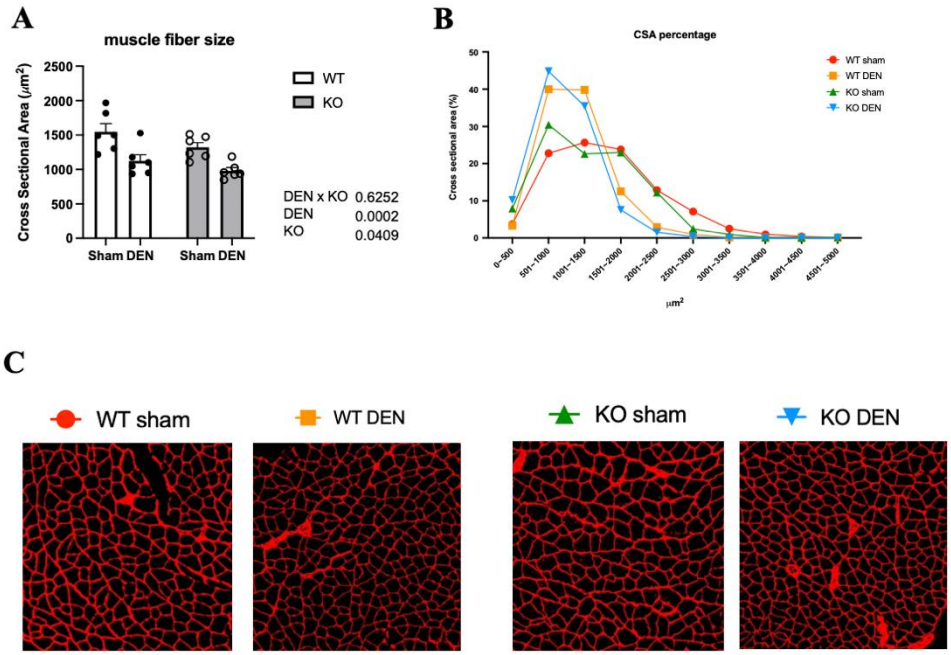

Figure 3-3 Muscle fiber sizes were smaller in ALDH2-deficient mice compared to WT mice regardless of DEN. (A) muscle fiber sizes decreased by DEN and KO, however, there was no interaction between DEN and KO. (B) the lines of DEN moved to the left side compared to sham. (C) There were no notable changes between genotypes. Data are presented as means ± SE. Two-way ANOVA was conducted to compare the 4 groups (the statistical results are shown on the right side of each figure).

ALDH2 deficiency did not change anabolic and catabolic metabolism in skeletal muscle after denervation-induced muscle atrophy

Since the muscle weight of gastrocnemius and plantaris increased significantly, I first measured anabolic markers using plantaris muscle. It is well known that denervation-induced muscle atrophy increases mTORC1 downstream markers such as phosphorylated p70S6K, phosphorylated rp-S6, and phosphorylated 4EBP-1 in many previous studies. However, the mechanism is not well known (You et al., 2021). In the present study, we found that denervation increased mTORC1 downstream markers (Fig 3-4A~C). Furthermore, previous studies have shown that puromycin increased after denervation (You et al. 2021, Yamada et al., 2020). Fig. 3-4D shows the same pattern as the previous study, however, ALDH2 deficiency showed an upward tendency.

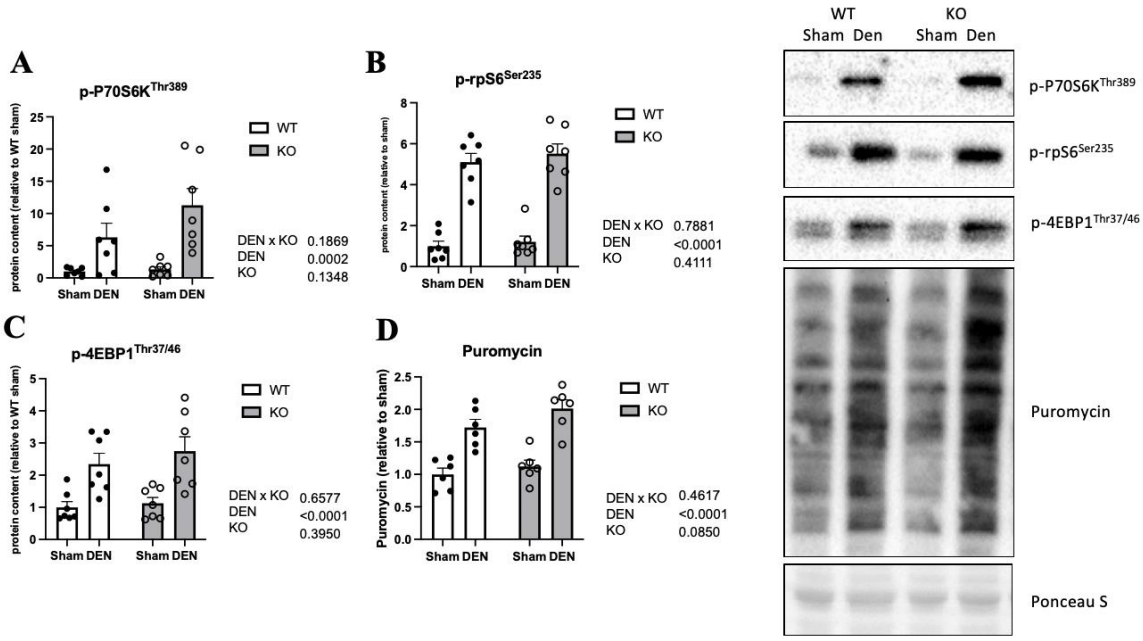

Figure 3-4 ALDH2 deficiency did not change the anabolic markers after DEN. (A-C) ALDH2 deficiency did not change the expressions of proteins involved in the mTORC1 signaling pathways. (D) ALDH2 deficiency showed a tendency to increase puromycin after DEN, but not significant. Data are presented as means ± SE. Two-way ANOVA was conducted to compare the 4 groups (the statistical results are shown on the right side of each figure).

I then examined catabolic markers using plantaris muscle as well. The previous study has shown that denervation-induced muscle atrophy significantly increases gene expression of Atrogin-1 and MuRF1 (Bodine et al., 2001). In the present study, denervation increased Atrogin-1 (Fig. 3-5A, $p<0.0001$) and MuRF1 (Fig. 3-5B, $p=0.0010$). However, there was no significant main effect of KO. Furthermore, it has been shown that denervation significantly increases the protein expression of LC3 and p62 (Triolo et al., 2022). I observed that denervation significantly elevated LC3-I and LC3-II (Fig. 3-5C and 3-5D, $p<0.0001$). A previous study has shown that 7 days of denervation augments total ubiquitin content (Baumann et al., 2016). In this study, denervation elicited a significant increase (Fig. 3-5F, $p<0.0001$).

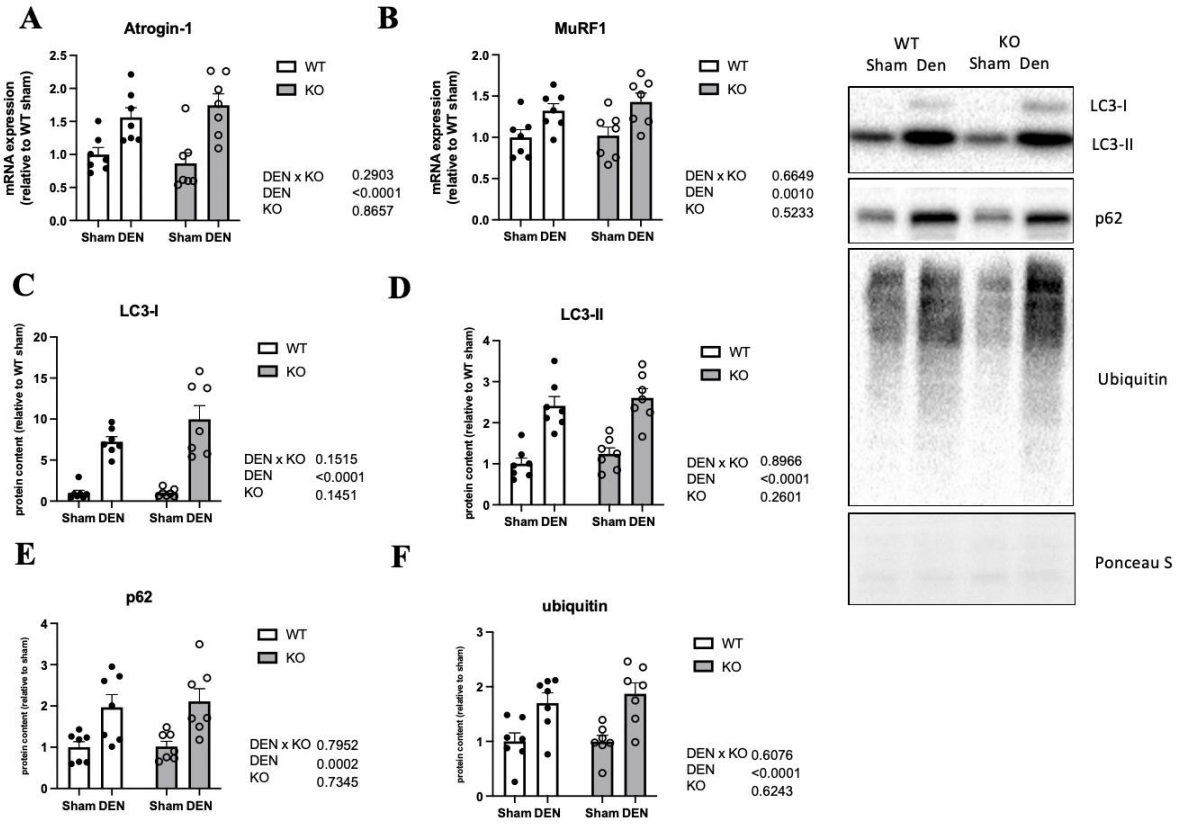

Figure 3-5 ALDH2 deficiency did not change the anabolic markers after DEN. (A-B) ALDH2 deficiency did not alter the gene expression of Atrogin-1 and MuRF1 after DEN. (C-F) ALDH2 deficiency did not change protein breakdown markers after DEN. Data are presented as means ± SE. Two-way ANOVA was conducted to compare the 4 groups (the statistical results are shown on the right side of each figure).

Downregulated NADH dehydrogenase complex assembly and mitochondrial respiratory chain complex I assembly-related genes might affect the mitochondrial respiratory function in ALDH2 deficiency mice skeletal muscle

As shown in the above experiments, ALDH2 deficiency is not involved in protein synthesis and breakdown after denervation. Thus, I next performed RNAseq analysis to determine whether there are differences between WT and KO and potential genes affecting decreased muscle mass other than protein synthesis and breakdown.

The first set of RNAseq was differential gene expressions between groups. I separated 2000 genes into 4 clusters based on the expression patterns using the K-means algorithm. The patterns of red (upregulation) and blue (downregulation) were different between sham and DEN groups and similar between genotypes (Fig. 3-6A). Fig. 3-6B. shows number of differentially expressed genes indicating upregulated and downregulated genes. To measure differential genes between genotypes, I compared KO sham vs WT sham and KO DEN vs WT DEN. Therefore, I observed 4 downregulated genes (Aldh2, Mvp, Banf1, and Fbxw7) and 2 upregulated genes (Mrpl28 and Lars2) in comparison of KO sham vs WT sham (Fig. 3-6C). It

indicates the different genes between genotypes without denervation. After denervation, I found only downregulated Aldh2 gene expression (Fig. 3-6D), but there was no other gene indicating the differences between genotypes without denervation disappeared after denervation.

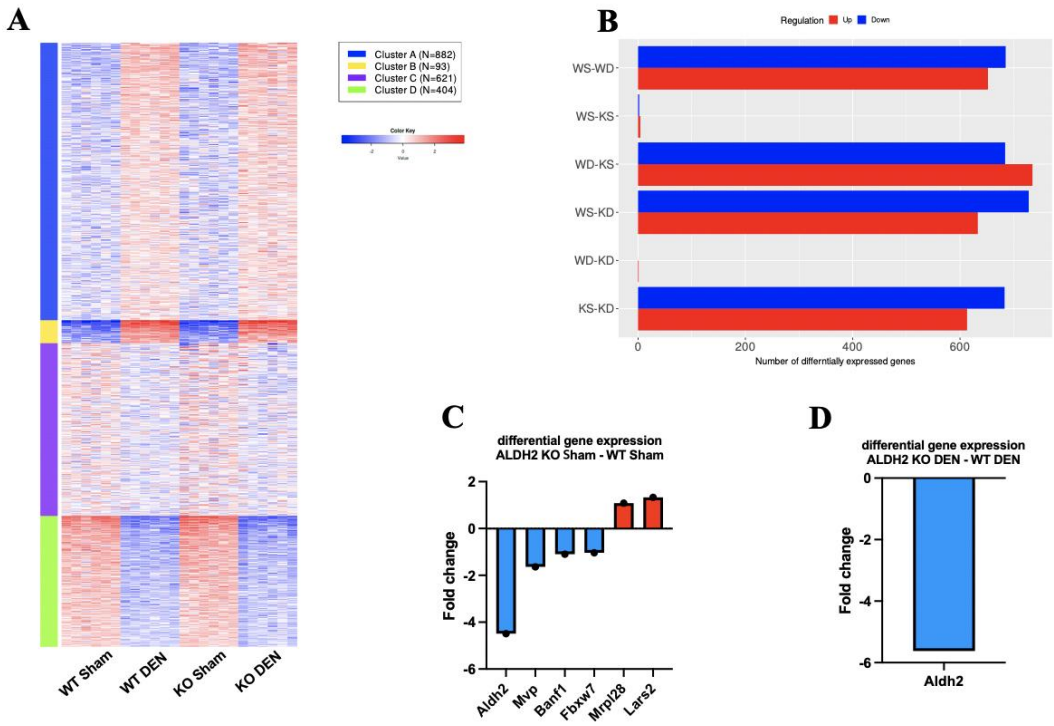

Figure 3-6 RNAseq analysis of differential gene expression. (A)K-means showing changes in the expression of gene between groups. 2000 genes were clustered into four groups. (B) The result of differential gene expression for each group comparison. (C) Six differentially expressed genes were found between WT sham and ALDH2 KO sham groups. (D) One differentially expressed gene (*Aldh2*) was found between WT DEN and KO DEN.

To determine whether those differentially expressed genes between sham and DEN in WT and KO groups show different trends, I next performed pathway analysis between WT DEN vs WT sham and KO DEN and KO sham and then I compared the results of those. I observed 12 pathways of WT comparison matched KO comparison. Other than those 12 pathways, there were notable pathways in KO comparison. It was remarkable that the genes related to NADH dehydrogenase complex assembly and Mitochondrial respiratory chain complex I assembly downregulated in KO comparison, differently from WT comparison (Fig. 3-7A and Fig. 3-7B).

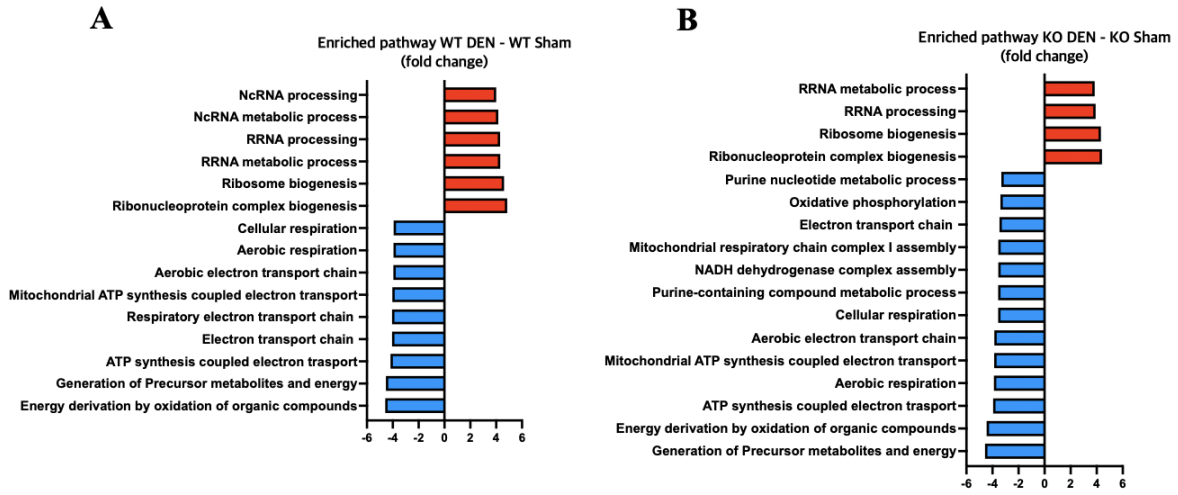

Figure 3-7 RNAseq analysis of enriched pathways. (A) Enriched pathways between WT sham and WT DEN. (B) Enriched pathways between KO sham and KO DEN.

Mitochondrial contents and dynamics were not affected by ALDH2 deficiency after denervation-induced muscle atrophy

Having obtained the results of RNAseq, I next speculated that ALDH2 deficiency might affect mitochondrial metabolism. As I mentioned in the introduction part, ALDH2 is located in the mitochondria and is responsible for the catalytic oxidation of aldehydes and lipid peroxidation-produced compounds such as 4HNE and MDA (Wu and Ren, 2019). To determine whether ALDH2 deficiency during denervation alters mitochondrial metabolism, I next measured mitochondrial contents and their dynamics. The protein contents of PGC-1α and COX IV were not different between WT and KO (Fig. 3-8A and Fig. 3-8B). Furthermore, there was no significant change in the markers of the mitochondrial OXPHOS subunit (Fig. 3-8C~G). These results are consistent with the above results of PGC-1α and COX IV. Next, I assessed mitochondrial dynamics by measuring protein markers of mitochondrial fission and fusion. Mfn2, Opa1, DRP1, and Fis1 remained similar between the WT and KO groups. However, denervation decreased significantly Mfn2 and showed an upward tendency of DRP1 and a downward tendency of Fis1 (Fig. 3-9A~D).

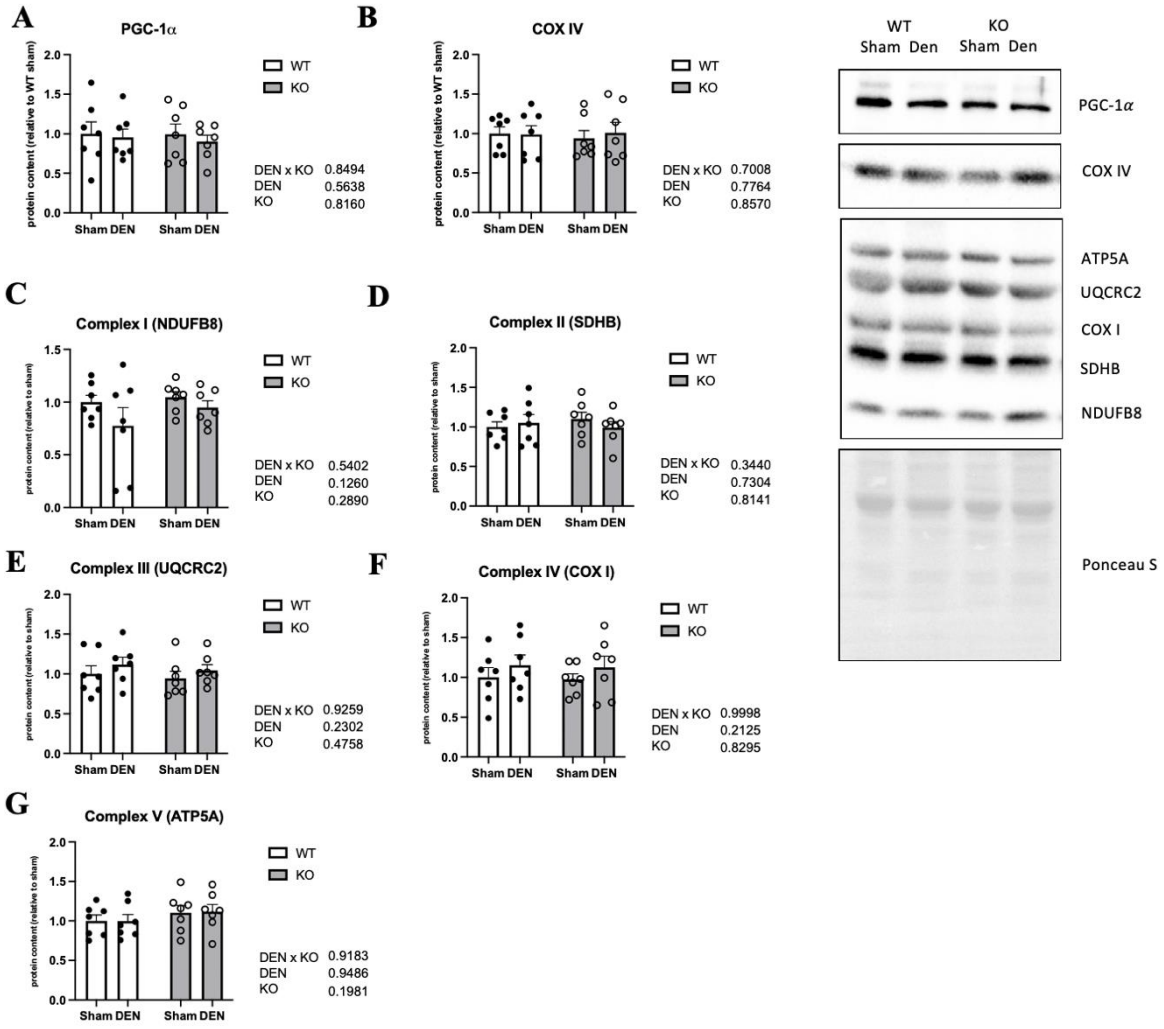

Figure 3-8 ALDH2 deficiency did not change mitochondrial contents after DEN. (A-B) PGC-1α and COX IV did not change in ALDH2-deficient mice after DEN. (C-G) ALDH2 deficiency did not change the OXPHOS proteins after DEN. Data are presented as means ± SE. Two-way ANOVA was conducted to compare the 4 groups (the statistical results are shown on the right side of each figure).

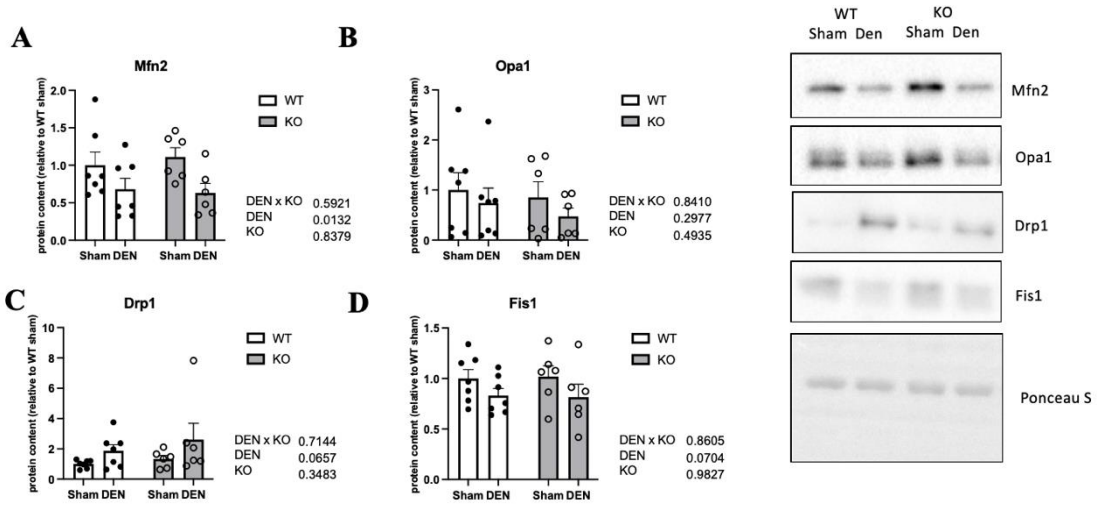

Figure 3-9 There were no changes in mitochondrial dynamics in ALDH2-deficient mice after DEN. (A-B) ALDH2 deficiency did not alter mitochondrial fusion-involved proteins after DEN. (C-D) ALDH2 deficiency did not change mitochondrial fission-involved proteins after DEN. Data are presented as means ± SE. Two-way ANOVA was conducted to compare the 4 groups (the statistical results are shown on the right side of each figure).

ALDH2 deficiency showed a tendency to decrease the function of mitochondrial respiration and increase ROS

Despite mitochondrial contents were not affected by ALDH2 deficiency, there is a possibility that the respirational function of mitochondria might be altered. To determine this, I next measured oxygen consumption rate (OCR) and ROS divided by OCR. Previous studies have shown that denervation decreases mitochondrial respiration (Singh and Hood, 2011, Muller et al., 2007). As I expected, denervation decreased significantly OCR in PM3 and GM3, but not SR3, and showed a tendency of interaction between denervation and ALDH2 deficiency in PM3 and GM3 (Fig. 3-10A, $p=0.0736$, Fig.3-10B, $p=0.0662$, Fig. 3-10C). Furthermore, GM3 decreased significantly in KO compared with the WT groups (Fig. 3-10B, $p=0.0098$).

In PM3 and GM3, the ROS divided by OCR significantly increased after denervation, but not in SR3 (Fig. 3-10D~F, $p<0.0001$). Moreover, ALDH2 deficiency elevated ROS with and without denervation in GM3, but not PM3 and SR3 (Fig. $p=0.0006$, Fig. 3-10E).

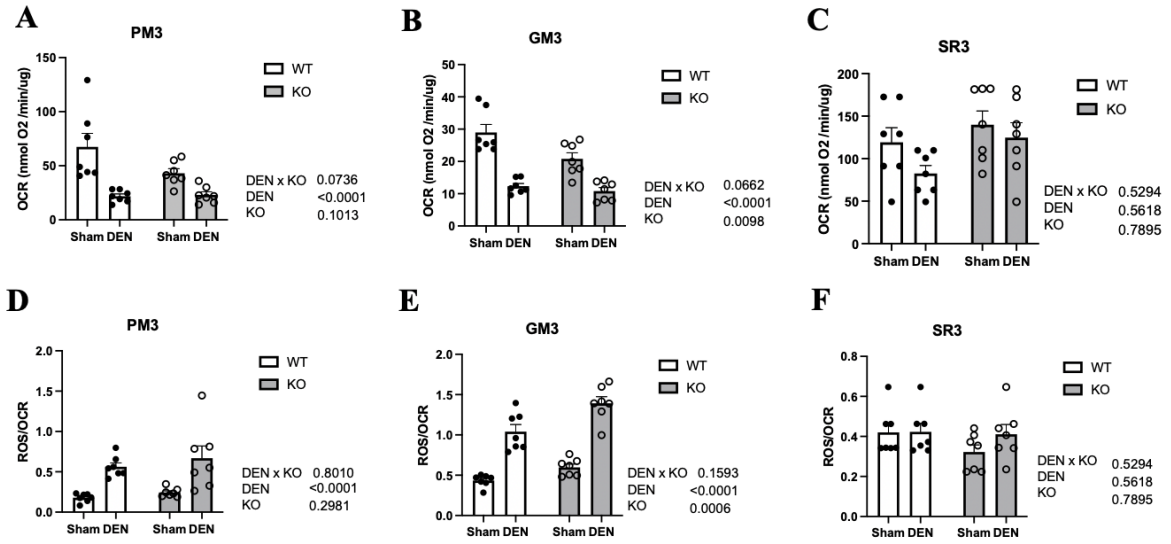

Figure 3-10 ALDH2 deficiency showed a tendency to decrease oxygen consumption rate (OCR) and increased ROS production after DEN. (A) OCR of Pyruvate + Malate stage 3 (PM3) showed a tendency to decrease in ALDH2 deficient mice after DEN. (B) ALDH2 deficiency showed a tendency to decrease OCR of Glutamate + Malate stage 3 (GM3) after DEN. Furthermore, GM3 was lower in KO compared to WT. (C) Succinate + Rotenone stage 3 did not show any different between groups. (D-F) ROS of GM3 significantly increased in KO mice, but not PM3 and SR3. Data are presented as means ± SE. Two-way ANOVA was conducted to compare the 4 groups (the statistical results are shown on the right side of each figure).

4-5. Discussion

The present chapter demonstrated that ALDH2 deficiency affects skeletal muscle adaptation during denervation-induced muscle atrophy. Specifically, I found that ALDH2 Knockout mice have smaller muscle sizes of gastrocnemius and plantaris with and without denervation. Furthermore, ALDH2 deficiency in plantaris muscle enhances denervation-induced muscle atrophy compared to WT, but not in gastrocnemius muscle. According to the pathway analysis in RNAseq between WT and KO groups, there is a possibility of that NADH dehydrogenase complex assembly and mitochondrial respiratory chain complex I assembly might affect the function of mitochondrial respiration. I next found that ALDH2 deficiency decreased mitochondrial respiratory function and increased ROS production after denervation.

I performed denervation surgery to induce muscle atrophy because it is possible to compare the sham and deserved muscle in a mouse, especially the whole-body knockout model, and to investigate the effect of muscle inactivity. I carefully decided the duration of intervention to avoid the extremely high effect of atrophy which might overwhelm the effect of ALDH2 deficiency. According to our recent study, 7 days of denervation was enough to

induce both muscle atrophy and changes in gene and protein expressions of ALDH2 (Jee et al., 2022). Furthermore, it has been recently reported that mitochondrial dysfunction and a decrease in muscle mass are most effective after 7 days of denervation compared to day 1 and day 3 (Triolo et al., 2022).

The most crucial finding of this study is that ALDH2 deficiency exacerbated denervation-induced muscle atrophy in plantaris muscle. According to a previous study using an aging-induced atrophy model, ALDH2 deficiency enhanced atrophy, especially oxidative muscle. The Authors suggested that age-related exacerbated muscle atrophy might be associated accumulation of oxidative stress via mitochondrial dysfunction (Kasai et al., 2022). Though the muscle types between the previous study and this study are a discrepancy, it coincides that both papers showed decreased muscle mass and mitochondrial dysfunction in ALDH2-deficient mice. According to a previous study, early stage of mitochondrial dysfunction negatively affects just in glycolytic muscle (EDL) not in oxidative muscle (SOL). The EDL muscle showed decreased complex I and complex I and II respiration in the presence of pyruvate but not glutamate. They suggested glycolytic muscle is more prone to an imbalance

of energy supply and oxidation (Warren et al., 2014). In the present study, I performed the denervation for 7 days and it might be insufficient to undergo the chronic stage of mitochondrial dysfunction such as the aging model, which takes 2 years up to sacrifice from birth. Thus, to investigate it, a further experiment with a longer duration of DEN is necessary.

The next finding of this chapter was that although the body weights were not different between genotypes, ALDH2 KO mice had smaller muscle sizes of gastrocnemius and plantaris with and without denervation. Herein, it is an important point to discuss whether ALDH2 KO mice originally have smaller muscle sizes regardless of denervation and whether the small muscle size of KO mice might affect the decrease of muscle size after denervation. According to the previous studies, there was no difference in muscle mass between genotypes (Wakabayashi et al., 2020, Kasai et al., 2022). Thus, it seems not possible that ALDH2 KO mice originally have smaller muscle sizes. Moreover, it has been reported that the denervation impacts the contralateral-innervated muscles resulting increasing proteasome activities (Liu and Thompson, 2019). Hence, I next suspected that it might be possible the proteasome activities in KO mice were stronger than in WT mice inducing the decrease in muscle size.

However, I did not find a significant difference in the ubiquitin-proteasome pathway in the present study.

The next objective was to look at the effect of denervation-induced adaptations on mitochondrial function in skeletal muscle. I found downregulated mitochondrial respiratory chain complex I assembly and NADH dehydrogenase complex assembly in the comparison of pathway analysis between KO sham and KO DEN, not in the WT comparison. Furthermore, I found that ALDH2 deficiency significantly increased ROS divided by OCR in Glutamate + Malate stage 3 (GM3) after DEN. Since I observed a decreased tendency in the OCR of PM3 and GM3 in KO compared to WT after DEN, the increased ROS seems highly related to decreased OCR in the KO DEN group. In a previous study, ALDH2 deficiency increased ROS accumulation and damaged mitochondrial networking (Wakabayashi et al., 2020). Furthermore, aged ALDH2 KO mice showed an increased tendency to ROS production compared to aged WT mice (Kasai et al., 2022). The present study showed that increased ROS production might be caused by the decreased respirational function of mitochondria. Referencing these two results in pathway analysis and OCR, I suggest that mitochondrial respiratory chain complex

I and NADH dehydrogenase-related pathways might affect the decrease of the function of mitochondrial respiration.

Finally, it should be discussed whether decreased mitochondrial function is related to decreased muscle mass. The concept of mitochondria dysfunction contributes to disuse muscle atrophy has been contemplated for nearly 50 years, the most mechanisms of signaling events remained unexplained until recently (Hyatt et al., 2019). Nevertheless, it has been reported that mitochondrial ROS trigger atrophy signaling pathways during prolonged muscle inactivity. For instance, unabated ROS production promotes expression of protein involved in the UPS and the autophagy system (Powers et al., 2012, Powers et al., 2016). Furthermore, Protein synthesis pathway can also be negatively impacted by level of ROS through of the initiation of mRNA translation (Shenton et al., 2006). In the present study, I did not find the alteration of protein expression of UPS and autophagy and the proteins related to protein synthesis. Thus, above possibilities are not appropriate to explain the relationship between mitochondrial dysfunction and decreased muscle mass in this research. As I mentioned above, the mechanism of dysfunctional mitochondria induces muscle atrophy is barely known. Whether decreased

muscle mass or dysfunctional mitochondria is a cause or consequence is the key factor to understanding the mechanism. Nevertheless, since ALDH2 deficiency induced both dysfunctions of mitochondria and a decrease in muscle mass, the function of ALDH2 in skeletal muscle should be more importantly addressed.

4-6. Conclusion

In chapter 3, I investigated whether ALDH2 deficiency alters denervation-induced muscle atrophy and muscle metabolism. As results, the muscle size of gastrocnemius and plantaris in KO significantly decreased compared to WT with and without DEN. Moreover, ALDH2 deficiency enhances denervation-induced muscle atrophy in plantaris. In the analyze of mitochondrial function, I found that ALDH2 deficiency showed a decreased tendency to mitochondrial respiration and increased ROS production in KO mice with and without DEN. I suggest ALDH2 deficiency adversely affects muscle metabolism during muscle atrophy by decreasing muscle mass and inducing mitochondrial dysfunction.

Chapter 4

Title

ALDH2 deficiency enhances mechanical overload-induced protein synthesis but not muscle mass

3-1. Abstract

Mitochondrial aldehyde dehydrogenase 2 (ALDH2) plays a major role in the detoxification of aldehydes. ALDH2 is highly expressed in organs that consume relatively large amounts of oxygen, including the liver, heart, brain, and skeletal muscle. ALDH2 mutations occur in severe chronic human diseases, such as Alzheimer's disease, cardiovascular diseases and cancer. In skeletal muscle, ALDH2 deficiency negatively affects the muscle metabolism during muscle atrophy. However, it has been never investigated whether ALDH2 deficiency alters muscle metabolism during muscle hypertrophy. I therefore examined whether ALDH2 deficiency alters mechanical overload-induced muscle protein synthesis and muscle mass in mouse skeletal muscle. Ten-week-old male ALDH2 knockout mice (background strain:

C57BL/6J) and wild-type (WT) littermates were used. Unilateral synergist ablation (SA) and sham surgery were performed and plantaris muscles were collected seven days later. I measured the rate of protein synthesis via SUnSET puromycin incorporation and mTORC1 signaling activity, which regulates protein synthesis, using western blotting. ALDH2 deficiency augmented the synergist ablation-induced increase in phosphorylation of p70S6K at Thr389, a functional readout of mTORC1 activity. Concomitantly, higher amounts of puromycin-labeled peptides were observed in ALDH2-deficient mice. In conclusion, ALDH2 deficiency increased mechanical overload-induced mTORC1 activity and protein synthesis rate but not muscle mass. I therefore suggest that ALDH2 deficiency modulates muscle metabolism during mechanical overload-induced muscle hypertrophy but its effect is insufficient to change muscle structure.

3-2. Introduction

Aldehyde dehydrogenase 2 (*ALDH2*) encodes a mitochondrial enzyme that metabolizes toxic acetaldehyde to non-toxic acetic acid. A polymorphism in *ALDH2* encodes an inactive form of the enzyme, termed ALDH2*2, and is highly prevalent among East Asian populations (~40%). This ALDH2 variant has greatly reduced activity for detoxification of the aldehyde group, causes a flushing response after consuming alcohol resulting in a flushed face, increased heart rate, and nausea (Wall et al., 1992, Jiang et al., 2017). The ALDH2*2 polymorphism is also involved in chronic diseases such as Alzheimer's disease (Chen et al., 2019), cardiovascular diseases (D'Souza et al., 2015), and several types of cancer (Li et al., 2016).

Despite associations of ALDH2*2 with several chronic diseases, the function of ALDH2 in skeletal muscle remains elusive. It has been recently reported that ALDH2 is highly expressed in skeletal muscle and its deficiency in ALDH2 knockout (KO) mice results in damage to skeletal muscle mitochondria, increased production of reactive oxygen species (ROS), and the mitochondrial depolarization (Wakabayashi et al., 2020, Jee et al., 2022).

Furthermore, ALDH2 KO enhances aging-induced muscle atrophy, especially in oxidative muscle fibers rich in mitochondria (Kasai et al., 2021). Mitochondrial dysfunction, including the generation of ROS, contributes to impaired protein synthesis and increased autophagy (Kumar et al., 2019); therefore, the recent findings indicate that ALDH2 deficiency alters muscle protein metabolism via impairment of mitochondria and, as a result, modifies chronic adaptation. ALDH2*2 transgenic mice showed a similar phenotype to ALDH2 KO mice, such as decreased muscle mass (Kobayashi et al., 2020).

ALDH2 may be involved in the chronic adaptation of skeletal muscle to various external stimuli because the gene and protein expression of ALDH2 are changed by denervation and mechanical overload as shown in chapter 2 (Jee et al., 2022). In the chapter 3, I found that ALDH2 deficiency adversely affects muscle metabolism during muscle atrophy using denervation model. To understand the effect of ALDH2 deficiency in both directions of muscle atrophy and hypertrophy, it seems necessary to investigate the effect of ALDH2 deficiency in the muscle hypertrophy as well. Especially in chapter 2, Aldh2 mRNA levels were significantly decreased, however, the ALDH2 protein level was not (Jee et al., 2022) indicating that the

changes in gene expression do not maintain until the protein level. To clarify this, I examined whether ALDH2 deficiency changes muscle protein synthesis and muscle mass during chronic adaptation to mechanical overload-induced muscle hypertrophy.

3-3. Materials and Methods

Ethical Approval

As shown in chapter 2.

Animals

As shown in chapter 3. ALDH2 KO mice (n=11) and WT littermates (n=13) were used for this study.

Genotyping

As shown in chapter 3.

Synergist Ablation (SA) Surgery

As shown in chapter 2. The mice were allowed to recover for 7 days. The both sides of plantaris

muscle were collected and immediately frozen in liquid nitrogen until further analysis.

Western Blotting

As shown in chapter 2.

Measurement of Muscle Cross-sectional Area

As shown in chapter 3.

Measurement of Muscle Protein Synthesis Rate

As shown in chapter 3.

RNA Extraction, Reverse Transcription, and Real-time Quantitative PCR

As shown in chapter 2.

Library preparation, RNA-seq, and Bioinformatics Analyses

As shown in chapter 3.

Statistical Analysis

Data are presented as means ± SEM. For comparisons of body weight and muscle weight change, the unpaired (WT vs KO) Student's *t*-test was conducted to compare two groups. Data were analyzed using the unpaired *t*-test (muscle weight change) and two-way ANOVA (KO × SA). Two-way ANOVA (KO × SA) was performed to compare four groups. If an interaction was observed, the Tukey-Kramer multiple-comparison test was performed. Statistical significance was defined as $p < 0.05$. A trend was defined as $p < 0.10$. All statistical analyses were performed using GraphPad Prism (ver. 9.0, Macintosh, GraphPad Software, La Jolla, CA, USA).

3-4. Results

ALDH2 deficiency does not affect muscle weight or muscle fiber size after SA-induced muscle hypertrophy

I examined the level of ALDH2 protein in WT and KO groups, which confirmed the absence of ALDH2 in KO mice. Compared with the WT sham group, the level of ALDH2 protein was not affected in the WT SA group (Fig. 4-1A). I then examined the levels of the ALDH family members, ALDH1A1 (cytoplasmic form) and ALDH1B1 (mitochondrial form), which are most highly expressed in skeletal muscle along with ALDH2 (Etienne et al., 2020). During the development of skeletal muscle hypertrophy in WT and KO mice, SA significantly decreased the level of ALDH1A1 protein (Fig. 4-1B, $p=0.0019$). In contrast, the level of ALDH1B1 was increased in WT mice after SA by 46.76% and by 40.80% in KO mice (Fig. 4-1C, $p<0.0001$, $p=0.002$). Importantly, these observations indicate that the level of ALDH2 protein did not change after SA, while the levels of ALDH1A1 and ALDH1B1 did. This is

consistent with the result of chapter 2.

Muscle weight increases significantly 7 days after SA (Marino et al., 2008, Mendias et al., 2017). To clarify whether there is a difference in the amount of muscle weight increase after SA between genotypes, I compared body weight, plantaris muscle weight, and the percentage change in muscle weight between WT and KO groups. There was no significant difference in body weight between genotypes (Fig. 4-1D). While SA significantly increased the plantaris muscle weight in both groups (6.9 ± 0.83 mg increase, $p<0.0001$), ALDH2 deficiency did not affect the muscle weight gain after SA (Fig. 4-1E). Furthermore, the percentage of muscle weight change was not significantly different between genotypes (Fig. 4-1F). I next assessed muscle fiber size after SA by measuring the cross-sectional area of the plantaris muscle. SA increased muscle size slightly but there was no significant difference between genotypes (Fig. 4-1G and 4-1H). Taken together, these results indicate that ALDH2 deficiency did not change muscle weight or muscle fiber size after SA-induced muscle hypertrophy.

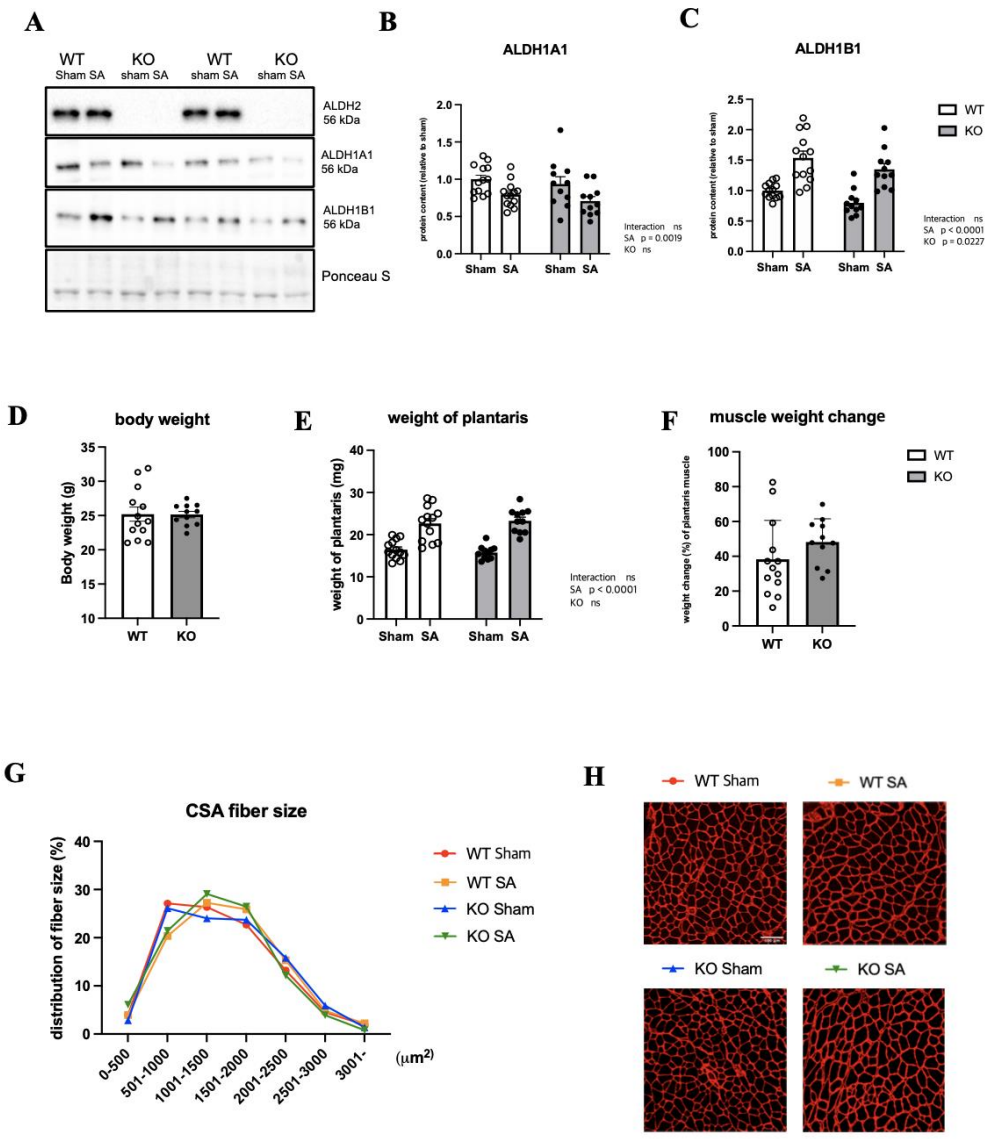

Figure 4-1 ALDH2 deficiency did not change muscle mass or muscle fiber size. (A-C) ALDH isoforms detected by western blotting. The detected ALDH2 band indicates the absence of ALDH2 in KO mice. (D) ALDH2 deficiency did not change body weight. (E) ALDH2 deficiency did not alter the plantaris muscle weight after SA. (F) There was no significant difference in muscle weight change between genotypes. (G) ALDH2 deficiency did not change the muscle fiber size after SA. (H) Representative images of muscle fiber size 7 days after SA. Data are presented as means ± SE. Unpaired t test were used to compare WT and KO groups. Two-way ANOVA was conducted to compare the 4 groups (the statistical results are shown on the right side of each figure).

ALDH2 deficiency augments muscle protein synthesis after SA-induced muscle hypertrophy

Although muscle weight and muscle fiber size were not changed by ALDH2 deficiency, it remained possible that changes in muscle protein levels after SA via altered protein synthesis and/or breakdown might be different between genotypes. Therefore, I next clarified changes in muscle protein synthesis after SA. SA can induce an increase in mammalian target of rapamycin complex 1 (mTORC1)-related muscle protein synthesis (Chale-Rush et al., 2009). In both WT and KO mice, SA increased mTORC1-related proteins (Fig. 4-2A~G). These results indicate that the SA surgery was successfully performed and induced muscle hypertrophy within 7 days. Importantly, p70S6K is phosphorylated (p-p70S6K), and therefore activated, more in the KO SA group than in the WT SA group (Fig. 4-2A, SA × KO; $p=0.024$). However, phosphorylated 4EBP1 (downstream of mTORC1) and phosphorylated rpS6 (downstream of p-p70S6K) were not consistent with the highly levels of p-p70S6K (Fig. 4-2B, 4-2C, and 4-2G).

The balance between the rate of protein synthesis and protein degradation is crucial in determining changes in skeletal muscle mass (Goodman et al., 2011). Several pieces of evidence indicate that the rate of protein synthesis is regulated by a kinase cascade of

phosphoinositide 3-kinase (PI3K), AKT, and mTORC1 (Schiaffino et al., 2021). Therefore, I next investigated the rate of protein synthesis by puromycin incorporation to understand the effect of increased p-p70S6K activation. Surprisingly, ALDH2 deficiency significantly increased the rate of protein synthesis after SA (Fig. 4-2H and 4-2I, $p=0.013$). These unexpected results demonstrate that ALDH2 deficiency increases both mTORC1-related muscle protein synthesis and the rate of muscle protein synthesis.

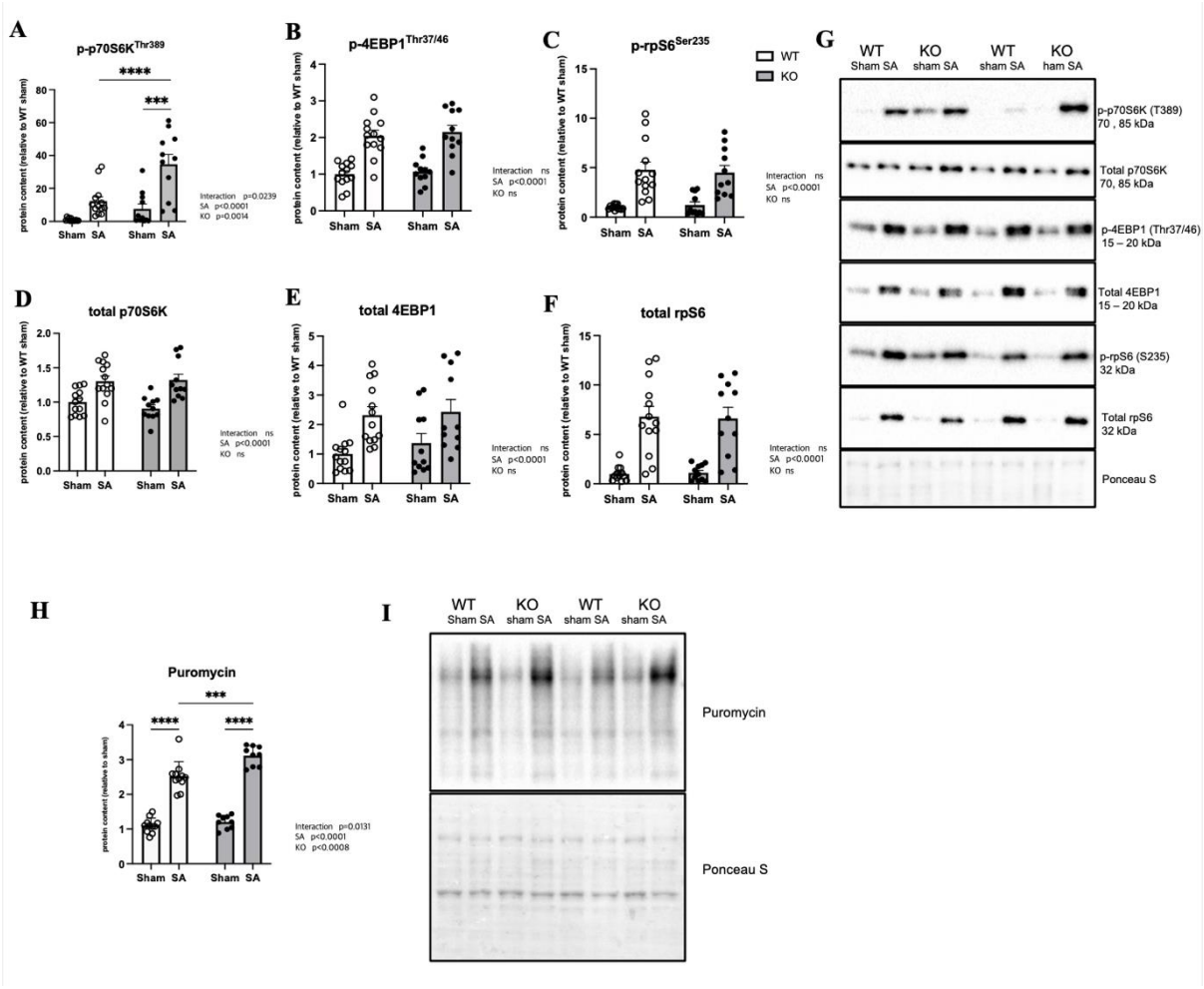

Figure 4-2 ALDH2 deficiency increased muscle protein synthesis after SA. (A-G) Levels of proteins involved in mTORC1 signaling after SA. ALDH2 deficiency significantly increased p-p70S6K after SA. (H-I) ALDH2 deficiency increased the rate of muscle protein synthesis after SA. Data are presented as means ± SE. Two-way ANOVA was conducted to compare the 4 groups (the statistical results are shown on the right side of each figure). ***, $p<0.001$; ****, $p<0.0001$.

ALDH2 deficiency does not change the protein and gene expression of catabolic markers in skeletal muscle after SA-induced muscle hypertrophy

As shown in the above experiments, ALDH2 deficiency is involved in an increased rate of muscle protein synthesis after SA. I also suspected that ALDH2 deficiency alters muscle protein breakdown during SA. Thus, I next investigated autophagy-related proteins, including LC3-I, LC3-II, ubiquitin-conjugates, and p62. SA induces a decrease in the LC3-II/LC3-I ratio and increases levels of p62 and ubiquitin-conjugates (Moriya et al., 2018). I found that the LC3-II/LC3-I ratio decreased significantly after SA. However, there was no difference between WT and KO mice (Fig. 4-3A~C, 4-3F). Furthermore, I observed increased levels of p62 and ubiquitin-conjugates after SA but no notable changes between genotypes (Fig 4-3D~F). Having obtained evidence indicating that ALDH2 deficiency does not affect muscle protein breakdown, I next examined the gene expression of *Fbxo32* and *MuRF1*, which are key regulators of muscle atrophy (Bodine et al., 2001). Chronic resistance training decreases *Fbxo32* and *MuRF1* gene expression (Zanchi et al., 2009). As shown in Fig. 4-3G and 4-3H, 4-3I observed that *Fbxo32* ($p<0.0001$) and *MuRF1* expression were decreased significantly

after SA ($p<0.0001$). Interestingly, I found that the gene expression of *Fbxo32* in the KO group was significantly lower compared with that in the WT group ($p=0.002$). Together, these observations indicate that ALDH2 deficiency did not change the protein levels or gene expression of catabolic markers after SA. However, ALDH2 deficiency lowered the level of *Fbxo32* without SA intervention.

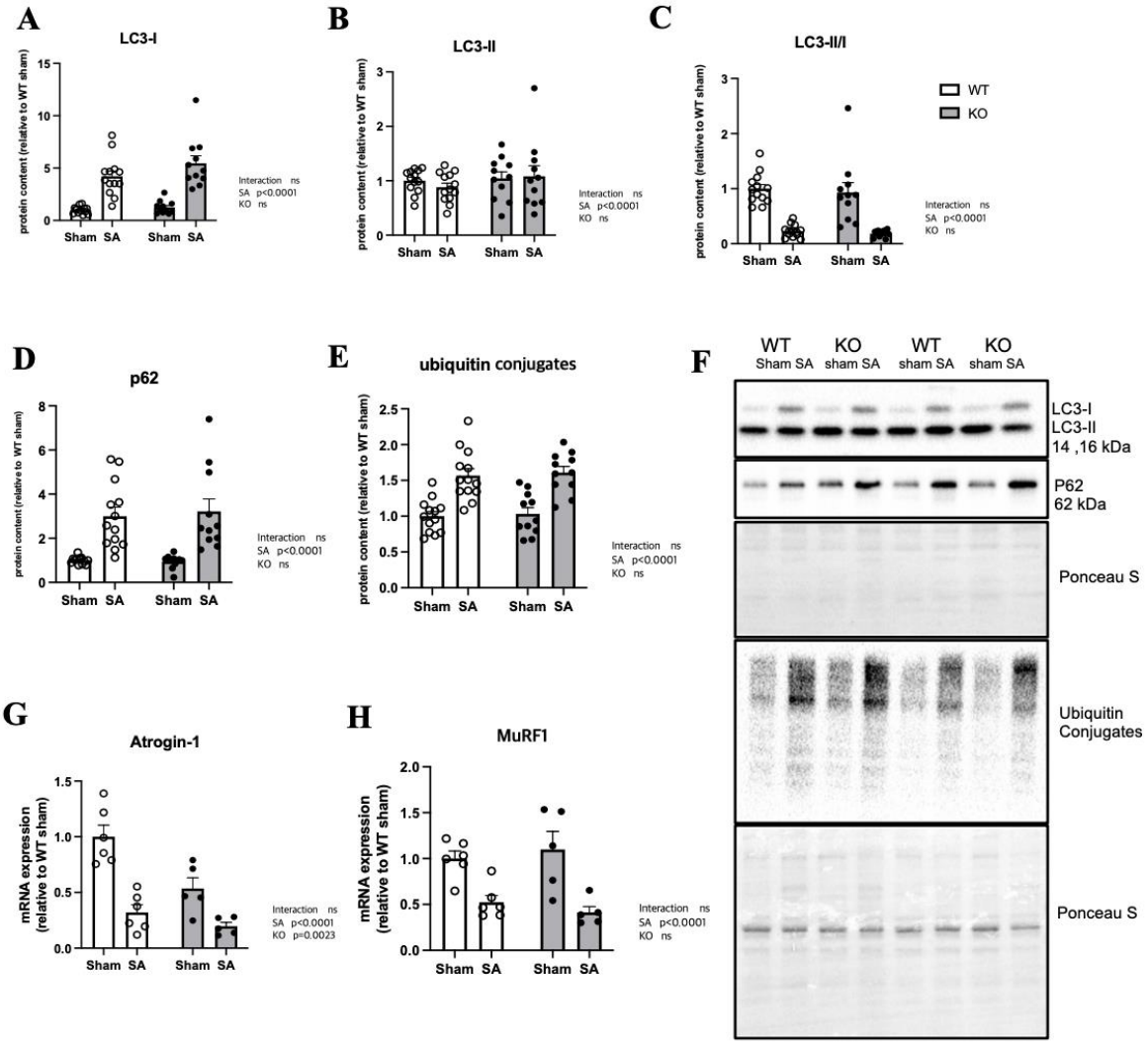

Figure 4-3 ALDH2 deficiency did not change muscle protein breakdown after SA. (A-C) ALDH2 deficiency did not alter the LC3II/I ratio after SA. (D-F) ALDH2 deficiency did not change levels of p62 or ubiquitin conjugates after SA. (G-H) SA significantly decreased Atrogin-1 and MuRF1 gene expression but there were no interactions between SA and genotypes. Data are presented as means ± SE. Two-way ANOVA was conducted to compare the 4 groups (the statistical results are shown on the right side of each figure).

SA-induced muscle hypertrophy attenuates the difference in skeletal muscle-related gene expression between ALDH2 WT and KO mice

Having obtained evidence indicating that ALDH2 deficiency increases muscle protein synthesis and maintains muscle protein breakdown after SA, I then investigated genes affecting muscle protein synthesis at the RNA level. I performed RNAseq to determine differences in gene expression between groups. I separated 2000 genes into four groups based on their expression patterns using the K-Means algorithm. The trends of upregulation and downregulation were different between sham and SA groups and similar between genotypes (Fig. 4-4A). I found six differentially expressed genes between the WT sham and the KO sham groups: *Aldh2*, *Ankrd1*, *Chac1*, *Cilp*, *Mustn1*, and *Tceal7* (Fig. 4-4B and 4-4C). ANKRD1 is involved in the myofibrillar stretch-sensor system interacting with myopalladin and titin, and its deletion causes muscular dystrophy with myositis (Witt et al., 2006). Furthermore, CILP interacts with mTOR (Choi et al., 2002) and MUSTN1 is related to lengthening and shortening contractions in skeletal muscle (Kostek et al., 2007). Surprisingly, the differential expression of these genes between WT and KO disappeared after SA (Fig. 4-4B and 4-4D). Taken together,

there was no difference in gene expression or pathways between genotypes after SA. However, SA-induced muscle hypertrophy attenuated the difference in gene expression between WT and KO mice.

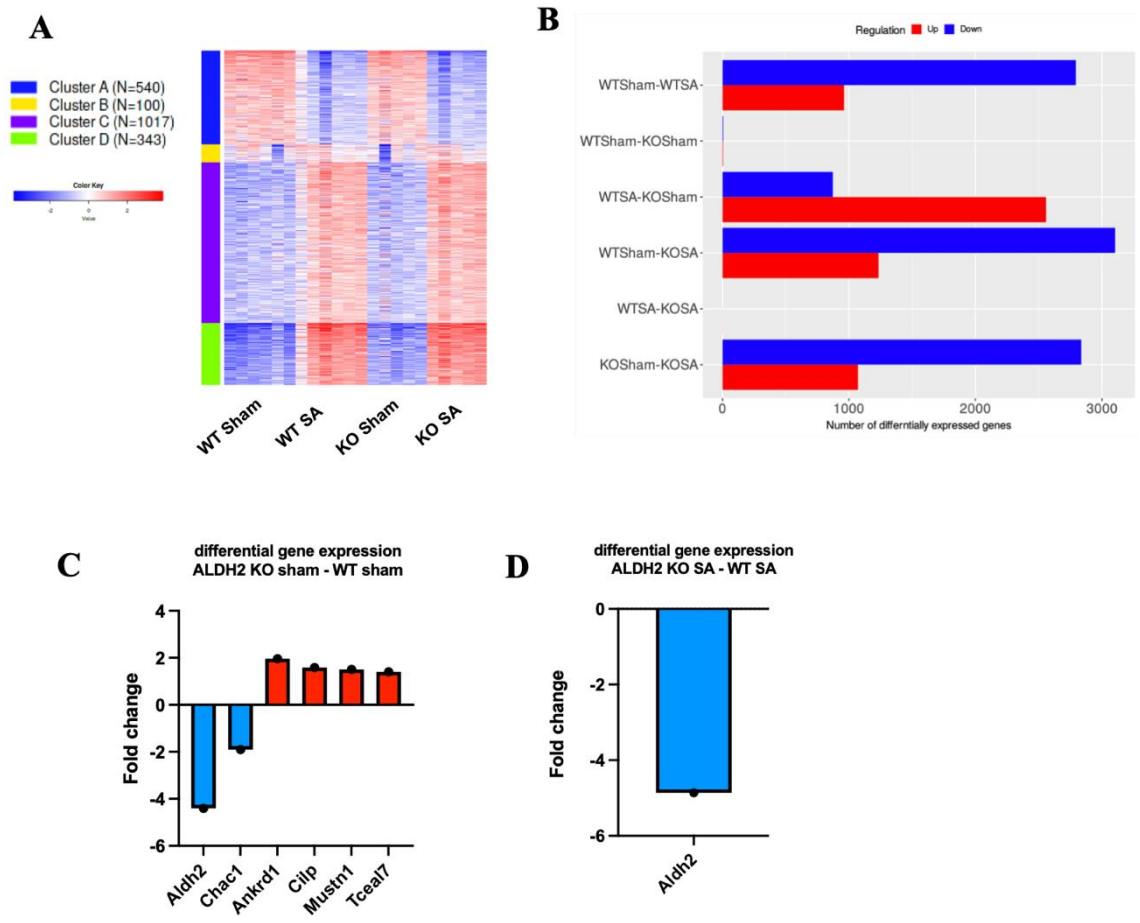

Figure 4-4 RNAseq analysis (A)K-means showing changes in the expression of genes between groups. 2000 genes were clustered into four groups. (B) The result of differential gene expression for each group comparison. (C) Six differentially expressed genes were found between WT sham and KO sham groups. (D) One differentially expressed gene (*Aldh2*) was found between WT SA and KO SA.

3-5. Discussion

In chapter 4, I investigated the effect of ALDH2 deficiency on muscle protein synthesis and muscle mass during chronic adaptation to mechanical overload-induced muscle hypertrophy. The key findings of this chapter can be briefly summarized as follows. First, ALDH2 deficiency did not alter muscle mass or muscle fiber size after SA. Second, ALDH2 deficiency promoted muscle protein synthesis by increasing the phosphorylation of p70S6K. Finally, RNAseq analysis of ALDH2 KO and WT mice did not identify any genes affecting muscle protein metabolism after SA.

Few studies have investigated the relationship between ALDH2 deficiency and muscle mass. According to a recent study, ALDH2 deficiency induced aging-induced muscle atrophy, especially in oxidative muscle compared with glycolytic muscle (Kasai et al., 2021). Furthermore, ALDH2*2 (rs671) mice have a smaller body and muscle size than wild-type mice (Kobayashi et al., 2021). These two studies suggest that ALDH2 deficiency modulates muscle metabolism and induces changes in muscle mass. Furthermore, chapter 3 showed that ALDH2 deficiency decreased muscle mass in gastrocnemius and plantaris with and without DEN. Thus,

it was necessary to examine whether ALDH2 deficiency alters the magnitude of muscle hypertrophy after inducing mechanical overload (i.e. resistance training). As results, I found that ALDH2 deficiency did not show any effects on the chronic adaptation of skeletal muscle mass after SA.

In contrast to no effect on muscle mass, I detected an increase in muscle protein synthesis after SA in ALDH2-deficient mice. It was also necessary to measure muscle protein breakdown to determine whether muscle protein synthesis increased (Zanchi et al., 2009, Wang et al., 2009). ALDH2*2 (rs671) transgenic mice show an increase in both anabolic and catabolic markers, indicating increased turnover. Therefore, I speculated that increased turnover may occur as in the above study. However, I found that ALDH2 deficiency did not increase the levels of markers of protein breakdown. Thus, the increased rate of muscle protein synthesis was not reflected by increased turnover in ALDH2 KO mice after SA.

In chapter 2, SA-induced muscle hypertrophy decreased *Aldh2* gene expression in early adaptation. However, it did not alter protein levels in early adaptation or gene and protein expression in chronic adaptation. It might be possible that an acute response against SA affects

chronic adaptation of muscle protein synthesis. Nevertheless, an increased base of protein synthesis did not trigger a change in muscle structure.

As observed in this chapter, ALDH2 deficiency increased muscle protein synthesis but not muscle mass. Discrepancies between protein metabolism and muscle mass have been reported several times previously. For instance, mTORC1-related muscle protein synthesis does not match the magnitude of increased muscle mass (Ogasawara et al., 2013) or the volume of training (Ogasawara et al., 2017) by Sprague-Dawley rats using an electric stimulator on the gastrocnemius muscle. Additionally, human biopsies show similar results to the rodent model (Mitchell et al., 2012, Mitchell et al., 2014). These results indicate that the acute measurement of muscle protein synthesis does not always reflect chronic training-induced muscle hypertrophy.

Furthermore, it has been also reported that raptor/mTORC1 is not necessary for a mechanical overload-induced increase in protein synthesis (You et al., 2019) or for maintaining muscle mass (Ham et al., 2020). Therefore, it seems necessary to separately consider our results of increased muscle protein synthesis using SUnSET and increased

phosphorylation of p70S6K (which is downstream of mTORC1). From this perspective, it might be possible that mechanical overload-induced muscle protein synthesis indicates not only muscle protein synthesis but also muscle recovery and remodeling. This concept is clear when endurance exercise and denervation alters mTORC1 signaling without hypertrophy (Castets et al., 2019). In chapter 2, I found that denervation-induced muscle atrophy upregulated ALDH2 expression at both protein and RNA levels in response to chronic adaptations (Jee et al., 2022). I suggest a possibility that the increased p-p70S6K in ALDH2-deficient mice after SA might be a response to overloaded muscle-induced muscle recovery and/or remodeling.

Synergist ablation is one of the most recognized procedures to induce muscle hypertrophy. Even though the molecular mechanisms that control the regulation of skeletal muscle remain poorly defined (Sandri, 2008), synergist ablation-induced muscle hypertrophy is ultimately driven by several growth regulatory events (Goodman et al., 2011). However, synergist ablation is a supraphysiologic model. The magnitude of hypertrophy is different from human resistance training. For example, the continuous activity of mice affects the magnitude

of muscle hypertrophy following synergist ablation, while human resistance training consists of several sessions of training and recovery. Therefore, synergist ablation induces 30–80% muscle hypertrophy in 2 weeks (Terena et al., 2017), while, human resistance training induces about 6% muscle hypertrophy in 8 weeks (Figueiredo et al., 2015). Hence, the effect size of SA depending on the duration was the main concern at the begging of this study. According to the previous studies, the magnitude of muscle hypertrophy on day 7 of SA is enough to compare the changes in muscle mass between groups (Terena et al., 2017). Furthermore, the gene expression level on day 7 of SA is peak and then it becomes slowly moderate (Chaillou et al., 2013). Though SA is typically used for more than 14 days of the experimental period, I tried to find the shortest experimental period (7 days) with enough effect size of SA (average 45.9% increase in both genotypes). Furthermore, unifying the duration with the denervation model of chapter 3 was also important to consider. Nevertheless, the effect of synergist ablation might have overwhelmed the effect of ALDH2 deficiency in the present study.

Although the aforementioned discrepancies in muscle metabolism and muscle mass were unexpected, the alteration of protein synthesis in ALDH2-deficient mice after SA was highly

congruent with previous studies suggesting ALDH2 deficiency to be related to muscle metabolism (Wakabayashi et al., 2020, Kasai et al., 2022, Kobayashi et al., 2021). What then is the key to solving this unexpected issue? There are at least three possibilities worthy of consideration. First, it is close to impossible to measure the balance of muscle protein synthesis and muscle protein breakdown with currently available technology. As mentioned above, it is controversial whether the increased muscle protein synthesis reflects muscle hypertrophy. Second, the effect of ALDH2 deficiency is probably insufficient to enable a comparison of changed muscle mass using the synergist ablation method. Third, it seems necessary to investigate whether ALDH2 contributes to regulating the mTORC1 signal and what is the exact mechanism. Up to now, the relationship between ALDH2 and mTORC1 signaling in skeletal muscle is unclear. However, the present research suggests the possibility that ALDH2 expression is related to the rapamycin-sensitive mTORC1 pathway. Thus, it might be worthy to examine it as a future study.

4-6. Conclusion

In conclusion, I showed that ALDH2 deficiency increased muscle protein synthesis after mechanical overload-induced muscle hypertrophy in mouse skeletal muscle, but not muscle mass. I did not detect any gene affecting muscle protein synthesis in ALDH2-deficient mice after SA by using RNAseq analysis. These results indicate that ALDH2 deficiency modulates muscle metabolism during mechanical overload-induced muscle hypertrophy but the effect of ALDH2 deficiency is insufficient to alter muscle structure.

Chapter 5

General discussion

5-1. Summary

The purpose of this thesis is to investigate as follows:

Since ALDH2 deficiency negatively affects muscle homeostasis by increasing ROS production and damaging mitochondrial function, it might be possible that ALDH2 deficiency adversely alters the adaptation of skeletal muscle, especially during muscle atrophy. Thus, the main purpose of this study is to examine the adaptation of skeletal muscle using the muscle atrophy and hypertrophy model.

1. To measure the adaptabilities of gene and protein expression of ALDH2 in different conditions of muscle activities such as muscle atrophy and muscle hypertrophy. (Chapter 2)
2. To examine whether ALDH2 deficiency alters denervation induced muscle protein breakdown and muscle atrophy using ALDH2 KO mice. (Chapter 3)

3. To investigate whether ALDH2 deficiency changes mechanical-overload induced muscle protein synthesis and muscle hypertrophy using ALDH2 KO mice. (Chapter 4)

In chapter 2, The key findings were that denervation-induced muscle atrophy increased both gene and protein expression of ALDH2 in chronic adaptation and synergist ablation-induced muscle hypertrophy decreased gene expression of *Aldh2* but not protein expression. From these results, I could speculate that ALDH2 might regulate muscle metabolism during muscle inactivity.

In chapter 3, I then investigated whether loss of ALDH2 function changes muscle mass and muscle metabolism during muscle atrophy using ALDH2 KO mice. As mentioned above, denervation-induced muscle atrophy alters the protein and gene expression of ALDH2 in chronic adaptation (7 days of DEN). Thus, I replicated the same experimental condition of chapter 2. As results of muscle mass, the gastrocnemius and plantaris muscle size were smaller in KO mice compared to WT mice with and without DEN. Furthermore, ALDH2 deficiency enhances muscle atrophy in plantaris muscle, but not in gastrocnemius. To clarify the cause of

the change in muscle mass, I analyzed mitochondrial function because previous studies suggest that ALDH2 deficiency damages mitochondria. Surprisingly, ALDH2 deficiency showed a decreased tendency to mitochondrial respiration, especially the substrate related to mitochondrial respiration chain complex I. Moreover, I also found increased ROS production in KO mice with and without DEN. These results of decreased mitochondrial function looked similar to the results of decreased muscle mass. Hence, I suggest ALDH2 deficiency adversely affects muscle metabolism during muscle atrophy.

In chapter 4, I next examined whether loss of ALDH2 function changes muscle hypertrophy and muscle metabolism during muscle hypertrophy. While denervation changes both gene and protein expression of ALDH2 in chronic adaptation in chapter 2, synergist ablation alters *Aldh2* gene expression in early adaptation and its expression did not maintain until protein expression. In chapter 4, ALDH2 deficiency increased phosphorylated p-70S6K and puromycin indicating increased muscle protein synthesis. However, its significant alteration was not sufficient to change muscle mass. Thus, I suggest the effect of ALDH2 deficiency is not overwhelming during muscle hypertrophy.

Taken together, the effect size of ALDH2 deficiency is substantial during muscle atrophy rather than during muscle hypertrophy. Furthermore, ALDH2 deficiency adversely affects muscle health during muscle atrophy by decreasing muscle mass and mitochondrial respiration and increasing mitochondrial ROS production. In the other words, the function of ALDH2 should be more importantly addressed, especially during muscle atrophy.

5-2 Future perspectives

Based on the work presented in this thesis, I have some recommendations for future research.

In the present study, I addressed denervation-induced muscle atrophy and synergist ablation-induced muscle hypertrophy to investigate the effect of ALDH2 deficiency on muscle adaptation. As I showed in chapters 3 and 4, it seems that the effect of ALDH2 deficiency is stronger in the muscle atrophy model than in the muscle hypertrophy model. Thus, it is necessary to focus on muscle adaptation on atrophy such as cachexia, COPD, Alzheimer's disease, aging, etc. Since ALDH2 deficiency is highly related to chronic diseases,

understanding other chronic diseases-induced atrophy models might explain the relationship between ALDH2 deficiency, muscle mass, and muscle metabolism.

In chapter 3, I found decreased muscle mass and mitochondrial function in ALDH2-deficient mice after DEN. However, its correlation between decreased muscle mass and mitochondrial dysfunction was not clarified. Since it is a long-time controversial topic and the mechanisms are still unclear, it seems necessary to investigate it separately. Furthermore, I found the increased p-p70S6K and puromycin in ALDH2-deficient mice after SA in chapter 4. There are several previous studies that rapamycin inhibits ALDH enzyme activity. So that it has been suggested that the mTORC1 signal might be regulated by ALDH (Cai et al., 2014, Mu et al., 2013). However, it has been never researched in the muscle cell. Thus, it might be necessary to inhibit mTORC1 expression using rapamycin to investigate the mechanism of how ALDH2 deficiency regulates mTORC1 signal.

Finally, I suggested that ALDH2 deficiency adversely affects muscle metabolism during muscle atrophy in rodents. I speculate that the gene polymorphism of ALDH2 in humans might show the same tendency. There are some human studies that ALDH2 mutation carriers are

prone to have weak grip strength compared to WT carriers. For instance, it might be interesting to examine whether elder people or patients who face muscle loss have weaker strength/power of skeletal muscle depending on ALDH2 polymorphism.

5-3. Possible role of ALDH2 in muscle adaptation

The ALDH2 mutation carriers are about 40 % of East Asia and 8% of the world population. What if there are differences in muscle metabolism between wild type and mutation? This question was the trigger for the start of this thesis and I found that the loss of the ALDH2 function is a strong disadvantage in circumstances of muscle disuse. Of course, further studies are necessary to understand the exact mechanism of enhanced muscle atrophy in ALDH2-deficient mice. Firstly, it should be researched when ALDH2 is overexpressed in skeletal muscle, and whether it positively affects muscle health or the opposite. secondly, it should be addressed whether specific compounds or substrates such as those that are strong anti-oxidant can rescue the disadvantages of loss of ALDH2 function. Third, if yes, whether those compounds and substrates can be used as clinical therapeutics. As I mentioned above, ALDH2

is an ethnic-specific enzyme. It might explain the correlations between chronic diseases and muscle loss which is a risk factor for severe diseases depending on polymorphisms. Hence, this basic knowledge from this thesis might contribute to personalized therapeutics.

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Abbreviations

4EBP1	Eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1
4HNE	4-hydroxy-2-nonenal
ALDH	Aldehyde dehydrogenase
AKT	Protein kinase B
Atrogin-1 (MAFbx)	Muscle Atrophy F-box gene
BCA	Bicinchoninic acid
COX IV	Cytochrome c oxidase subunit 4
CSA	Cross-Sectional Area
DEN	Denervation
DNA	Deoxyribonucleic acid
DRP1	Dynamin-related protein 1
elF4E	Eukaryotic translation initiation factor 4E
FDR	False discovery rate
Fis1	Mitochondrial fission protein 1
FoxO	Forkhead box transcription factors
GM	Glutamate + Malate
IGF1	Insulin-like growth factor-1
kDa	Kilodaltons
KEGG	Kyoto Encyclopedia of Genes and Genomes
KO	Knock Out
LC3	Microtubule-associated protein 1A/1B-light chain 3
MDA	Malondialdehyde
Mfn2	Mitofusin-2
mRNA	messanger Ribonucleic acid
mTORC	Mammalian target of rapamycin complex
Murf1	Muscle-specific RING finger protein 1
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide

NRF2	Nuclear factor erythroid 2–related factor 2
OCR	Oxygen Consumption Rate
Opa1	Optic atrophy 1
OXPHOS	Oxidative phosphorylation
p62	Sequestosome-1
p70S6K	P70 ribosomal protein S6 kinase
PCR	Polymerase Chain Reaction
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3K	phosphatidylinositol-3 kinase
PM	Pyruvate + Malate
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
rpS6	Ribosomal protein S6
SA	Synergist Ablation
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SEM	Standard error of mean
SR	Succinate + Rotenone
SUnSET	Surface sensing of translation
UPS	Ubiquitin-proteasome system
WT	Wild type

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List of publications

1. Jee, E., Tamura, Y., Kouzaki, K., Kotani, T., & Nakazato, K. (2022). Effect of different types of muscle activity on the gene and protein expression of ALDH family members in C57BL/6J mouse skeletal muscle. Applied Physiology, Nutrition, and Metabolism, (ja).