学位論文（要約）

Passive repetitive stretching is associated with greater muscle mass and cross-sectional area in sarcopenic muscle

（他動的反復ストレッチによるサルコペニア筋の筋量および筋断面積の増加）

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Background: Age-associated loss of muscle mass and function, known as sarcopenia, is characterized by a progressive decline in muscle fiber number and size, a shift in fiber-type composition, and modification of adverse metabolic parameters. Despite the growing recognition of clinical importance, sarcopenia is poorly managed by routine treatment. It is imperative to identify effective countermeasures that induce hypertrophy of senescent muscle and combat progression of sarcopenia. The regulation of skeletal muscle mass and fiber size is largely determined by the dynamic turnover between protein synthesis and degradation. Muscle hypertrophy occurs when the overall rate of protein synthesis exceeds the rate of protein degradation while muscle atrophy, occurs when protein degradation rates exceed protein synthesis. Insulin-like growth factor 1/Akt/mammalian target of the rapamycin (IGF1/Akt/mTOR) signaling primarily controls muscle protein synthesis. Akt stimulates protein synthesis by activating mTOR and inhibits protein degradation through Forkhead box O (FoxO)-mediated ubiquitin-ligases including Muscle Atrophy F-box (MAFbx)/atrogin-1, and Muscle Ring Finger 1 (MuRF1) downregulation. Myostatin has been extensively studied as another potential mediator of sarcopenia owing to its potent negative effects on cell growth and intracellular catabolic and anabolic signaling pathways. In addition, skeletal muscle hypertrophy can be induced by the activation of muscle satellite cells. When activated, a surge of Myogenic regulatory factors (MRFs), including Myoblast determination protein 1 (MyoD), Myogenic factor 5 (Myf5), and myogenin, is required owing to the role of MRFs in driving the differentiation of myoblasts to mature myotubes. Passive stretching, a type of mechanical stimulus, is widely used in rehabilitation medicine to prevent muscle shortening, maintain the range of joints, and muscle flexibility. Evidence has shown that stretching applied to cultured skeletal muscle cells provides

mechanical stimuli and triggers cellular biomarkers essential for muscle growth. However, recent studies in vivo investigated the effect of static and low-frequency stretching using immobilization approaches on skeletal muscles with very limited hypertrophied outcomes. It has been observed that induction of myogenesis is more effective in fast, repetitive stretching than static stretching. However, the mechanisms underlying muscle hypertrophy resulting from passive repetitive stretching remain poorly understood. Moreover, aging processes alter mechano-transduction, indicating a perturbed load-induced plasticity for aged muscle hypertrophy. Whether passive repetitive stretching is effective in improving muscle mass and fiber area in senescent muscles remains unknown.

Methods: This study mainly consisted of two experiments. The *Experiment 1* using 6-weeks-old Institute of Cancer Research (ICR) mice was performed to investigate the effect passive repetitive stretching on healthy skeletal muscle mass and myofiber morphology and decipher the regulatory elements of action behind. The *Experiment 2* using 35-weeks- old Senescence-Accelerated Mouse Prone-8 (SAM-P8) mice was performed to further elucidate that whether or not passive repetitive stretching exerts a hypertrophic effect against sarcopenic atrophy. In order to isolate active isometric contractile activity during stretching duration, I anesthetized the mice with isoflurane solution using inhalation anesthesia equipment. I stretched the right gastrocnemius muscles of the animals 15 times/min for 15 min daily, 5 days a week for 2 weeks in both two experiments. Contralateral unstretched muscles were used as controls. Twenty-four hours after the final stretching session, the gastrocnemius muscles of both legs of the mice were removed under deep anesthesia. The mean muscle fiber cross-sectional area (CSA) and the number of myonuclei were determined by the hematoxylin

and eosin (H&E) staining. Paired box-7 (Pax7) + cells were detected by immunohistochemistry (IHC) staining. The messenger ribonucleic acid (mRNA) expression of MRFs and signaling molecules involved in muscle protein synthesis and degradation were detected by real time polymerase chain reaction (PCR). The phosphorylation level of Akt, ribosomal protein S6 kinase beta-1 (p70S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) were measured with western blotting.

Results: In the *Experiment 1*, I found that the gastrocnemius muscle weight of the stretched side increased as compared with unstretched side. Histological analysis showed that the mean gastrocnemius muscle fiber CSA of the stretched side was larger than that of the unstretched side. The mRNA expression of p70S6K increased while 4E-BP1 expression decreased on the stretched side as compared with the unstretched side. The mRNA expression of MAFbx, MuRF1 and myostatin was decreased on the stretched side as compared with the unstretched side. There is no difference in the Pax7, Myf5, expression while MyoD expression decreased and myogenin increased on the stretched side as compared with the unstretched side. Passive repetitive stretching increased the phosphorylation level of p70S6K. I found there was no significant difference in the number of Pax7+ cells between the stretched and unstretched sides by immunohistochemistry. The number of myonuclei per fiber showed no difference in stretched and unstretched muscles. In *Experiment 2*, I found that the gastrocnemius muscle weight and fiber CSA of the stretched side was greater compared with that of the unstretched side. Passive repetitive stretching increased the mRNA expression level of Akt, p70S6K, 4E-BP1, Myf5, myogenin, MuRF1.The phosphorylation level of p70S6K was significantly increased in stretched muscles as compared with unstretched

ones. The Pax7+ cells and myonuclei content doesn't differ between stretched and unstretched muscles.

Discussion: Akt signaling enhances protein synthesis primarily via activation of mTORC1, the two critical downstream effectors of which are the translational suppressor 4E-BP1 and the ribosomal protein p70S6K. The activation of mTOR1 mediates the phosphorylation of S6 kinase (S6K) and the inhibition of 4E-BP1. In the *Experiment 1*, I found that the mRNA expression of p70S6K significantly increased while 4E-BP1 expression decreased in the stretched side compared to the unstretched contralateral limb. I provided further evidence that in vivo repetitive stretching strongly increased the phosphorylation level of p70S6K in both *Experiment 1* and *Experiment 2*. The stretch-activated Akt/mTOR signaling pathway involved in skeletal muscle protein synthesis is intact in aged mice. Overexpression of MuRF1 and MAFbx results in muscle atrophy. In the *Experiment 1*, I observed a decreased expression of MuRF1 and MAFbx after two weeks of passive repetitive stretching. Inhibition of MAFbx and MuRF1 in stretched muscles would help to maintain muscle mass and fiber size. In the *Experiment 2*, however, I found that MuRF1 mRNA expression was elevated, indicating the involvement of the cellular degradation pathway in aged skeletal muscle adaptation to passive stretching. Another potential contributor to the increase in muscle mass and myofiber CSA is the significant decrease in myostatin expression as observed in the *Experiment 1*. Myostatin inhibits muscle hypertrophy via negative regulation of myogenesis and protein synthesis; therefore, decreased levels of myostatin permit increased protein synthesis and muscle growth. Myostatin expression was not affected by stretching in the *Experiment 2*. I also assumed that the hypertrophic or suppressed atrophic observations in passive repetitive stretching may result from satellite cells

activation. Pax7 expression has been recognized as a marker of satellite cells. However, there was no significant difference in the number of Pax7+ cells between the stretched and unstretched muscles in both ICR and SAM-P8 mice. In the *Experiment 1*, the mRNA expression of Pax7 remained unchanged while myogenin was largely upregulated and MyoD was reduced in stretched muscles compared to unstretched ones. In the *Experiment 2*, the mRNA expression of Pax7 was undetected, which indicates a contraction of the satellite cells content and impaired cell function associated with aging. The expression of MyoD was unchanged, whereas the Myf5 and myogenin mRNA expressions were upregulated in the stretched side when compared with the unstretched side. Finally, the number of nuclei per fiber was measured in each muscle section to determine whether or not stretching result in satellite cell activation and incorporation of new nuclei. As a result, the stretched and unstretched muscles did not differ in both ICR and SAM-P8 mice. These findings suggest the hypertrophic or suppressed atrophic observation in stretched muscles are mainly attributable to the protein turnover rather than triggering satellite cell activation and fusion. Future studies are required to determine the time course of the acute effect of passive repetitive stretching on the expression of key factors implicated in the regulation of protein synthesis and also myogenesis pathway to further elucidate the mechanisms and optimize the stretching protocols.

Conclusion: I propose that passive repetitive stretching for 2 weeks induce muscle hypertrophy via regulation of Akt/mTOR and MuRF1/MAFbx pathway as well as Myostatin in healthy ICR mice. In addition, passive repetitive stretching proved to be effective in preserving and improving muscle mass and fiber area in sarcopenic muscle in SAM-P8 mice.