博士論文（要約）

Analysis of indoxyl sulfate toxicity on skeletal muscles

(インドキシル硫酸が骨格筋に及ぼす影響解析)

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Introduction

In the aging society, sarcopenia is attracting attention because of its strong association with increased risk of falls, muscle functional decline, morbidity, and mortality. Sarcopenia is defined as a condition characterized by loss of skeletal muscle strength, and quality or quantity. Many factors are involved in sarcopenia, and in general, age-related muscle loss is classified as primary sarcopenia, and that associated with a decreased activity, inadequate nutrition, or chronic disease are classified as secondary sarcopenia. In those sarcopenic conditions, the imbalance between muscle protein synthesis and proteolysis is involved and caused the reduction of skeletal muscle mass and function. Moreover, the physiological and morphological changes in skeletal muscle with advancing age and debilitating diseases are characterized by overall declines in size and number of skeletal muscle fibers, mainly the fast-twitch muscle fibers. The reduction of fast-twitch muscle fibers, which are capable of rapid muscle contraction and powerful movement, correlates to the risk of falls and hip fractures. Therefore, to prevent fast-twitch muscle reduction is one of the target-to-treat factors for protecting patient's quality of life especially in the aging society. Furthermore, to explore the therapeutic intervention for sarcopenia, it is necessary to realize the mechanisms in each sarcopenic condition.

In patients with chronic kidney disease (CKD), sarcopenia is also strongly associated with increased morbidity and mortality, which is a so-called uremic sarcopenia. Past epidemiological studies have demonstrated that many factors possibly contribute to the increased incidence of uremic sarcopenia, including aging, restricted protein intake, insufficient exercise, energy deficiency, inflammation, metabolic acidosis, lack of natural vitamin D, increased circulating angiotensin II and even diuretic treatment. Preventing and treatment for uremic sarcopenia such as medical nutritional and exercise therapy have been investigated. However, pharmacological approaches to overcome uremic sarcopenia has not been established yet. Accumulation of uremic toxins in CKD also exerts negative effects on skeletal muscle mass and function. Uremic toxins are usually filtered and excreted by healthy kidneys but are accumulated in the body when renal function has deteriorated. Among the uremic toxins, the protein-bound uremic toxins, such as indoxyl sulfate (IS) and p-cresyl sulfate are difficult to remove by hemodialysis because of their strong binding to serum albumin and are considered directly harmful to various organs such as the cardiovascular system and kidney. In the context of uremic sarcopenia, the studies for uremic toxin-induced sarcopenia are only a few, and the mechanism and treatment have not been fully elucidated yet. So, I hypothesized that the accumulation of representative uremic toxin, indoxyl sulfate (IS) might have a direct negative effect on skeletal muscle, and I aimed to explore the mechanism and the potential treatment for IS-induced sarcopenic effects.

Results and Discussion

I first examined the effect of IS on the mouse skeletal muscle mass and strength. Uremic mouse models have been already established such as the subtotal nephrectomy model. However, in these models, the serum levels of not only IS but also another protein-bound toxin are possibly increased. Therefore, to see the pure effects of IS toxicity, I established the protocol using IS-injected half nephrectomized mice. In this protocol, IS (600 mg/kg/day) was intraperitoneally administered to half nephrectomized mice for one week. After these interventions, four limbs grip test representing the instantaneous strength is dominantly impaired in the IS group. The mouse body weight tends to decrease in the IS group, and the gastrocnemius (GC) muscle, which is composed of mainly fast twitch muscle, also tends to decrease in IS group. In this condition, the other organ dysfunctions were undetectable such as renal failure, anemia, and cardiac hypertrophy.

Past reports demonstrated that secondary sarcopenia due to debilitating diseases showed a predominant decrease in fast-twitch muscle fibers. Moreover, IS treatment-induced impaired instantaneous muscle strength with the tendency of decreased GC muscle rich in fast-twitch muscle. Therefore, I performed immunofluorescent staining with fast and slow-twitch myosin heavy chain antibodies in mouse GC muscle, and evaluated each type of cross-sectional area. As the result, IS induced a predominant decrease in cross-sectional area of fast-twitch muscle fiber and decrease in protein expression of fast-twitch myosin heavy chains by western blotting.

Muscle quantity is mainly regulated by protein synthesis and proteolysis. To detect the mechanism of IS induced muscle atrophy, I first evaluated gene expression associated with classic ubiquitin-proteasome pathways, such as myostatin, atrogin-1, and MuRF-1. In the mouse GC muscle with IS treatment, these atrophic gene expressions were not remarkedly increased. Therefore, I conducted in vivo experiments of puromycin assay for analyzing protein synthesis, which is so-called the surface sensing of translation (SUnSET) technique. At the end of the mouse experiment, puromycin solution was intraperitoneally injected and performed a sacrifice and removed GC muscle. As the result of western blotting, the protein expression of puromycin labeled peptides was seen only in puromycin injected mice group, and was decreased in IS injected group. So, IS treatment may restrict protein synthesis rather than proteolysis.

Next, to explore the potential treatment for uremic sarcopenia, I focused on the ergogenic drug β2 adrenergic receptor (AR) agonist, clenbuterol. β2-AR agonists promote skeletal muscle hypertrophy and are used as anabolic drugs to increase skeletal muscle mass. Moreover, β2-AR agonists induce slow-to-fast MHC isoform transition. Therefore, IS induced instantaneous muscle weakness and predominant decrease on fast-twitch muscle fibers might ameliorate by β2-AR agonist treatment. I first investigate the clenbuterol effects against the IS induced sarcopenic changes on mouse skeletal muscle *in vivo*. Clenbuterol was intraperitoneally injected

in the IS injection model as described above. Clenbuterol treatment increased body weight and GC muscle mass even in mice treated with IS. Furthermore, clenbuterol treatment increased MHC expression in the skeletal muscle of mice treated with IS. However, clenbuterol treatment could not restore IS-induced muscle weakness.

Past reports showed that reactive oxygen species (ROS) play a pivotal role in skeletal muscle atrophy. Furthermore, IS induces ROS accumulation in several cells and organs. So next, I evaluated the effect of IS on mouse C2C12 myotube size and ROS accumulation. Co-administration of albumin and IS were needed to make the same condition in vivo, and past systematic review urged this method. Then, IS treatment on fully differentiated myotubes induced the decrease in myotube length and diameter. In this condition, I also evaluated the ROS accumulation by using CM-H2DCFDA, a ROS sensitive fluorescent dye to detect mainly hydrogen peroxide. IS induced ROS accumulation in C2C12 myotubes and this change was suppressed by L-ascorbic acid treatment. However, interestingly, β2 AR agonist clenbuterol also reduced IS-induced ROS generation in skeletal myotube, which has not been reported before.

From the results of *in vitro* experiments, I next evaluated ROS accumulation in mouse GC muscle treated with IS and clenbuterol by using immunofluorescent staining with 4-HNE to detect lipid peroxidation derived from ROS generation. As the same in vitro result, 4-HNE expression was significantly increased in the IS group and tends to be suppressed by clenbuterol treatment.

Conclusion

In summary, IS has the potential to decrease skeletal muscle size and strength, predominantly in fast-twitch myofibers via the ROS generation. Against that, clenbuterol treatment attenuates IS-induced ROS generation, reduction of mouse skeletal muscle mass and MHC protein expression although muscle strength is not positively affected. For uremic sarcopenia, further investigations and the discovery of a new drug effective for both muscle strength and quantity are awaited.