Akt1-Mediated Fast/Glycolytic Skeletal Muscle Growth Attenuates Renal Damage in Experimental Kidney Disease

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ABSTRACT
Muscle wasting is frequently observed in patients with kidney disease, and low muscle strength is associated with poor outcomes in these patients. However, little is known about the effects of skeletal muscle growth per se on kidney diseases. In this study, we utilized a skeletal muscle-specific, inducible Akt1 transgenic (Akt1 TG) mouse model that promotes the growth of functional skeletal muscle independent of exercise to investigate the effects of muscle growth on kidney diseases. Seven days after Akt1 activation in skeletal muscle, renal injury was induced by unilateral ureteral obstruction (UUO) in Akt1 TG and wild-type (WT) control mice. The expression of atrogin-1, an atrophy-inducing gene in skeletal muscle, was upregulated 7 days after UUO in WT mice but not in Akt1 TG mice. UUO-induced renal interstitial fibrosis, tubular injury, apoptosis, and increased expression of inflammatory, fibrosis-related, and adhesion molecule genes were significantly diminished in Akt1 TG mice compared with WT mice. An increase in the activating phosphorylation of eNOS in the kidney accompanied the attenuation of renal damage by myogenic Akt1 activation. Treatment with the NOS inhibitor L-NAME abolished the protective effect of skeletal muscle Akt activation on obstructive kidney disease. In conclusion, Akt1-mediated muscle growth reduces renal damage in a model of obstructive kidney disease. This improvement appears to be mediated by an increase in eNOS signaling in the kidney. Our data support the concept that loss of muscle mass during kidney disease can contribute to renal failure, and maintaining muscle mass may improve clinical outcome.


Loss of skeletal muscle mass is a hallmark of patients with CKD. Muscle wasting in CKD is induced by the changes in amino acid and lipid metabolism, reduced food intake, endocrine dysfunction, reduced physical activity, and altered anabolic intracellular signaling pathways. Muscle wasting is independently associated with increased mortality in CKD. Numerous studies demonstrate that supervised exercise therapy has beneficial effects, including improvement of peak VO₂, BP control, and quality of life in patients with CKD. On the basis of these observations, exercise training is now recommended as a complementary therapeutic intervention for patients with CKD.

Regarding the mode of exercise, studies show that conventional aerobic endurance training is beneficial for patients with CKD. In addition to endurance training, resistance training is reported to be beneficial for patients with CKD. For example, resistance training is highly effective in enhancing mitochondrial content in patients with moderate-to-severe CKD. Resistance training is effective...
against the catabolism of a low-protein diet and uremia as well as systemic inflammation in patients with predialysis CKD.\textsuperscript{11,12} Another study also showed that resistance training combined with aerobic training produces greater effectiveness compared with aerobic exercise alone in patients with CKD who receive dialysis.\textsuperscript{13} These studies clearly demonstrate the beneficial effects of exercise training on kidney diseases; however, the mechanisms are largely unknown. Although exercise may be a reasonable treatment for patients with CKD, it is not clear whether improvements in muscle mass \textit{per se} can affect kidney function or whether the beneficial effects of exercise are the consequence of general systemic improvements, such as an accompanying increase in cardiovascular function.

Akt is a serine-threonine protein kinase that is activated by various extracellular stimuli through the phosphatidylinositol 3 kinase pathway and mediates a variety of cellular responses such as metabolism, growth, and proliferation.\textsuperscript{14} In skeletal muscle, Akt is preferentially activated by resistance training and plays a central role in fast/glycolytic fiber hypertrophy.\textsuperscript{15} Consistent with these observations, muscle atrophy in CKD is associated with reduced Akt signaling in skeletal muscle tissue.\textsuperscript{16} We previously generated conditional, skeletal muscle–specific Akt1 transgenic (TG) mice that can induce functional skeletal muscle growth in the absence of exercise training by using the Tet-On system of gene activation.\textsuperscript{17} We previously used these mice as a model to demonstrate that Akt1-mediated skeletal muscle growth improves metabolic parameters in obese mice\textsuperscript{17} and attenuates cardiac remodeling after myocardial infarction.\textsuperscript{18} In this study, we utilized these mice to assess the effects of skeletal muscle growth, independent of exercise, on renal damage in a model of kidney disease.

**RESULTS**

**Myogenic Akt1 Activation Inhibits the Muscle Atrophy Program after Renal Injury**

To investigate the effects of skeletal muscle growth on renal pathology, renal injury was induced by unilateral ureteral obstruction (UUO) in Akt1 transgenic (Akt1 TG) and wild-type (WT) control mice at 7 days after doxycycline treatment (Figure 1A). As shown in Figure 1B, obstructed kidneys exhibited global renal atrophy and thinning at 1 week after surgery. At this time point, mice were euthanized to assess the degree of renal damage. In this tissue-specific conditional TG system, the Akt1 transgene was detected only in skeletal muscle, was upregulated at 7 days after UUO compared with control Akt1 TG mice after transgene induction.\textsuperscript{18} As shown in Figure 1D, activation of Akt1 signaling in myofibers led to an increase in skeletal muscle mass, assessed by gastrocnemius muscle weight at 14 days after doxycycline treatment. Skeletal muscle weight was not different between sham- and UUO-treated mice at 7 days after surgery in WT mice (data not shown). However, the expression of atrogin-1, an atrophy-related gene in skeletal muscle, was upregulated at 7 days after UUO in WT mice but not in Akt1 TG mice (Figure 1E). It was previously reported that the increase in atrogin-1 expression in skeletal muscle of a CDK mouse model is associated with decreased Akt1 signaling.\textsuperscript{16} As shown in Figure 1F, Akt1 phosphorylation in skeletal muscle tissue was significantly decreased in WT mice at 7 days after UUO compared with control mice. Restoring Akt signaling by forced activation of Akt1 significantly decreased atrogin-1 expression in UUO-treated Akt1 TG mice (Figure 1E). These results suggest that the skeletal muscle atrophy program was initiated before the appearance of muscle atrophy.
wasting, and could be blocked by myogenic Akt1 activation in this renal injury model.

**Akt1-Mediated Skeletal Muscle Growth Attenuates Renal Fibrosis and Tubular Injuries after UUO**

Renal tissue sections were stained with periodic acid–Schiff (PAS) to evaluate tubular injuries in WT and skeletal muscle–specific Akt1 TG mice. As shown in Figure 2A, PAS-stained histologic sections revealed that the renal tubular injury score was increased by 1 week of UUO in WT mice, but this score was significantly lower in Akt1 TG mice. The fibrotic area was evaluated by Masson’s trichrome staining of tissue sections. Renal interstitial fibrosis induced by UUO was significantly decreased in Akt1 TG mice compared with WT mice (Figure 2B). Little tubular injuries and renal fibrosis could be detected in intact kidneys of either Akt1 TG or WT mice. Western blot analysis of total kidney lysates revealed that protein expression of collagen I in the injured kidneys was significantly reduced in Akt1 TG mice compared with WT mice (Figure 2C). Consistent with these observations, the upregulation of TGF-β, connective tissue growth factor, collagen I and III, and fibronectin gene expression in the injured kidneys was significantly decreased in Akt1 TG mice compared with WT mice (Figure 2D). Akt1 activation in skeletal muscle did not affect these gene expressions in the contralateral intact kidney.

Doxycycline, used to activate the transgene in this model, is reported to interfere with the production of fibrosis in pulmonary diseases.19 To exclude the possible involvement of doxycycline independent of the Akt activation on renal fibrosis, we provided normal water or doxycycline-treated water to WT mice 7 days before UUO surgery, and assessed fibrosis-related gene expression 7 days after surgery. There was no significant difference in fibrosis-related gene expression (TGF-β, collagen I and III, α-smooth muscle actin [α-SMA], and smooth muscle protein 22-α) between these groups (data not shown).

**Akt1 TG Mice Display Reduced Inflammatory Cell Infiltration into Injured Kidneys**

Macrophage infiltration into the kidney was assessed by immunohistologic staining with F4/80. The number of F4/80-positive macrophages was significantly increased in WT kidneys at 7 days after UUO; however, the infiltration of these cells was significantly decreased in Akt1 TG mice (Figure 3A). These findings were confirmed by quantitative real-time PCR of transcripts using specific primers for mouse F4/80 (Figure 3B). UUO surgery led to an increase in the expression of inflammatory-related genes such as IL-6, IL-1β, TNF-α, and monocyte chemoattractant protein-1 in injured kidneys of WT mice, and this upregulation was attenuated in Akt1 TG mice at 7 days after surgery (Figure 3C). The upregulation of the adhesion molecule genes intracellular adhesion molecule-1 and vascular adhesion molecule-1 by UUO was also decreased in Akt1 TG mice compared with WT mice (Figure 3D). In the contralateral uninjured kidney, the low levels of inflammatory cell infiltration and inflammatory-related gene expression were unaffected by Akt transgene induction in skeletal muscle.

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**Figure 2.** Akt1-mediated skeletal muscle growth attenuates renal fibrosis and tubular injuries after UUO. (A) Representative images of PAS-stained kidney sections (top). Quantitative analysis of renal tubular injury in WT and Akt1 TG mice at 1 week after UUO (bottom). (B) Representative images of Masson’s trichrome–stained kidney sections (top). Quantitative analysis of renal fibrosis in WT and Akt1 TG mice at 1 week after UUO (n=4 mice per experimental group) (bottom). (C) Representative immunoblots of collagen I and α-tubulin protein expression in 1 week after UUO (top). Quantitative analysis of immunoblots (n=3 mice per experimental group) (bottom). (D) TGF-β, CTGF, collagen I and III, and fibronectin mRNA expression in WT and Akt1 TG mice at 1 week after UUO (n=11–14 mice per experimental group). Results are presented as the mean±SEM. CTGF, connective tissue growth factor.
Myofibroblast Activation and Renal Cell Apoptosis Was Decreased by Akt1 Activation in Skeletal Muscle

Activated myofibroblasts appear to play a crucial role in renal fibrosis. Therefore, we evaluated myofibroblast activation by immunohistology and quantitative real-time PCR. As shown in Figure 4A, the α-SMA–positive cell area per field was significantly increased 1 week after UUO in WT mice. However, these changes were significantly attenuated in the Akt1 TG mice. Upregulation of α-SMA and smooth muscle protein 22-α gene expression in response to 1 week of UUO was also attenuated in Akt1 TG mice compared with WT mice (Figure 4B). Akt1 activation in skeletal muscle did not affect the expression of these genes in the intact kidney.

Apoptosis of renal cells is a hallmark of AKI. The number of terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling–positive cells was significantly increased in injured kidneys at 7 days after UUO in WT mice; however, the frequency of terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling–positive cells was significantly decreased in Akt1 TG mice (Figure 5A). Consistent with these results, the upregulation of cleaved caspase-3 by UUO surgery was significantly reduced in Akt1 TG mice (Figure 5B). By contrast, Akt1 activation in skeletal muscle had no effect on the low levels of renal cell apoptosis and caspase-3 activation in the intact kidneys of these mice.

Akt1-Mediated Skeletal Muscle Growth Preserves Renal Function in the Cisplatin Nephrotoxicity Model

We performed a cisplatin nephrotoxicity (CN) model to determine whether Akt1 overexpression in skeletal muscle is...
effective in a mouse model in which creatinine clearance decreased. Akt1 TG mice and WT mice were injected with 20 mg/kg cisplatin in PBS intraperitoneally 11 days after doxycycline treatment and euthanized 72 hours later (Figure 6A). Skeletal muscle mass of Akt1 TG mice increased at 14 days after doxycycline treatment in the CN model (Figure 6B). As shown in Figure 6C, serum BUN levels were increased in cisplatin-treated WT mice compared with control mice. However, this change was significantly diminished in Akt1 TG mice. Furthermore, a decrease in creatinine clearance at 72 hours after cisplatin administration was reduced in Akt1 TG mice compared with WT mice, although these differences did not reach statistical significance (Figure 6D). These results suggest that skeletal muscle growth protects against renal dysfunction in this model.

Myogenic Akt1 Activation Altered the Cytokine Expression Profile

Kidney disease increases the production of cytokines in skeletal muscle and these cytokines can influence metabolic responses of muscle and, potentially, the kidney.22,23 Various cytokines have renoprotective effects and can provide beneficial effects on kidney fibrosis.24,25 To evaluate cytokine production in skeletal muscle in this model, we performed a cytokine array using skeletal muscle tissue of both UUO-treated WT mice and UUO-treated TG mice. This analysis demonstrated that the expression of many cytokines was upregulated in Akt1 TG mice compared with WT mice (Figure 7A). These upregulated cytokines included not only renoprotective cytokines (e.g., IL-2 and IL-10) but also those that potentially have adverse effects (e.g., TNF-α).

It is reported that a decrease in adiponectin, an adipose tissue–derived cytokine, attenuates the regulation of monocyte-to-fibroblast transition with suppression of renal fibrosis.26 In this study, serum adiponectin levels of Akt1 TG mice were significantly lower than those of WT mice (Figure 7B), suggesting that decreased serum adiponectin in Akt1 TG mice might be involved in suppression of renal fibrosis. Collectively, these data suggest that the effect of skeletal muscle growth on kidney fibrosis can be mediated by multiple factors including these cytokine candidates.

Protective Effects of Akt1-Mediated Muscle Growth on Renal Damage Is Blocked by Treatment with a Nitric Oxide Synthase Inhibitor

Nitric oxide synthase (NOS) and endothelial nitric oxide synthase (eNOS) signaling were previously shown to play a central role in renal protection.27–29 To investigate the mechanism by which Akt1-mediated skeletal muscle growth attenuates renal damage after UUO surgery, we analyzed eNOS signaling in kidney tissue. As shown in Figure 8A, there was a trend toward increased eNOS phosphorylation at Ser1177 after 1 week after UUO in WT mice, but this was not statistically significant. However, phosphorylation of eNOS was significantly augmented in the injured side of the kidney in Akt1 TG mice. There was no significant difference in eNOS phosphorylation in the intact side of the kidney between WT and Akt1 TG mice.

To examine whether augmented renal eNOS activation in Akt1 TG mice plays a causal role in renal protection in the situation of acute renal injuries induced by UUO, WT and Akt1 TG mice were treated with Nω-nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor, for 2 weeks (Figure 8B). Akt1-mediated gastrocnemius muscle growth was not affected by L-NAME treatment (Figure 8C). Systolic BP was significantly increased in both WT and Akt1 TG mice at 1 or 2 weeks after L-NAME treatment, but there was no difference in this parameter.
between WT and Akt1 TG mice (Figure 8D). As shown in Figure 8E, upregulation of inflammatory-, fibrosis-, and myofibroblast differentiation-related gene expression at 1 week after UUO was not different between WT and Akt1 TG mice with chronic L-NAME treatment. These results indicate that the protective effects of Akt1-mediated muscle growth on renal injuries were dependent on the activation of eNOS-derived nitric oxide (NO).

**DISCUSSION**

Muscle wasting is one of the hallmarks of patients with CKD. Thus, therapeutic intervention involving exercise is now recommended for these patients. However, it is unclear whether the beneficial effects of exercise are caused by systemic improvements and there has been little investigation regarding the effect of skeletal muscle mass per se on renal pathology, partially because of the lack of an appropriate animal model that can induce skeletal muscle growth in an inducible manner. In this study, we utilized conditional, skeletal muscle–specific Akt1 TG mice and demonstrated that Akt1-mediated skeletal muscle growth, independent of exercise, attenuated renal damage in a mouse model of obstructive renal injury. Akt1 TG mice exhibited attenuated renal fibrosis, inflammation, myostatin, which is one of the major mediators of muscle atrophy, is upregulated in CKD. Pharmacologic inhibition of myostatin reversed muscle wasting in a mouse model of CKD; however, these studies did not examine the effects of muscle growth on renal pathology. Furthermore, exercise training studies cannot exclude the possibility that favorable effects on the kidney are secondary to the systemic effects of exercise, such as changes in the cardiovascular system. Thus, we utilized inducible, skeletal muscle–specific Akt1 TG mice as a model of resistance training and analyzed renal pathology in the UUO model.

In patients with CKD, impaired IGF-1 signaling is observed in skeletal muscle and kidneys. Intermittent IGF-1 therapy in patients with advanced CKD preserves renal function. It is reported that increasing serum IGF-1 levels is positively associated with CKD in a representative sample of United States adults. The chronic uremic state impairs basal signaling through the mammalian target of rapamycin anabolic pathway, an abnormality that may contribute to muscle wasting. These data suggest the presence of IGF-1 resistance in patients with CKD. In our mouse model, Akt1 can be activated regardless of IGF-1 resistance, because a myristoylated (constitutive active) form of Akt1 is used. Because the transgene produced renoprotective effects including reducing interstitial fibrosis...
and inflammation after UUO, overcoming IGF-1 resistance and activating downstream pathways in skeletal muscle could be a reasonable therapeutic strategy for renal diseases. On the other hand, activation of downstream pathways of IGF-1, including Akt1 and extracellular signal-regulated kinase (ERK) in the kidney, was not different between Akt1 TG mice and WT mice (data not shown), indicating that the renoprotective effect of skeletal muscle growth observed in this study was independent of IGF-1 signaling in kidney tissue.

Reduced production of NO in the kidney is one of the characteristics of CKD; thus, increasing endogenous NO production is a reasonable strategy. NO production was significantly suppressed in rats with CKD, and both running and swimming training highly upregulated the NO level. Treadmill exercise was shown to increase NOS in the kidney and heart in the rat model of myocardial infarction. Our data show that Akt1 activation in skeletal muscle increased eNOS phosphorylation in the kidney. Because the Akt1 transgene was restricted in skeletal muscle and was not detected in kidney homogenates, activation of renal eNOS may have been induced by the endocrine regulators that are induced by myogenic Akt1 activation. We previously reported several factors secreted by muscle that are increased by Akt1 overexpression. In the UUO model, we found that myogenic activation of Akt1 upregulated serum levels of stromal cell-derived factor-1, IL-10, and IL-17, which are known to activate eNOS. Further studies will be required to determine whether these factors are involved in renal eNOS activation and renoprotective properties of Akt1-mediated skeletal muscle growth.

During muscle wasting, atrophy-related genes that function to promote proteolytic degradation are upregulated in skeletal muscle. Atrogin-1 is robustly upregulated in skeletal muscle in the rodent model of CKD. In this study, atrogin-1 gene expression was upregulated before apparent skeletal muscle wasting, suggesting that the muscle atrophy program begins at an early stage of kidney disease. Furthermore, Akt1 activation in skeletal muscle significantly attenuated UUO-induced atrogin-1 gene induction. These results indicate that interventions that promote skeletal muscle Akt activation could reverse the muscle wasting program that is initiated by kidney dysfunction.

In conclusion, Akt1-mediated fast/glycolytic skeletal muscle growth reverses muscle wasting and reduces renal damage in a model of UUO. The improvements in kidney function appear to be mediated by an activation of eNOS signaling in the kidney. Our data suggest that interventions that promote the growth or maintenance of fast/glycolytic skeletal muscle growth could have utility in the treatment of kidney diseases.

**CONCISE METHODS**

**Skeletal Muscle–Specific Conditional Akt1 TG mice**

MCK-rtTA TG mice were crossed with TRE-myrAkt1 TG mice to generate double TG mice (Akt1 TG mice). The muscle creatine kinase promoter construct used in the driver line is mutated and transgene expression is expressed in a subset of muscle, but no expression occurs in the kidney.
experiments, mice were given 1 mg/ml L-NAME (Dojindo) in drinking water at 7 days before UUO. The BP of conscious mice was measured by the tail-cuff method (MK-2000ST; Muromachi Kikai Co., Tokyo, Japan) every week.

Experimental Protocol
Control and Akt1 TG mice were subjected to UUO at 10–12 weeks of age. Mice were anesthetized, an incision was made in the abdominal midline, and the left proximal ureter was exposed and ligated with 6-0 silk. The contralateral nonobstructed kidney served as the control. Doxycycline treatment was started at 7 days before surgery until the experiments were terminated. Mice were euthanized 7 days after UUO. Mice were anesthetized and bilateral kidneys and skeletal muscles were rapidly excised. Kidneys were fixed in 4% paraformaldehyde for immunohistochemical analysis, and kidneys and skeletal muscle were flash-frozen in liquid nitrogen for further RNA and protein analyses. We used a CN model to evaluate the effect of Akt1-mediated skeletal muscle growth in kidney function. Mice were administered 20 mg/kg of cisplatin (Sigma-Aldrich) by a single intraperitoneal injection. Mice were euthanized 72 hours after the administration of cisplatin, and tissue and blood were collected for further analysis. Doxycycline treatment was started at 11 days before cisplatin administration. All procedures were performed in accordance with the Kumamoto University animal care guidelines (approval reference no. B25-121), which conformed to the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (publication no. 85-23, revised 1996).

Histologic Analyses
Kidney tissues were fixed with 4% paraformaldehyde and embedded in paraffin. Kidney sections were stained with PAS and Masson's
Figure 8. Protective effects of Akt1-mediated muscle growth on renal damage is blocked by the treatment with NOS inhibitor. (A) Representative immunoblots of phospho-eNOS, total eNOS, and α-tubulin protein expression in 1 week after UUO (left). Quantitative analysis of immunoblots (right). (B) Schematic illustration of experimental protocol of L-NAME and doxycycline treatment time course. (C) Gastrocnemius muscle weight/tibial length in WT and Akt1 TG mice at 2 weeks after doxycycline treatment. (D) Time course of systolic BP in WT and Akt1 TG mice before and 1 and 2 weeks after L-NAME treatment. (E) Gene expression analysis in WT and Akt1 TG mice at 1 week after UUO (WT, n=15; TG, n=6). Results are presented as the mean±SEM. DOX, doxycycline; TL, tibial length; MCP-1, monocyte chemoattractant protein-1; CTGF, connective tissue growth factor.
Table 1. Primer sequences used for quantitative real-time PCR

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<th>Gene</th>
<th>Forward Primer</th>
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<td>α-SMA</td>
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<td>Fibronectin</td>
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CTGF, connective tissue growth factor; ICAM-1, intercellular adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1; SM-22, smooth muscle protein 22α; VCAM-1, vascular adhesion molecule-1.

trichrome by standard procedure, and all histologic analyses were made in the cortex. Tuberointerstitial injury scores were graded as follows: 0, 0%; 1, 11%–25%; 2, 26%–50%; 3, 51%–75%; and 4, 76%–100%, as previously described.51 To evaluate interstitial fibrosis, kidney sections were stained with Masson’s trichrome and the percentage of fibrosis area was quantified digitally. Myofibroblasts and macrophage infiltration were assessed by α-SMA and F4/80 staining, respectively. Kidney sections stained with Masson’s trichrome, α-SMA, and F4/80 were quantified using Lumina Vision analysis software (version 2.2). These analyses were performed by two investigators in a blinded manner.

Quantitative Real-Time PCR
Total RNA was prepared using a Qiagen RNeasy fibrous mini-kit, using the protocol supplied by the manufacturer, and cDNA was produced using PrimeScript RT-PCR Systems (Takara, Otsu, Japan). Quantitative real-time PCR was performed as previously described.18 Transcript expression levels were determined as the number of transcripts relative to those for 18S, and were normalized to the mean value from the control

Cytokine Array Analyses
The expression profile of 40 cytokines was analyzed with a mouse cytokine expression array (R&D Systems, Minneapolis, MN). Blocking, hybridization of the array filters, washing conditions, and chemiluminescent detection steps were performed according to the manufacturer’s instructions.

Statistical Analyses
All data are presented as the mean±SEM. Comparisons between two groups were made using the t-test. Differences among more than two groups were analyzed using one-way ANOVA followed by a Bonferroni post hoc test. The significance level of a statistical hypothesis test was 0.05.

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DISCLOSURES
None.

REFERENCES


