Review

Pathogenesis of uremic sarcopenia based on metabolic alteration

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Abstract Sarcopenia, the degenerative loss of skeletal muscle mass, is associated with increased morbidity and mortality for patients with chronic kidney disease (CKD), where it is specifically referred to as uremic sarcopenia. However, the details of the abnormal metabolic processes induced by uremic toxins remain unclear. Recently, we clarified the pathogenic mechanisms of uremic sarcopenia using liquid chromatography-mass spectrometry (MS) and MS imaging, which revealed that uremic toxin indoxyl sulfate accumulates in the muscle tissue of CKD model mice. Moreover, capillary electrophoresis MS-metabolomics of a muscle cell line suggested that indoxyl sulfate induces metabolic alterations such as upregulation of glycolysis, including the pentose phosphate pathway, for protection against oxidative stress. This altered metabolic flow leads to downregulation of the tricarboxylic acid cycle resulting in an ATP shortage. In a clinical study, plasma indoxyl sulfate levels were associated with skeletal muscle mass reduction in CKD patients. In this review, I discuss the known pathogenic mechanisms of uremic sarcopenia induced by the uremic toxin indoxyl sulfate with a focus on the consequent metabolic alteration and mitochondrial dysfunction.

Key words: Uremic toxin, mass spectrometry imaging, mass spectrometry, metabolomics

Introduction

Chronic kidney disease (CKD), which is a consequence of lifestyle-related diseases such as diabetes and hypertension, is a major health problem worldwide associated with the risk of end-stage renal disease and cardiovascular disease. Although cardiovascular disease is the primary cause of death for patients with CKD[1,2], other complications also substantially contribute to the high mortality rate. Skeletal muscle is the major tissue of energy consumption in the body, and is also the primary site of glucose disposal. Patients with CKD typically suffer from uremic sarcopenia, which is the progressive loss of skeletal muscle mass and strength[3,4]. Sarcopenia is observed in all CKD stages, and the reduction of muscle strength is associated with a poor prognosis[5]. Thus, uremic sarcopenia is a serious complication of CKD that requires medical treatment. Although inflammation, hormonal and immunological changes, metabolic acidosis, and reduction of protein intake are all known to be involved in the pathogenesis of sarcopenia in CKD, the specific pathogenic mechanisms of uremic sarcopenia remain unclear. Accordingly, there is currently no efficacious therapy for uremic sarcopenia.

CKD progression results in the accumulation of uremic toxins in the circulation, some of which cannot be adequately removed by dialysis therapy, including protein-bound uremic toxins[6]. These accumulated uremic toxins are involved in the various complications of CKD such as hypertension, cardiovascular disease, neurological impairment, and bone disorders[7−11]. Recently, we revealed the pathogenic mechanisms of uremic sarcopenia using

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mass spectrometry (MS)[12]. In this review, I summarize the insight gained into these pathogenic mechanisms with a focus on the effects of metabolic alterations and mitochondria dysfunction occurring in the muscle cells due to the impact of the accumulation of uremic toxins.

Uremic Sarcopenia

Sarcopenia refers to the reduction in muscle mass and function, which is associated with the physiological aging. Uremic sarcopenia, which represents muscle wasting in CKD patients, is associated with increased morbidity and mortality[4]. Uremic sarcopenia is common with an overall prevalence of ～50% in patients undergoing dialysis[4,13]. Multiple factors such as inflammation, hormonal changes, myocellular changes, immunological changes, protein energy wasting, renin-angiotensin system changes, muscle protein balance changes, and uremic changes are known to contribute to uremic sarcopenia[4]. The ATP-dependent ubiquitin-proteasome system (UPS) pathway is recognized to be involved in one of the most important forms of muscle loss and is characterized as the primary cause of muscle mass degradation in CKD patients[14]. Inflammation, metabolic acidosis and insulin resistance, which are complications of CKD, activate the UPS to degrade muscle protein[15]. Insulin/insulin-like growth factor I (IGF-I) signaling plays an important role in the regulation of cell growth, cell proliferation, development, and maintenance of several tissues within the human body.

Satellite cells, located under the basal lamina of myofibers, maintain muscle mass. They are activated by muscle injury and they express the transcription factors MyoD and myogenin, which trigger cell proliferation and differentiation during repair. Impairment of insulin/IGF-I signaling and satellite cell function leads to muscle wasting. In a rodent CKD model, metabolic acidosis, excess angiotensin II, and inflammation were found to be the factors causing muscle wasting via impairment of insulin/IGF-I signaling[5,16–19]. In addition, satellite cell function was found to be impaired in a rodent CKD model[20]. Although the activation of UPS, impairment of the insulin/IGF-I pathway, and impairment of satellite cell function are considered pathogenic mechanisms of uremic sarcopenia, no potential agent for therapy or prevention is yet available for muscle wasting in CKD. Therefore, novel strategies with regard to the therapy or prevention of uremic sarcopenia are required.

Uremic Toxins and Sarcopenia

Several waste products accumulate in the circulation due to the reduction of renal function[6], and those with a negative impact on biological functions are known as uremic toxins[21]. Uremic toxins are classified into three groups[22,23]: low-molecular-weight solutes (<500 Da), protein-bound low-molecular-weight solutes (<500 Da), and intermediate-sized molecules (>500 Da). These uremic toxins contribute to the pathogenesis of CKD-related complications such as hypertension, cardiovascular disease, neurological disorders, and bone disorders[7–10]. Although uremic toxins have been generally considered to be linked to the pathogenesis of uremic sarcopenia, the details remain unclear. Our preliminary examination of this association suggested that plasma indoxyl sulfate (IS) and methylguanidine levels were negatively correlated with the skeletal muscle mass level of CKD patients. Based on these results, we further examined the toxic effects of these two uremic toxins on myoblast cell line C2C12 using MTT assays, and found that only IS had a toxic effect on the muscle by restricting cell growth[12]. Recently, Enoki et al. examined the effect of uremic toxins on the C2C12 cells[24]. They specifically focused on six protein-bound uremic toxins, IS, indole acetic acid, p-cresyl sulfate, hippuric acid, kynurenic acid, and 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid, which are known to accumulate in the circulation under a condition of CKD due to a high affinity for serum albumin[25]. They found that IS had the strongest anti-proliferative effect for C2C12 cells and induced skeletal muscle atrophy by increasing the production of atrogin-1 and myostatin through inducing muscular oxidative stress-mediated inflammation[24]. IS is an end-product of tryptophan metabolism and has been associated with CKD progression[26]. Nishikawa et al. found that IS might be involved in the impairment of exercise capacity through oxidative stress, and that the removal of uremic toxins by using an oral adsorbent AST-120 improved exercise capacity in CKD mouse models[27]. However, the specific effects of IS toxicity on muscle cells remain unclear. Thus, we further examined the association between IS and sarcopenia using an adenine-induced CKD mouse model[12] that is characterized by a significant increase in the plasma IS level. To evaluate the relationship between IS and skeletal muscle changes in a CKD condition, we compared the cross-sectional area of the anterior tibia muscle and intramuscular IS between control and CKD mice. The cross-sectional area of the anterior

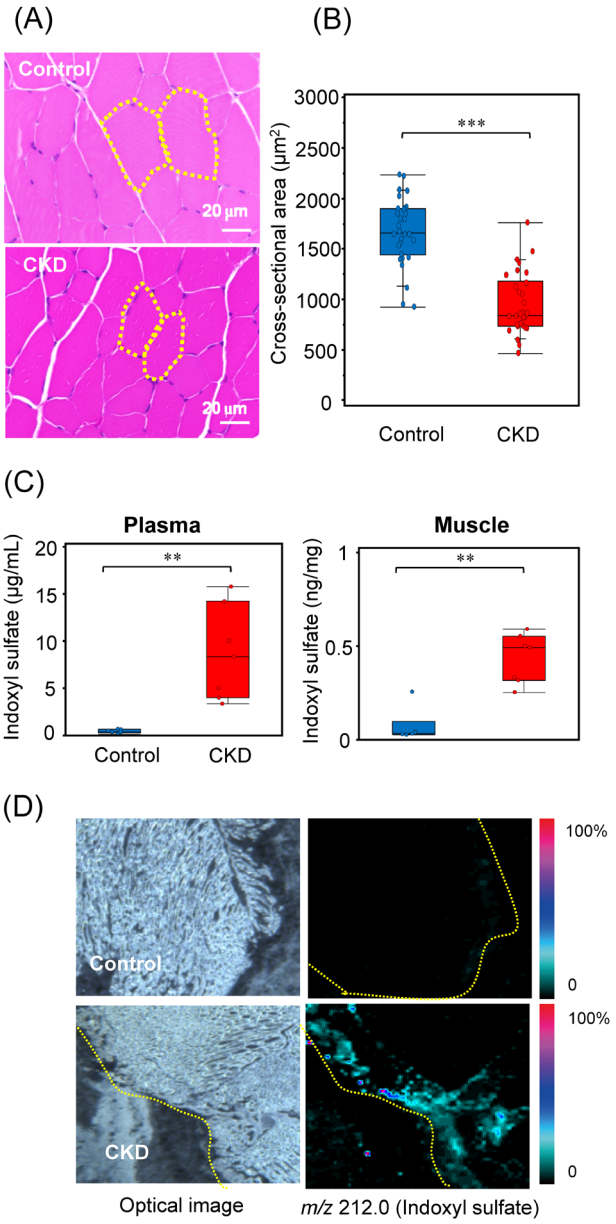

Fig. 1. Comparison of cross-sectional area and indoxyl sulfate levels in muscle tissues between healthy and chronic kidney disease (CKD) mice.
(A) Hematoxylin and eosin staining of the anterior tibia muscle. (B) Comparison of the cross-sectional area between control and CKD mice. (C) Indoxyl sulfate accumulation in plasma and muscle. Modified from Ref. 28. (D) Mass spectrometry imaging-based cellular uptake of indoxyl sulfate into CKD mice. Reproduced from Ref. 12 with permission.

tibia muscle was significantly smaller in CKD mouse model than that in control mouse (Fig. 1A, 1B). IS accumulation in muscle tissues was examined using liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Fig. 1C)[28]. Methods of sample preparation and measurement for LC-MS/MS were as follows: Muscle tissue was perfused before harvesting to flush out blood. Fifty-milligram of muscle tissue was mixed with 800 μL of 0.1% formic acid methanol containing 2.5 μg/mL IS-d_4, and homogenized for 30 s at 6000 rpm at room temperature using a Percellys 24 lysing and homogenization system (M&S Instruments Inc., Osaka, Japan). After homogenization, sample was sonicated for 15 min and then centrifugated at 16,400×g for 20 min at 4°C. An equal volume of 0.1% formic acid was added to the supernatant, and the mixture was analyzed by LC-MS/MS. For plasma, 150 μL of 0.1% formic acid methanol containing 2.5 μg/mL IS-d_4, and vortex for 1 s. After vortexing, sample was sonicated for 15 min and then centrifugated at 16,400×g for 20 min at 4°C. An equal volume of 0.1% formic acid was added to the supernatant, and the mixture was analyzed by LC-MS/MS. Quantitative analysis of IS was performed using a Nanospace SI-II HPLC platform (Shiseido, Tokyo, Japan) coupled to a TSQ Quantiva mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA), and operated in the negative mode. 100×2.0 mm Capcell Pak C18 MG-III 3 μm column (Shiseido) was used. 10 mM ammonium acetic acid and acetonitrile were used as mobile phase for gradient elution. Quantification analyses by MS/MS were performed by selected reaction monitoring (SRM) mode. SRM condition and collision energy were as follow: m/z 212→80, 21 eV for IS and m/z 216→80, 30 eV for IS-d_4. Plasma IS level was 20-fold higher in the CKD group than in the control group, and muscle IS level was 6.2-fold higher in the CKD group than in the control group (Fig. 1C). In addition to LC-MS/MS analysis, we visualized IS on muscle tissue by MS imaging (Fig. 1D). Methods of sample preparation and measurement for mass spectrometry imaging were as follows: Muscle tissues were sectioned at 10-μm thickness with a cryostat and thaw-mounted onto ITO-coated glass slide. 9-aminoacridine (9-AA) was used as a matrix. Matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS, AXIMA® Confidence, Shimadzu) equipped with a 377 nm N_2 laser was used for spectrometry analysis. Mass spectra were acquired with the laser frequency in the negative and scanning mass range from m/z 50 to m/z 1000 at high-resolution mode. Metabolites were identified with the MS/MS spectrum from results of IS chemical standard analysis. IS signal was detected only on muscle tissue of CKD group (Fig. 1D). Our results indicated that the skeletal muscle was atrophied and IS accumulated in the muscle of CKD mice.

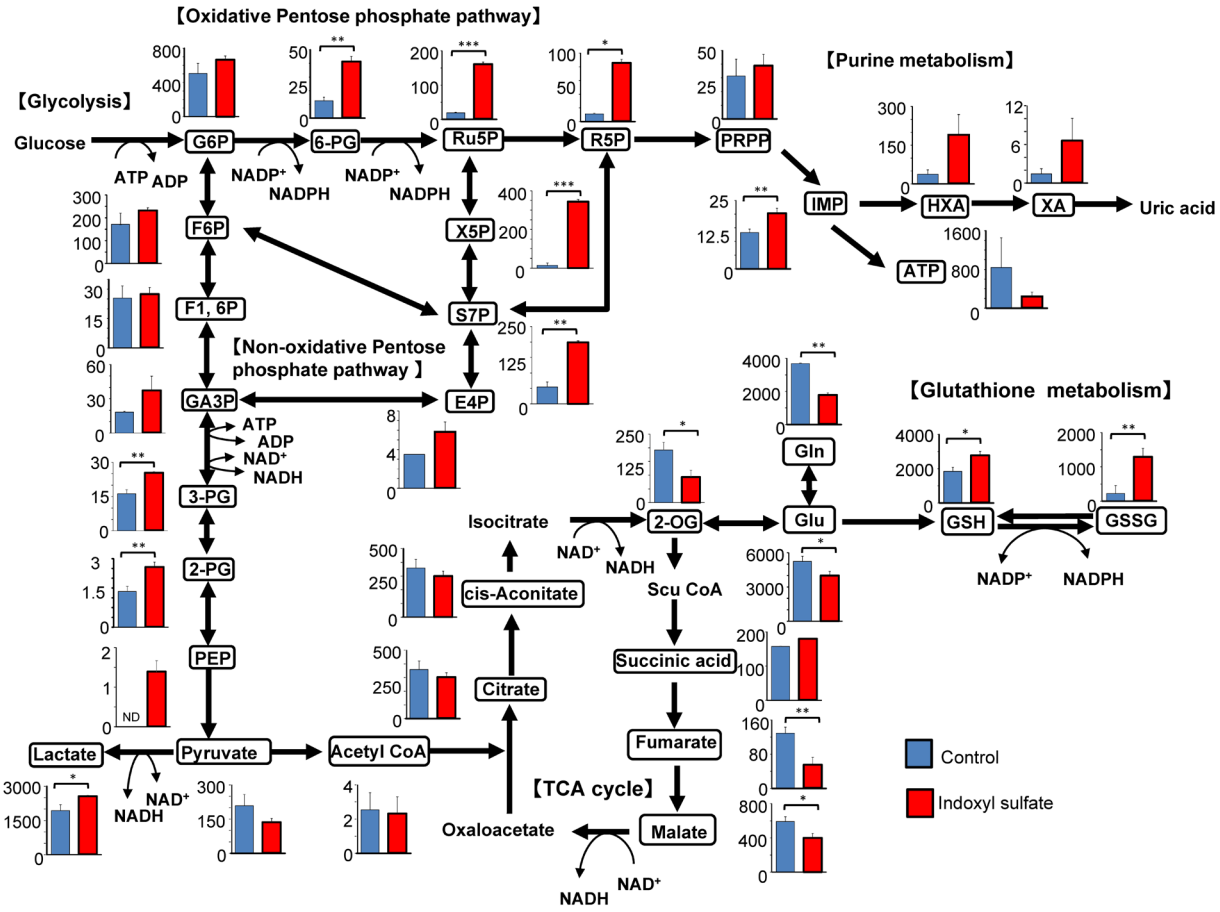

Fig. 2. Comparison of metabolites involved in glycolysis, pentose phosphate pathway, tricarboxylic acid cycle, glutathione metabolism, and purine metabolism in C2C12 cells.

The relative changes of metabolite levels between non-treated (control) and indoxyl sulfate-treated C2C12 cells extracts are shown. Data are mean±standard deviation, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.0001$ compared to the control based on Welch *t*-test. The vertical axis shows the absolute value (pmol/10^6 cells). G6P: glucose 6-phosphate; F6P: fructose 6-phosphate; F1,6P: fructose 1,6-diphosphate; GA3P: glyceraldehyde 3-phosphate; 3-PG: 3-phosphoglyceriate; 2-PG: 2-phosphoglycerate; PEP: phosphoenolpyruvate; 6-PG: 6-phosphogluconate; Ru5P: ribulose 5-phosphate; R5P: ribose 5-phosphate; PRPP: phosphoribosyl pyrophosphate; X5P: xylulose 5-phosphate; S7P: sedoheptulose 7-phosphate; E4P: erythrose 4-phosphate; IMP: inosine monophosphate; HXA: hypoxanthine; XA: xanthine. Reproduced from Ref. 12 with permission.

CKD and Metabolic Alteration

A common feature of CKD is alteration of metabolic processes and pathways in the body. In particular, CKD is associated with a negative nitrogen balance, and metabolic acidosis is observed in CKD patients when the glomerular filtration rate decreases to below 20−25% of the normal level[29]. In CKD metabolic acidosis, muscle proteolysis is stimulated by activating the ATP-dependent UPS pathway. Impairment of carbohydrate oxidation caused by protein degradation signaling in muscle cells ultimately contributes to sarcopenia[30]. Therefore, metabolic alteration in the tissues plays an important role in CKD progression. Recently, Liu et al.[31]revealed metabolic changes of the kidney occurring during renal fibrosis in a unilateral ureteral obstruction (UUO) rat renal failure model using MALDI-MS imaging. They identified 21 metabolites involved in glycolysis, tricarboxylic acid (TCA) cycle, ATP metabolism, fatty acids metabolism, as well as antioxidants and metal ions that changed in the UUO rat kidney. In addition, hippuric acid, which is a protein-bound uremic toxin, accumulated in the UUO rat kidney. In our study[12], we investigated the effects of IS on intracellular metabolic alterations in skeletal muscle using C2C12 cells and capillary electrophoresis-mass spectrometry (CE-MS). CE-MS based target quantitative analysis was performed at Human Metabolome Technologies, Inc. (Yamagata, Japan). To measure the levels of metabolites in the C2C12 cells, an aliquot of methanol containing internal standards was added to 1×10^6 cells to

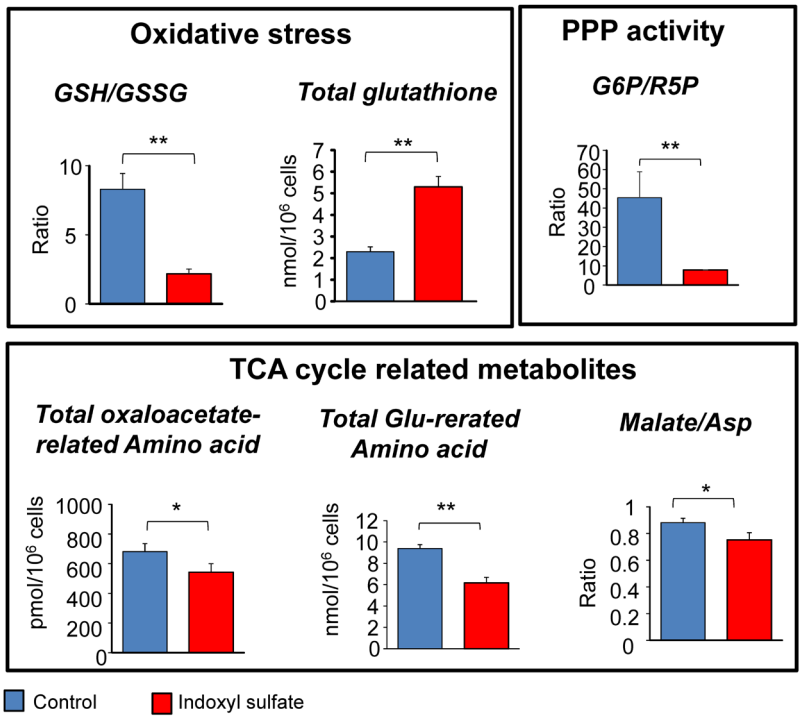

Fig. 3. Comparison of parameters of oxidative stress, pentose phosphate pathway activity, and tricarboxylic acid cycle metabolites in C2C12 cells.

The GSH/GSSG ratio, total glutathione (GSH + 2GSSG) level, glucose 6-phosphate (G6P) /ribose 5-phosphate (R5P), total oxaloacetate-related amino acid, total Glu-related amino acid content, and malate/Asp ratio for the non-treated (control) and indoxyl sulfate-treated C2C12 cells extracts are shown. Data are mean±standard deviation; * $p<0.05$, ** $p<0.01$, compared with the control by Welch *t*-test. Reproduced from Ref. 12 with permission.

extract cellular metabolites. Resultant cellular extract was centrifuged, and upper aqueous layer was centrifugally filtered through a Millipore 5-kDa cutoff filter to remove proteins. The filtrate was used for CE-MS analysis. Cationic compounds were measured in the positive mode of CE-TOFMS (Agilent CE-TOFMS system, Fused silica capillary, i.d. 50 μm×80 cm), and anionic compounds were measured in the positive and negative mode of CE-MS/MS (Agilent CE system and Agilent 6400 TripleQuad LC/MS, Fused silica capillary, i.d. 50 μm×80 cm). CE-MS analysis revealed that similar metabolic alterations found in CKD could be induced by IS stimulation to C2C12 cells (Fig. 2), which resulted in an increase in the metabolites involved in glycolysis, the pentose phosphate pathway (PPP), and glutathione metabolism and a decrease of the metabolites involved in the TCA cycle, glutamine, and glutamate. These metabolic alterations were further confirmed with parametric analysis (Fig. 3). The results of metabolomics analyses in C2C12 cells suggested that IS upregulated glycolysis, PPP, and glutathione metabolism and downregulated the TCA cycle and glutamine anabolism.

Oxidative Stress-Induced Metabolic Alteration

As described above, the MS-metabolomics study identified upregulation of the glutathione metabolism pathway and PPP in C2C12 cells following IS stimulation, which suggested that IS might induce oxidative stress. To test this hypothesis, we performed electron spin resonance (ESR) analysis using the spin-trap reagent 5,5-dimethyl-1-pyrroline-N-oxide (DMPO). The ESR spectrum of the IS-stimulated C2C12 cells culture medium showed a typical spectrum of hydroxyl radicals trapped by DMPO, confirming that IS induces the generation of reactive oxygen species in C2C12 cells. We further examined the molecular mechanisms driving this IS-induced upregulation of PPP in C2C12 cells. In an oxidative condition, glutathione disulfide (GSSG) is reduced to glutathione by GSSG reductase at the expense of NADPH. The major functions of PPP include the production of NADPH for protection against oxidative damage[32]. Nuclear factor (erythroid-2-related factor) -2 (Nrf2) is one of the main transcriptional activators in response to oxidative stress[33], and additionally regulates the expression of genes in the PPP such as glucose-6-phos-

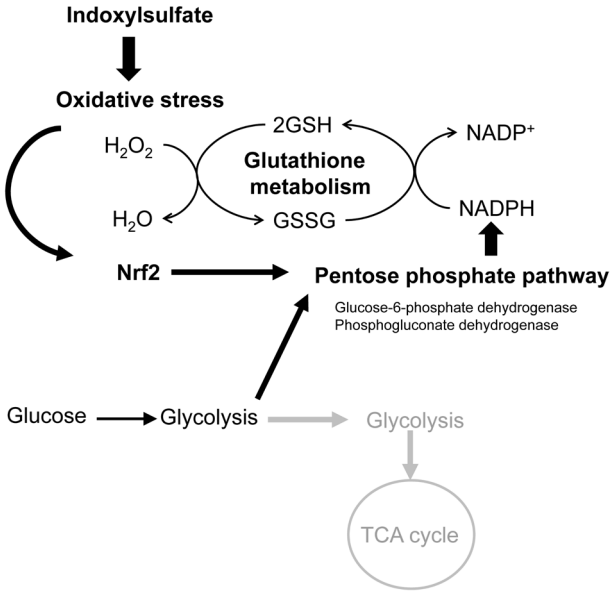

Fig. 4. Hypothesized mechanisms of metabolic alteration caused by oxidative stress induced by indoxyl sulfate in muscle cells.
The cellular requirement for NADPH increase due to indoxyl sulfate-induced oxidative stress in muscle cells. The cells change the metabolic flow to adjust the cellular requirements via pentose phosphate pathway activation to produce NADPH.

phate dehydrogenase (G6PD) and phosphogluconate dehydrogenase (PGD)[34]. Indeed, we found that IS stimulation induced Nrf2 protein expression and increased the levels of enzymes (G6PD and PGD) related to NADPH production in the PPP in C2C12 cells. These results indicated that the cellular requirement for NADPH increased along with the accumulation of IS-induced oxidative damage in muscle cells, and thus the cells altered the metabolic flow to make these adjustments for NADPH through PPP activation (Fig. 4).

Sarcopenia and Mitochondrial Dysfunction

In addition to the effects of IS on the PPP, MS-metabolomics also revealed that the TCA cycle was downregulated in C2C12 cells following IS stimulation, which suggests mitochondrial dysfunction accompanied by oxidative damage. The skeletal muscle is highly metabolic and requires a vast amount of mitochondria for sufficient ATP production. Thus, mitochondrial abnormalities result in a decrease of ATP production, leading to an ATP shortage in muscle cells. A study in CKD rats showed that the expression levels of mitochondrial biogenesis genes were decreased[27]. In addition, the numbers of mitochondria-rich type I fibers were decreased in CKD mice[35]. Moreover, a clinical study indicated low levels of mitochondrial enzymes in the muscle biopsy of patients undergoing dialysis, and decreased levels of mitochondrial oxidative enzymes, synthesis of muscle contractile mixed muscle proteins, myosin heavy chain, and mitochondrial proteins were observed in the muscle biopsy of CKD patients[4]. Thus, our results were in line with these previous studies. IS induced mitochondrial dysfunction such as a reduction of ATP production and induction of mitochondrial network disintegration in C2C12 cells[12]. Overall, these results indicate that the mitochondrial dysfunction induced by IS plays an important role in the pathogenesis of uremic sarcopenia.

Toward a Better Understanding of Uremic Sarcopenia in CKD Patients

Several factors are considered to be involved in the development of uremic sarcopenia, such as inflammation, metabolic acidosis, reduced protein intake, physical inactivity, excess angiotensin II, abnormalities in insulin/IGF-I, myostatin expression, reduction in satellite cell function, and hormonal, immunological, and myocellular changes[4]. As outlined in this review, we and others have demonstrated the development of sarcopenia in CKD mice or rats[24,27,35], including the reduction of body and skeletal muscle weight, mitochondria dysfunction, and exercise capacity reduction. However, animal CKD conditions are not necessarily equivalent to human CKD conditions. In our clinical study, plasma IS levels were found to be significantly increased and associated with the skeletal muscle mass reduction in CKD patients[12]. Although these results suggest that IS is involved in skeletal muscle mass reduction, further clinical studies are needed to validate whether similar metabolic changes are observed in the muscle tissues of CKD patients.

In conclusion, in CKD conditions, accumulated IS induces oxidative stress in muscle cells and alters the metabolic flow toward an excess anti-oxidative response of the PPP through Nrf2 activation, resulting in a decrease of ATP production leading to sarcopenia (Fig. 5). Targeting these metabolic alterations and mitochondrial dysfunction induced by IS in muscle tissues may be useful for the prevention or treatment of uremic sarcopenia. A recent clinical study found that AST-120 continuously reduced circulating IS levels and improved oxidative stress in maintenance hemodialysis patients[36]. Another clinical study reported that the oral administration of probiotics reduced uremic

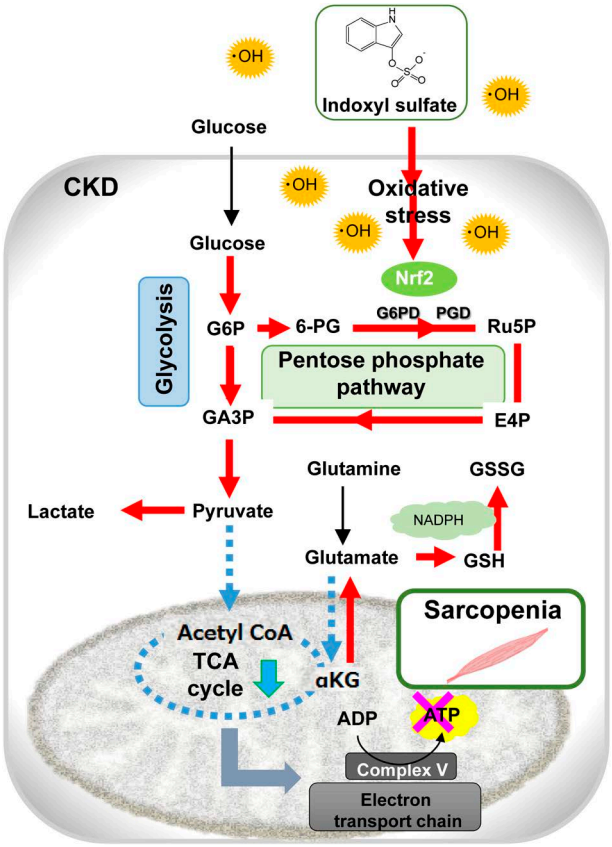

Fig. 5. Hypothesized pathogenic mechanisms of uremic sarcopenia induced by indoxyl sulfate.
Indoxyl sulfate induces the production of reactive oxygen species in muscle cells and the metabolic flow shifts toward the pentose phosphate pathway to protect the cells from oxidative stress through Nrf2. The tricarboxylic acid cycle is downregulated due to the metabolic alteration, and results in an ATP shortage leading to sarcopenia. CKD, chronic kidney disease; G6P, glucose-6-phosphate; GA3P, glyceraldehyde 3-phosphate; 6-PG, 6-phosphogluconate; Ru5P, ribulose 5-phosphate; E4P, erythrose 4-phosphate; G6P, glucose-6-phosphate; PGD, phosphogluconate dehydrogenase; αKG, α-ketoglutarate.

toxins by inhibiting bacterial growth in continuous hemodialysis patients[37]. Removal or reduction of accumulated IS in muscle tissues through the modulation of circulating IS levels by the use of AST-120 or prebiotics/probiotics may be an efficacious therapeutic option for uremic sarcopenia.

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Conflict of Interest

The author declares no competing interest.

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