Improvement of Physical Decline Through Combined Effects of Muscle Enhancement and Mitochondrial Activation by a Gastric Hormone Ghrelin in Male 5/6Nx CKD Model Mice

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Because a physical decline correlates with an increased risk of a wide range of disease and morbidity, an improvement of physical performance is expected to bring significant clinical benefits. The primary cause of physical decline in 5/6 nephrectomized (5/6Nx) chronic kidney disease model mice has been regarded as a decrease in muscle mass; however, our recent study showed that a decrease in muscle mitochondria plays a critical role. In the present study, we examined the effects of a gastric hormone ghrelin, which has been reported to promote muscle mitochondrial oxidation, on the physical decline in the chronic kidney disease model mice, focusing on the epigenetic modulations of a mitochondrial activator gene, peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α). Ghrelin treatment improved a decline in exercise endurance of 5/6Nx mice, associated with an increase in both of the muscle mass and mitochondrial amount. The expression level of PGC-1α was decreased in the skeletal muscle of 5/6Nx mice, which was associated with an increase in the methylation ratio of the cytosine residue at 260 base pairs upstream of the initiation point. Conversely, ghrelin treatment demethylated the cytosine residue and increased the expression of PGC-1α. A representative muscle anabolic factor, IGF-1, did not affect the expression of PGC-1α and muscle mitochondrial amount, although it increased muscle mass. As a result, IGF-1 treatment in 5/6Nx mice did not increase the decreased exercise endurance as effectively as ghrelin treatment did. These findings indicate an advantage of ghrelin treatment for a recovery of physical decline.

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formance in the CKD model mice; although previous interven-
tions have been mainly aiming at an enhancement of
muscle mass. In fact, a high-protein diet increases muscle
mass and strength, and might be beneficial for a physical
decline; however, a decrease in muscle mitochondrial
activity, that impaired exercise endurance and glucose tol-
erance, was demonstrated in mice with chronic adminis-
tration of excessive dietary protein (10). The representa-
tive muscle anabolic hormones, GH and the downstream
effectors, IGF-1, have been shown to increase muscle mass
in several sarcopenic animal models; however, improve-
ment of exercise endurance, which well reflects muscle
mitochondrial function (11–13), has not been easily
yielded (14–17).

In these contexts, a novel intervention to effectively
improve both muscle mass and muscle mitochondrial ac-
tivity has been expected for a recovery of physical decline.
Ghrelin is a stomach-derived GH-releasing peptide hor-
mone that stimulates appetite through activation of the
hypothalamic appetite center to evoke a hunger feeling (18,
19). Ghrelin treatment has been shown to increase muscle
mass (20–22), and in addition, recent study revealed that
ghrelin have a potential to promote muscle oxidative me-
tabolism (23). These facts made us hypothesize that gre-
lin might exert combined actions on muscle as a muscle
tenancer and a muscle mitochondrial activator for a re-
cover of physical decline.

A decrease in muscle mitochondria in aging or CKD
has been shown to be associated with a chronic reduc-
tion in the expression of a representative mitochondrial
activator gene, peroxisome proliferator-activated re-
ceptor (PPAR)-γ coactivator-1α (PGC-1α); however, the
regulatory mechanism has not been clarified
enough. Recently, an epigenetic modification of the
promoter region in upstream of initiation point of the
gene was revealed to critically control the expression
(24–26). Among them, the cytosine residue at 260 base
pairs upstream (C-260) was demonstrated to be a rep-
resentative methylation site, that decreased the gene ex-
pression (24). Therefore, the alteration in the methyl-
ation ratio of C-260 associated with 5/6Nx and ghrelin
treatment was examined as a possible mechanism for
the mitochondrial regulation.

In these contexts, the usefulness of ghrelin treatment for
a recovery of physical decline was examined by using
5/6Nx CKD model mice, in comparison with IGF-1 treat-
ment. The study focus was the effects of ghrelin and IGF-1
on muscle mitochondria and epigenetic modification of
PGC-1α gene, which are possible contributors on the
physical performance.

Materials and Methods

Materials, animals, and cells
Male C37Bl/6 mice were purchased from Charles River Lab-
oratories and maintained under specific pathogen-free condi-
tions. 5/6 nephrectomy was performed by heminephrectomy and
polectomy at 6 (1/2 nephrectomy) and 7 (remaining 1/3 polec-
tomy) weeks old, as previously reported (9). Mice were admin-
istered with acylated ghrelin (AG) (0.1 nmol/g body weight
(BW), 4373-second; Peptide Institute) or recombinant IGF-1
(0.1 nmol/g BW, I3769; Sigma-Aldrich) ip 3 times per week from
8 weeks old, unless otherwise noted. The dose was similar to
previous study (27). C2C12 cells, which are mouse-derived cul-
tured myocytes, were acquired from RIKEN Bio Resource Cen-
ter. Cells were grown to 50% confluency in DMEM (11995-065;
Life Technologies) supplemented with 10% fetal bovine serum
(FBS), and the cells were differentiated into myocytes by incu-
bation with 1% FBS for 7 days before the experiments. Primary
cultured skeletal muscle cells were isolated using a previously
reported procedure (28). Briefly, skeletal muscle was taken from
the hind limbs of 8 weeks old adult mice. To isolate myoblasts by
enzymatic digestion, the muscle was preserved in mixture of
0.1% collagenase II (17101-015; Life Technologies) and 2-U/mL
dispase II (17105-041; Life Technologies), according to the manu-
facturer’s instructions, and was incubated in a shaker bath
(37°C, 60 min). The slurry was filtered through 70-μm nylon mesh
(332360, Falcon cell strainer; Corning), then centrifuged. The pellets were preserved in DMEM supplemented with 10% FBS, and the same treatment was repeated twice. After preplating on a culture dish for 30 minutes, nonattached cells were trans-
ferred to a collagen-coated dish (354450, BioCoat Collagen I
100 mm Dish; Corning).

Differentiated cells were treated with DMEM with or without
AG (10nM, 100nM, or 1000nM), des-acylated ghrelin (DAG)
(100nM, 4437-second; Peptide Institute), recombinant IGF-1
(10nM, 100nM, or 1000nM), or TNF-α (1 ng/mL, T7539;
Sigma-Aldrich) for 24 hours before analysis, unless otherwise
noted. The dose was determined in accordance with previous
study (9, 29). All animal experiments were approved by the local
ethics committee and were done in accordance with domestic law
on the protection of laboratory animals, which is based on the
National Institutes of Health Guide for the Care and Use of
Laboratory Animals.

Small interfering RNA (siRNA) transfection in
myocytes
C2C12 cells were treated with Opti-MEM Reduced-Serum
Medium (31985-070; Life Technologies) and incubated for 24
hours. A mixture of siRNA targeting PGC-1α (10nM of final
density; MSS207867, MSS207868, and MSS276436; Stealth
RNAi siRNA, Life Technologies) or a negative control (12935-
300, Stealth RNAi Negative Control Medium GC Duplex;
Life Technologies) was transfected by using Lipofectamine
RNAiMAX (13788; Life Technologies) according to the manu-
facturer’s instructions. AG (100nM) or saline was added to the
medium after the transfection.
Analysis of mitochondrial amount, mitochondrial electron transport complex (ETC) activity, and oxygen use in C2C12 cells

To evaluate mitochondrial amount, mitochondrial DNA copy number was determined by quantitative PCR analysis (Applied Biosystems 7500 Fast Real-Time PCR system; Applied Biosystems), by using primers specific for the mtDNA encoded 16S ribosomal RNA gene and the nuclear DNA encoded hexokinase 2 gene, as described previously (30). The primer sequences are described in Supplemental Table 1. To evaluate mitochondrial ETC activity, mitochondria from a fixed number of cells were isolated, and mitochondrial nicotinamide adenine dinucleotide dehydrogenase activity and cytochrome c oxidase (COX) activity were measured using commercially available kits (700930, Mitochondrial complex I activity assay kit; Cayman Chemical and KC010100, Mitochondria Isolation kit and KC310100, Mitochondria Activity Assay kit; BioChain), according to the manufacturer’s instructions.

The signal intensity was read by a microplate reader (Synergy 4; BioTek). Oxygen use of C2C12 cells was measured using an extracellular flux analyzer (XF24; Seahorse Bioscience). Microscopic analysis of C2C12 cells was performed with fluorescent dyes. C2C12 cells were prepared on a cover glass (C015001, Micro Cover Glass; Matsunami Glass), which was coated with 0.1% gelatin-PBS and then placed in a 24-well plate. Mitochondria and nuclei of the cells were stained with fluorescent dyes (mitochondria: M-7514, Mito Tracker Green FM, Life Technologies and nuclei: 62249, Hoechst 33342 Fluorescent Stain, Thermo Fisher Scientific) and visualized under a fluorescence microscope (LSM 710; Carl Zeiss).

Analysis of physical performance of mice

To analyze physical performance, 5/6Nx mice were evaluated for grip power and running distance at 12–16 weeks old. Grip power was measured using a dynamometer for mice (MK-380M, Muromachi kikai). Each mouse was put on a metal mesh and pulled horizontally. The power of traction when the mouse released the mesh was defined as grip power. Running distance was measured by using a treadmill for mice (LE8710M; PanLab), according to a previous report (9). Briefly, mice were forced to run on the motor-driven treadmill until they were exhausted, which was defined as the point at which they kept still on an electrical shocker plate (mild stimulation of 0.2 mA, equivalent to medically used electric therapy equipment) at the end of the treadmill for more than 30 seconds. The treadmill was set at a 10% incline; the speed was 18 cm/s initially and was increased by 3 cm/s every 2 minutes. The average running time until exhaustion for wild-type mice was approximately 30 minutes (corresponding to 600 m of running).

Analysis of biological parameters of mice

Blood samples were collected from the orbital venous plexus at 16 weeks old. Measurements of serum creatinine, TNF-α, and urine creatinine were performed by a standard method (SRL). Creatinine clearance (CCr) was calculated by the formula: CCr (µL/min) = [urinary creatinine (mg/dL) × urine volume (µL/d)] ÷ serum creatinine (mg/dL) ÷ (24 × 60). Plasma levels of AG and DAG were measured by using commercially available kits (97751, Active Ghrelin ELISA kit and 97752, Desacyl-Ghrelin ELISA kit; Mitsubishi Chemical Medience), according to the manufacturer’s instructions.

Analysis of muscle histology and mitochondria of the 5/6Nx mice

Mice were killed at 16 weeks old, and tissue samples were harvested, weighed, crushed, and frozen at −80°C until analysis. For histological examination, the gastrocnemius muscle was embedded in OCT compound (Sakura Finetek), frozen, and sliced. The frozen sections of the gastrocnemius muscle were stained with succinate dehydrogenase (SDH) for measurements of muscle fiber size and classification of fiber type into I (slow oxidative), Ila (fast oxidative), or llb (fast glycolytic) as a previously described (9). Briefly, microscopic images of muscle were captured (IX81; Olympus) and the tissue images were digitized as gray-level images on a computer assisted image-processing system (Photoshop CS6; Adobe systems). A gray level value of 0 was equivalent to 100% transmission of light (%T), and that of 255 was equivalent to 0% T. The optical density value of all the muscle fibers was determined on the basis of the gray-level images and classified into the 3 groups: I (%T, 100%–80%), Ila (%T, 80%–40%), and llb (%T, 20%–0%). Mitochondrial amount and ETC enzymatic activity of the muscle sample were measured using the same method as was used in the in vitro experiment.

Estimation of expression levels by real-time PCR

The mRNA levels of mitochondria related genes in the quadriceps muscle of 5/6Nx mice were evaluated by real-time PCR. The expression levels of ghrelin and GH secretagogue receptor (GHS-R) were evaluated in the skeletal muscle, the stomach, and the kidney. Total RNA was extracted by using a commercially available kit (74104, RNasey minikit; QIAGEN) and cDNA was prepared by reverse transcription (RR037A, Primescript RT reagent kit; Takara Bio). Subsequent real-time PCR was performed by using Applied Biosystems 7500 Fast Real-Time PCR system and SYBR Premix Ex Taq II (RR820A; Takara Bio), according to the manufacturer’s instructions. The mRNA levels of PGC-1α, mitochondrial transcription factor A (Tfam), COX subunit IV (COX IV), ATP synthase, PPAR-δ, troponin I, sirtuin 1 (SIRT1), uncoupling protein (UCP)2 and UCP3, ghrelin, and GHS-R (3 types of primers were used: those for common region of GHS-R1a and GHS-R1b, GHS-R1a-specific region, and GHS-R1b-specific region) were examined and the results were corrected by the levels of 18S rRNA. The primer sequences are described in Supplemental Table 1.

Analysis of protein levels by Western blotting

Western blot analysis was performed to evaluate the AMP-activated protein kinase (AMPK) activity in the quadriceps muscle. Total protein extracts (10 μg) from the quadriceps muscle were separated by polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes using a dry blotting system (Transblot turbo; Bio-Rad), and incubated with the primary antibodies for phospho-AMPK (P-AMPK)α (Thr172) or AMPKα, the detailed information of the antibodies is described in Table 1. Immuno-labeled proteins were detected using a chemiluminescence kit (ECL Prime; GE Healthcare) and a lumino-image analyzer (LAS 4000; Fuji Film). The density of the blot was estimated using imaging

[Note: The rest of the text is cut off and not fully transcribed.]
software (MultiGauge, Fuji Film). Results of densitometry analysis of P-AMPK were adjusted by those of total AMPK (P-AMPK/AMPK) to estimate AMPK activity.

Analysis of C-260 methylation at the promoter region of PGC-1α gene

To investigate the methylation ratio of C-260 at the promoter region of PGC-1α gene in C2C12 cells or the quadriceps muscle of 5/6Nx mice, methylation-specific PCR (MSP) analysis and bisulfite genomic sequence (BGS) analysis were performed. For MSP analysis, total DNA of the cells or the quadriceps was extracted using a commercially available kit (69504, DNeasy Blood and Tissue kit; QIAGEN), and fragmented by using a restriction enzyme, BamHI (1010A; Takara Bio). Bisulfite treatment was performed using a commercially available kit (59104, Epitect Bisulfite kit; QIAGEN), according to the manufacturer’s instructions. MSP analysis was performed using the 96-Well GeneAmp PCR system 9700 (Applied Biosystems) and Episcop MSP kit (R100A; Takara Bio), according to the manufacturer’s instruction. PCR products were separated on a 3% agarose gel. Electrophoresis was performed by using the submarine electrophoresis system (Mupid-2plus; Advance), and FAS-III (Toyobo) instructions. PCR products were cloned using the TA cloning system and positive clones were sequenced according to a previously described protocol (31). Densitometry analysis of the captured images was performed using ImageJ (http://imagej.nih.gov/ij/). For BGS analysis, bisulfite-treated DNA was amplified (2 rounds) using primers that recognize the promoter region of PGC-1α gene containing C-260. The primer sequences are described in Supplemental Table 1. PCR products were cloned using a TA cloning system and positive clones were sequenced with a genetic analyzer.

Statistical analysis

All data are expressed as mean ± SE. Comparison of the means between 2 groups was performed by Student’s t test. For comparisons of more than 2 groups, ANOVA was used to evaluate significant differences among groups. When a difference was confirmed, it was further examined by Fisher’s protected least significant difference method. P < .05 was considered to be statistically significant.

Results

Plasma level of ghrelin and expression level of ghrelin receptor, GHS-R, in the skeletal muscle were increased in 5/6Nx CKD model mice

The effect of renal failure on plasma level of ghrelin and a ghrelin-specific receptor (GHS-R), was evaluated by examining the 5/6Nx CKD model mice. Plasma levels of 2 forms of ghrelin: AG (which can bind to GHS-R) and DAG (which cannot bind to GHS-R but possibly exerts biological effects through unidentified receptors) were elevated in 5/6Nx mice when compared with sham mice (Figure 1, A and B). Ghrelin was strongly expressed in the stomach and weakly expressed in the skeletal muscle and the kidney (at 1:1000 of the level in the stomach) (Figure 1C). In contrast, GHS-R was sufficiently expressed in the skeletal muscle (at 1:10 of the level in the stomach or the kidney). Remarkably, the muscular expression of GHS-R, as well as plasma ghrelin level, was increased in 5/6Nx mice (Figure 1D). These findings suggested that ghrelin would exert a greater effect in 5/6Nx mice than sham mice.

Furthermore, the expression of 2 splicing variants of GHS-R was examined (GHS-R1a and GHS-R1b). GHS-R1a is a functional receptor, whereas GHS-R1b is a truncated variant, which is incapable of binding ghrelin and therefore nonfunctional. Both GHS-R1a and GHS-R1b were expressed in the skeletal muscle and GHS-R1b was dominant, which is a finding similar to a previous report (32). The expression of both receptors increased in parallel in the skeletal muscle of 5/6Nx mice (Figure 1E). They were also detectable in C2C12-cultured myocytes and the expressions were increased in differentiated myotubes more than in undifferentiated myoblasts (Figure 1F).

Ghrelin treatment improved exercise endurance of 5/6Nx mice, associated with an increase in muscle mitochondrial amount

The effect of ghrelin treatment on physical decline in 5/6Nx mice, which is associated with a decrease in muscle mitochondrial amount, was investigated. 5/6Nx mice that had undergone 5/6 nephrectomy at 6 and 7 weeks were ip administered AG (0.1 nmol/g BW; 3 times per wk) from 8 weeks old. In addition, a group of 5/6Nx mice were administered IGF-1 (0.1 nmol/g BW; 3 times per week). The grip power of 5/6Nx mice was similar to that of sham mice, and was increased in both ghrelin- and IGF-1-treated 5/6Nx mice (Figure 2A). Meanwhile, reduced running distance of 5/6Nx mice was effectively improved by ghrelin treatment; however, IGF-1 treatment insufficiently improved the distance (Figure 2B). Histological analysis of

Table 1. Antibody Table

<table>
<thead>
<tr>
<th>Peptide/Protein Target</th>
<th>Name of Antibody</th>
<th>Manufacturer, Catalog Number, and/or Name of Individual Providing the Antibody</th>
<th>Species Raised in; Monoclonal or Polyclonal</th>
<th>Dilution Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-AMPK</td>
<td>Phospho-AMPKα (Thr172) antibody</td>
<td>Cell Signaling Technology, 2531</td>
<td>Rabbit; monoclonal</td>
<td>1:1000</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMPKα antibody</td>
<td>Cell Signaling Technology, 2532</td>
<td>Rabbit; monoclonal</td>
<td>1:1000</td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td>Antirabbit IgG</td>
<td>GE Healthcare, NA934–1ML</td>
<td>Donkey; polyclonal</td>
<td>1:5000</td>
</tr>
</tbody>
</table>

Remarkably, the muscular expression of GHS-R, as well as plasma ghrelin level, was increased in 5/6Nx mice (Figure 1D). These findings suggested that ghrelin would exert a greater effect in 5/6Nx mice than sham mice.
the gastrocnemius muscle was performed by using SDH staining (Figure 2C) and an electron microscope (EM) (Figure 2D). Muscle weight and fiber size were similar in the 5/6Nx mice and sham mice. Both ghrelin and IGF-1 increased muscle weight and fiber size of 5/6Nx mice (Figure 2, E and F).

Mitochondrial amount and ETC activities in the skeletal muscle of 5/6Nx mice were decreased. They were improved by ghrelin treatment (Figure 2, G and H); however, no significant improvement of them was observed in IGF-1-treated 5/6Nx mice. Expression of GHS-R1a and GHS-R1b in the quadriceps muscle of 5/6Nx mice (E) and C2C12-cultured myocytes (F) that were estimated by primers designed to specifically recognize GHS-R1a or GHS-R1b. Expression levels of GHS-R1a in sham mice and undifferentiated C2C12-cultured myocytes (corresponding to myoblasts) were assigned as 100%, respectively; n = 8 in each group for in vivo experiments and n = 4 independent experiments for in vitro studies; *, P < .05; **, P < .01 vs sham mice or undifferentiated C2C12-cultured myocytes.
Ghrelin treatment, which was not induced by IGF-1 treatment.

Ghrelin treatment increased mitochondrial amount in C2C12-cultured myocytes through an increase in PGC-1α expression

The effect of AG and IGF-1 on mitochondrial amount and the expression of PGC-1α gene were evaluated by using ghrelin or IGF-1-treated differentiated C2C12-cultured myocytes. Both PGC-1α expression and mitochondrial amount were dose-dependently increased in ghrelin-treated cells from the dose of 10nM, although IGF-1 did not affect them (Figure 4, A and B). A recent report demonstrated that PGC-1α expression was regulated by the methylation ratio of the cytosine (C-260) at the promoter region (24). The significant increase in the expression of PGC-1α and the mitochondrial amount by ghrelin treatment was also observed in primary cultured mouse skeletal muscle cells (Figure 4C). Therefore, we examined the methylation ratio in the ghrelin-treated cultured myocytes. MSP analysis showed that the methylation ratio of C-260 was reduced in ghrelin-treated cells (Figure 4, D and E).

To investigate whether the effect of ghrelin on mitochondrial amount was dependent on PGC-1α, the siRNA for PGC-1α which suppresses the expression was used. Expression level of PGC-1α was not increased by ghrelin in the siRNA-treated C2C12 cells (Figure 4F). Mitochondrial amount, ETC activity and oxygen use were increased in ghrelin-treated cells; however, these increases were attenuated in the siRNA-treated cells (Figure 4, G–J). These findings indicated that ghrelin treatment affected mitochondrial amount and oxygen use of the myocytes at least partially through an increase in the expression of PGC-1α, which was associated with demethylation of C-260 at the promoter region.

Ghrelin treatment alleviated the increase in serum TNF-α and methylation of C-260 at the promoter region of PGC-1α gene in the skeletal muscle of 5/6Nx mice

Because our previous report showed that inflammatory cytokines, such as TNF-α and IL-6, decreased mitochondrial amount in cultured myocytes in association with a decrease in PGC-1α expression, the effect of ghrelin on TNF-α levels were examined (9). Ghrelin suppressed serum TNF-α level and TNF-α expression in the muscles, spleen, kidney, and aorta of the 5/6Nx mice; however, it did not suppress that in the heart. The stronger suppression in the spleen suggested the potency of ghrelin to lower the production of TNF-α from immune cells. IGF-1 treatment did not suppress TNF-α expressions in any organs tested (Figure 5, A–C). The alteration in methylation ratio of C-260 at the promoter region of PGC-1α gene in the skeletal muscle of ghrelin-treated 5/6Nx mice was evalu-

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Table 2. Serum Creatinine Level and Creatinine Clearance of the Mice Used in the Present Study

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>5/6Nx</th>
<th>5/6Nx + ghrelin</th>
<th>5/6Nx + IGF-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum creatinine (mg/dL)</td>
<td>0.142 ± 0.014</td>
<td>0.272 ± 0.023a</td>
<td>0.288 ± 0.023</td>
<td>0.304 ± 0.016</td>
</tr>
<tr>
<td>Ccr (µL/min)</td>
<td>248 ± 27.9</td>
<td>116 ± 14.2a</td>
<td>133 ± 11.4</td>
<td>131 ± 10.7</td>
</tr>
</tbody>
</table>

n = 8 in each group.

a P < .01 between sham and 5/6Nx.
ated by MSP and BGS analysis, as a potential mechanism underlying the mitochondrial action of ghrelin. MSP analysis showed that the methylation ratio was increased in the skeletal muscle of 5/6Nx mice, and this augmented methylation was suppressed by ghrelin treatment, but not by IGF-1 (Figure 5, D and E). BGS analysis showed a compatible reduction in methylation ratio of C-260 at the promoter region of PGC-1α gene in the skeletal muscle of 5/6Nx mice (Figure 5F). These findings indicated that ghrelin treatment increased PGC-1α expression and mitochondrial amount in the skeletal muscle of 5/6Nx mice, associated with epigenetic modulation of PGC-1α gene.

The effect of AG and DAG on mitochondrial amount, PGC-1α expression and methylation ratio of C-260 at the promoter region of PGC-1α gene were evaluated using TNF-α-treated C2C12 cells. TNF-α decreased expression level of PGC-1α and mitochondrial amount, associated with an increase in methylation ratio of the C-260. Both AG and DAG restored these changes in cultured myocytes induced by TNF-α (Figure 5, G–J). The findings indicated that both AG and DAG are responsible for the mitochondrial effects of ghrelin.

**Discussion**

Ghrelin is a 28-amino acid peptide hormone which was originally identified as an endogenous ligand for the GHS-R (33). It is released mainly from neuroendocrine cells in the stomach during fasting (34) and exerts a potent orexigenic effect by stimulating hypothalamic arcuate nucleus. Serine 3 of ghrelin is n-octanoylated, and this AG binds to the GHS-R and exerts biological effects by increasing intracellular Ca2⁺ levels. The degradation product DAG is unable to bind to the GHS-R nor activate it; however, recent reports strongly suggest that DAG exerts unique activities through an unidentified receptor (35, 36).

In addition to the effect on appetite stimulation, ghrelin has been shown to regulate muscle mass and metabolism. Ghrelin treatment has been shown to increase muscle mass in sarcopenic animals due to cachexia and unloading (20–22). The muscle-specific ubiquitin ligases, atrogin-1 and muscle RING finger protein 1, which promote muscle degradation, have been shown to be suppressed by ghrelin (37, 38). Both AG and DAG were reported to induce muscle fusion and hypertrophy as to increase muscle mass (39, 40). In recent reports, muscle metabolism has been demonstrated to be regulated by ghrelin. Administration of ghrelin in rats stimulated fat oxidation in the gastrocnemius muscle associated with increased expression of PPAR-γ (41). Ghrelin treatment increased muscle mitochondrial function in rats with CKD (23). These muscular effects of ghrelin indicate possible beneficial effects on...
physical decline in CKD; however, detailed effects of ghrelin on physical performance have not been elucidated so far.

The present study revealed that ghrelin treatment in 5/6Nx mice effectively restored the decreased exercise endurance, when compared with a representative muscle enhancer IGF-1, associated with dual increases in muscle mass and mitochondria. The increase in muscle mitochondrial amount induced by ghrelin was consistent with the increased expression of PGC-1α and other mitochondrial regulators including ATP synthase, COX IV, SIRT1, UCP2, and UCP3. In contrast, IGF-1 treatment did not cause these changes nor did it sufficiently improve the reduced exercise endurance of 5/6Nx mice; even though the muscle mass and strength were increased. The novelities of current study can be summarized as follows. 1) Ghrelin showed superiority in increasing physical decline in 5/6Nx mice compared with IGF-1. 2) The advantage of ghrelin was corresponded with the activation of muscle mitochondria; because muscle mitochondrial function has been shown to closely correlate with exercise endurance (11–13). And 3) a key factor for the mitochondrial activation by ghrelin was a reduction in the methylation ratio of the promoter region of PGC-1α gene.

Although the regulatory mechanism for the PGC-1α expression has not been adequately clarified, recent reports identified that an epigenetic modification of the promoter region, especially of the C-260 of the initiation point (C-260), regulated the expression of PGC-1α and mitochondrial amount (24–26). These epigenetic modifications of genes can cause chronic changes in gene expression that might lead to a pathological condition. For example, uremic toxins due to renal failure have been shown to epigenetically modify genes for profibrotic cytokines and thus augment renal fibrosis that contributes to the progression of CKD (42).

We found that the overmethylation of C-260 in promoter region of PGC-1α gene was a mechanism for a chronic decrease in muscle mitochondria of 5/6Nx mice. DNA methylation in the promoter of PGC-1α has been demonstrated to be augmented by several substances including palmitate, oleate and TNF-α (24). Among them, TNF-α is a representative inflammatory cytokine that is increased from an early stage of CKD. Therefore, TNF-α
is a possible causative factor for a loss of muscle mitochondria in the 5/6Nx mice. In fact, TNF-α has been shown to decrease mitochondria in an in vitro experiment using cultured myocytes (9, 43).

Conversely, ghrelin treatment demethylated C-260, as to recover the muscle PGC-1α expression and mitochondrial amount in the 5/6Nx mice. Two distinct mechanisms by which ghrelin could regulate the DNA methylation were identified in the present study. First, a suppression of serum TNF-α level observed in ghrelin-treated 5/6Nx mice. Second, a direct effect on the methylation shown in ghrelin-treated C2C12 cells. Previous reports also demonstrated that ghrelin reduced TNF-α level in in vivo experiments, which was up-regulated by endotoxin infusion (44) or high-fat diet (45). On the other hand, IGF-1 treatment did not affect the DNA methylation, PGC-1α expression nor muscle mitochondrial amount. The difference of these mitochondrial effects between ghrelin and IGF-1 would explain the advantage of ghrelin treatment for a recovery of physical decline.

Recently, we reported that 5/6Nx modulates muscle physiology by decreasing muscle mitochondria through increasing circulating inflammatory cytokines and oxidation products that suppress the expression of PGC-1α (9). We used 5/6Nx mice as an experimental model of physical decline for several reasons. 1. 5/6Nx easily induces physical decline. A decrease in exercise endurance becomes apparent 4 weeks after the mice are performed 5/6Nx. It
is an easier way than preparing aging mice for an experimental model of physical decline. 2. Physical decline in an early stage of 5/6Nx mice is characterized by a decrease in muscle mitochondria and exercise endurance (9). Because the focus of present study was mitochondrial actions of ghrelin, we preferred an experimental model with remarkable mitochondrial dysfunction. 3. Physical decline in CKD patients has been a critical medical problem which strongly predicts cardiovascular events. Therefore, an intervention that can improve physical performance in CKD is clinically expected. For these reasons, we examined an effect of ghrelin treatment using 5/6Nx mice. The effect of ghrelin on physical performance should be further examined in other mice models of physical decline, such as aging mice, disuse sarcopenic mice or myodystrophic mice, because 5/6Nx mice are not a standard model of physical decline.

In summary, we revealed that ghrelin treatment effectively improved physical decline of 5/6Nx mice through the combined effects to enhance muscle mass and mitochondrial amount. The mitochondrial actions of ghrelin associated with epigenetic modification of muscle PGC-1α expression were suggested to be responsible for the beneficial effects. The findings indicate an advantage of ghrelin treatment for a recovery of physical decline in CKD.

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