Ghrelin treatment improves physical decline in sarcopenia model mice through muscular enhancement and mitochondrial activation

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Abstract. Chronic kidney disease (CKD) impairs physical performance in humans, which leads to a risk of all-cause mortality. In our previous study, we demonstrated that a reduction in muscle mitochondria rather than muscle mass was a major cause of physical decline in 5/6 nephrectomized CKD model mice. Because ghrelin administration has been reported to enhance oxygen utilization in skeletal muscle, we examined the usefulness of ghrelin for a recovery of physical decline in 5/6 nephrectomized C57Bl/6 mice, focusing on the epigenetic modification of peroxisome proliferator activated receptor gamma coactivator-1α (PGC-1α), a master regulator of mitochondrial biogenesis. The mice were intraperitoneally administered acylated ghrelin (0.1 nmol/gBW; three times per week) for a month. Muscle strength and exercise endurance were measured by using a dynamometer and treadmill, respectively. Mitochondrial DNA copy number was determined by quantitative PCR. The methylation levels of the cytosine residue at 260 base pairs upstream of the translation initiation point (C-260) of PGC-1α, which has been demonstrated to decrease the expression, was evaluated by methylation-specific PCR and bisulfite genomic sequencing methods after the ghrelin administration. Ghrelin administration improved both muscle strength and exercise endurance in the mice and was associated with an increase in muscle mass and muscle mitochondrial content. Ghrelin administration decreased the methylation ratio of C-260 of PGC-1α in the skeletal muscle and increased the expression. Therefore, ghrelin administration effectively reduced the physical decline in 5/6 nephrectomized mice and was accompanied with an increased mitochondrial content through de-methylation of the promoter region of PGC-1α in the muscle.

Key words: Ghrelin, Physical performance, Chronic kidney disease, Mitochondria, Sarcopenia

Physical decline is an important therapeutic target for the patients with chronic kidney disease

Patients with chronic kidney disease (CKD) have a lower physical performance than those without CKD. Observational studies on changes in renal function, muscle strength, and walking speed have revealed that the appearance of microalbuminuria and even a mild decrease in the glomerular filtration rate constitute risks of physical decline [1]. In patients with mild CKD, all-cause mortality by declining physical performance, especially slowed walking speed or decreased walking distance, was approximately 2.5 times higher than those without physical decline [2]. Declining physical activity also indicated a strong cardiovascular mortality risk, and the intensity was the same as the risk derived from hypertension or diabetes [3]. Therefore, maintaining the physical performance of patients with CKD is an important part of their long-term treatment.

To date, the main cause of physical decline in patients with CKD is thought to be a reduction in skeletal muscle mass (i.e., sarcopenia), which accompanies protein catabolism [4]. In fact, the prevalence of sarcopenia is higher in patients with CKD from the early stages [5]. Hemodialysis patients experience an approximately 10% greater decrease in skeletal muscle mass than those with moderate-to-severe renal dysfunction [6]. However, Wilhelm-Leen et al. indicated that physical decline in patients with CKD may not necessarily be due to sarcopenia [1]. The study also showed the
decline in physical performance to be unrelated to the occurrence of potential factors, such as anemia, hypertension, or acidosis, etc. Thus, the factors that diminish the physical performance of patients with CKD are currently unclear.

**The mechanism of physical decline in CKD**

Therefore, to elucidate the mechanisms of physical decline in CKD, we examined 5/6 nephrectomized mice, a mild CKD model [7, 8]. Male C57Bl/6 mice had undergone 5/6 nephrectomy at 6 and 7 weeks old. Grip power and running distance were evaluated as muscle strength and exercise endurance at 8, 12, and 16 weeks old, by using a dynamometer and a treadmill, respectively. Further, at 16–20 weeks, muscle mass, skeletal muscle mitochondrial content, and mitochondria-related molecules were evaluated.

Whereas the running distance in 5/6 nephrectomized mice was approximately 60% of that seen in sham mice, grip power remained the same. Both mitochondrial content and activity in the skeletal muscle of 5/6 nephrectomized mice were approximately 70%. In contrast, muscle mass did not decrease.

Skeletal muscle mitochondria are regulated by AMP-activated protein kinase (AMPK) [9]. The activation of peroxisome proliferator activated receptor gamma coactivator-1α (PGC-1α) and mitochondrial transcription factor A (Tfam) increases both mitochondrial content and the levels of the electron transport enzymes cyclooxygenase IV (COX IV) and ATP synthase [10]. Western blotting and quantitative PCR showed a decrease in the levels of phosphorylated AMPK, PGC-1α, and Tfam in the skeletal muscle of 5/6 nephrectomized mice (approximately 60%, 50%, and 60%, respectively), accompanied with a decrease in the levels of COX IV and ATP synthase.

Creatinine clearance in 5/6 nephrectomized mice was approximately 40% of that seen in sham mice. In CKD, inflammatory cytokine levels are known to increase, beginning at the early stages of the disease [11]. The blood concentration of tumor necrosis factor-α (TNF-α), a typical inflammatory cytokine, in 5/6 nephrectomized mice was approximately five times of that seen in sham mice. Therefore, we assume that inflammatory cytokines affect the expression of mitochondrial regulatory molecules. Based on this assumption, both TNF-α and interleukin-6 (IL-6) were added to the C2C12-cultured myocytes for 24 hours (from 0.01 to 1 ng/mL). Both TNF-α and IL-6 decreased the PGC-1α expression levels and mitochondrial content in a concentration-dependent manner, which recovered in the presence of the cytokine inhibitors SPD-304 and D7715A7, respectively.

The above results indicated that following 5/6 nephrectomy, mitochondrial content and exercise endurance declined prior to reductions in muscle mass and muscle strength. The elevation in inflammatory cytokine levels associated with renal failure was suggested to be responsible for the reduction in mitochondrial content. Our additional long-term observations indicated that in addition to mitochondrial content and exercise endurance, muscle mass and muscle strength also declined in 5/6 nephrectomized mice at 50 weeks. Furthermore, although a high-protein diet enhanced muscle mass and muscle strength, we found that exercise endurance was decreased by a high-protein diet and accompanied with metabolic changes in the mitochondria [8].

**Therapeutic effects of ghrelin on the physical performance of CKD model mice**

Our results therefore suggest that physical decline associated with renal failure is caused by a decrease in mitochondrial content of the skeletal muscle. To improve physical performance, we focused on ghrelin, a peptide hormone derived from the gastrointestinal tract, because ghrelin administration has been reported to enhance oxygen utilization in skeletal muscle [12] (Fig. 1). Acylated ghrelin, an active form of ghrelin, was intraperitoneally administered at 0.1 nmol/gBW three times a week to 8-week-old 5/6 nephrectomized mice. One month later, the 12-week-old mice were evaluated for their muscle strength, exercise endurance, muscle mass, and mitochondrial content. These results were compared with the effects of IGF-1 (intraperitoneally administered at 0.1 nmol/gBW three times a week), which is a typical muscle anabolic factor.

Grip power was significantly higher in mice administered with ghrelin or IGF-1. Running distance increased significantly after ghrelin administration and was approximately 120% of that seen in 5/6 nephrectomized mice, but insufficient improvement was noted after IGF-1 administration. Both ghrelin and IGF-1 administration increased muscle mass. In contrast,
mitochondrial content after ghrelin administration was approximately 120% of that seen in 5/6 nephrectomized mice, but not after IGF-1 administration. The expression of phosphorylated AMPK and PGC-1α also increased after ghrelin administration (approximately 120% compared with 5/6 nephrectomized mice), but not after IGF-1 administration. The renal function of 5/6 nephrectomized mice did not change after the administration of either ghrelin or IGF-1. The serum TNF-α level after ghrelin administration was approximately 70% of that seen in 5/6 nephrectomized mice, but not after IGF-1 administration. Thus, the muscle strength and exercise endurance of 5/6 nephrectomized mice improved after ghrelin administration, accompanied with increased muscle mass and mitochondrial content. On the other hand, mitochondria were not activated after IGF-1 administration, and improvements in exercise endurance were insufficient.

To clarify the key molecule for mitochondrial regulation after ghrelin administration, we focused on PGC-1α. C2C12-cultured myocytes were transfected with PGC-1α siRNA, and the effects on mitochondrial content and oxygen consumption after the addition of 100 nM ghrelin for 24 hours were evaluated. PGC-1α expression after ghrelin administration was approximately 150% of that seen in control cells, accompanied with an increase in both mitochondrial content and oxygen consumption (approximately 140% and 130%, respectively). However, PGC-1α expression under the PGC-1α knockdown by siRNA did not increase after ghrelin administration. Improvements of mitochondrial content and oxygen consumption were also insignificant under these conditions. These results suggest that the addition of ghrelin to cultured myocytes increases mitochondrial content and oxygen consumption by promoting PGC-1α expression.

To further examine a regulatory mechanism of PGC-1α expression by ghrelin, the methylation of the gene promoter region was examined. The methylation of C-260, a cytosine residue 260 bases upstream of the transcription initiation point, which is a well-known methylation site that regulates PGC-1α expression [13], was evaluated by methylation-specific PCR (MSP) and bisulfite genomic sequencing (BGS). Using MSP in mouse skeletal muscle, the ratio of C-260 methylation of 5/6 nephrectomized mice was approximately 125% of that seen in sham mice. The ratio decreased after ghrelin administration (by approximately 80% compared with 5/6 nephrectomized mice) but did not change after IGF-1 administration. Using BGS, the methylation ratio in 5/6 nephrectomized mice was also found to decrease after ghrelin administration. In our experiment with cultured myocytes, the increased ratio of C-260 methylation caused by TNF-α decreased after the addition of either acylated ghrelin or des-acylated ghrelin (by approximately 80% compared with

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**Fig. 1** Improvement of physical decline by ghrelin administration

The level of ghrelin, a peptide hormone mainly released from the stomach, increased after fasting and decreased after feeding. Our previous study showed increased serum ghrelin levels after 5/6 nephrectomy in mice. Ghrelin has been reported to cause muscle hypertrophy, anti-inflammation, and enhancement of oxygen utilization. These alterations will improve the physical decline in CKD.
the cells after the addition of TNF-α). Further, both PGC-1α expression and mitochondrial content showed significant differences, reflecting changes in methylation rates.

In summary, we discovered that ghrelin administration improved muscle strength and exercise endurance, accompanied with increases in both muscle mass and mitochondrial content in 5/6 nephrectomized mice. In contrast, IGF-1 administration did not affect mitochondrial content, and improvements in exercise endurance were limited; although it improved muscle mass and muscle strength. From these results, it appears that ghrelin readily improves physical performance by increasing skeletal muscle mass and mitochondrial content in CKD. Further, the mechanism of action of ghrelin appears to be the enhancement of PGC-1α expression, associated with a reduction in promoter methylation (Fig. 2). These findings had been published in our previous paper [14].

Acknowledgments

This work was supported by Grants-in-Aid for Scientific Research of Japan Society for the Promotion of Science (JSPS). The grant numbers were 26860644 (for M.T.), 23791063, 26460920 (for K.M.), and 21390286 (for H.I.).

Disclosure

The authors declare no conflicts of interest.

Fig. 2 Mechanism of physical decline in CKD model mice and the role of ghrelin administration

Mitochondrial content and exercise endurance declined prior to reductions in muscle mass and muscle strength after 5/6 nephrectomy, associated with an increase in inflammatory cytokine levels. In addition to mitochondrial content and exercise endurance, muscle mass and muscle strength also declined in the late phase. Ghrelin readily improves physical performance by increasing skeletal muscle mass and mitochondrial content. Furthermore, the mechanism of action of ghrelin appears to be the enhancement of PGC-1α expression, associated with a reduction in promoter methylation.
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