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Suppression of muscle wasting by the plant-derived compound ursolic acid in a model of chronic kidney disease

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Abstract

Background Muscle wasting in chronic kidney disease (CKD) and other catabolic disorders contributes to morbidity and mortality, and there are no therapeutic interventions that regularly and safely block losses of muscle mass. We have obtained evidence that impaired IGF-1/insulin signalling and increases in glucocorticoids, myostatin and/or inflammatory cytokines that contribute to the development of muscle wasting in catabolic disorders by activating protein degradation.

Methods Using *in vitro* and *in vivo* models of muscle wasting associated with CKD or dexamethasone administration, we measured protein synthesis and degradation and examined mechanisms by which ursolic acid, derived from plants, could block the loss of muscle mass stimulated by CKD or excessive levels of dexamethasone.

Results Using cultured C2C12 myotubes to study muscle wasting, we found that exposure to glucocorticoids cause loss of cell proteins plus an increase in myostatin; both responses are significantly suppressed by ursolic acid. Results from promoter and ChIP assays demonstrated a mechanism involving ursolic acid blockade of myostatin promoter activity that is related to CEBP/δ expression. In mouse models of CKD-induced or dexamethasone-induced muscle wasting, we found that ursolic acid blocked the loss of muscle mass by stimulating protein synthesis and decreasing protein degradation. These beneficial responses included decreased expression of myostatin and inflammatory cytokines (e.g. TGF-β, IL-6 and TNFα), which are initiators of muscle-specific ubiquitin-E3 ligases (e.g. Atrogin-1, MuRF-1 and MUSA1).

Conclusions Ursolic acid improves CKD-induced muscle mass by suppressing the expression of myostatin and inflammatory cytokines via increasing protein synthesis and reducing proteolysis.

Keywords Cachexia; Myostatin; Chronic kidney disease; Muscle wasting; Ursolic acid

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Introduction

Cachexia is characterized as the loss of muscle proteins that was stimulated by chronic diseases.[1] For example, chronic kidney disease (CKD) causes muscle wasting leading to increased morbidity and mortality.[2] Potential stimuli of muscle wasting in CKD include impaired insulin/IGF-1 signalling, excess glucocorticoids production, up-regulation of myostatin expression and systemic inflammation.[3,4] Unfortunately, the development of therapeutic interventions that block muscle wasting is still at initial stages.[5,6] We examined whether a herbal compound, ursolic acid, will block muscle wasting by inhibiting myostatin expression and inflammatory pathways.

Myostatin belongs to the TGF-β superfamily and is produced primarily in skeletal muscle. It functions to limit skeletal muscle overgrowth during both development stage and adulthood.[7] A pathophysiological role for myostatin is suggested by reports that its expression in muscle increases in response to CKD, disuse atrophy, thyroid hormone treatment and exposure to microgravity.[8] In addition, myostatin infusion into mice caused a decrease in body weight and muscle mass. In contrast, gene deletion or loss-of-function mutations in

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myostatin are associated with a marked increase in the muscle mass of mice, sheep, cattle or humans. Myostatin inhibition also improved muscle growth and strength in models of muscle atrophy. For example, Camporez *et al*. reported that treatment of young and old mice with an anti-myostatin antibody (ATA 842) increased muscle mass and muscle strength in both groups.[9] Thus, suppressing myostatin could prevent muscle wasting in certain catabolic disorders.

Evidence of inflammation has emerged as a compelling biological pathway causing muscle wasting. For example, the transcription factor NF-κB, a key regulator of inflammatory responses, is believed to stimulate ubiquitin E3 ligase MuRF-1acting to increase proteolysis by the ubiquitin-proteasome system (UPS).[10] Other pro-inflammatory cytokines, such as tumour necrosis factor-α (TNFα) and interleukin-6 (IL-6), and interferon-γ (IFN-γ) may trigger muscle wasting by activating NF-kB or/and increasing signal transducer and activator of transcription (STAT3).[11] We recently demonstrated that CKD-induced pro-inflammatory conditions will stimulate muscle-protein degradation by activating STAT3/CEBP-δ pathways. The response is to up-regulate myostatin leading to muscle wasting.[12]

Structurally, ursolic acid is a pentacyclic triterpenoid and is found in apple peels, basil leaves, prunes and cranberries.[13] It exerts beneficial effects in animal models of diabetes and obesity,[14] including improved glucose and lipids metabolism. Regarding muscle protein metabolism, Kunkel *et al*. examined mouse models of starvation or denervation and reported that ursolic acid reversed muscle atrophy by mechanisms involving enhanced IGF/PI3K signalling. The modulation required IGF-1 or insulin to activate their receptors and downstream substrates.[15] Consequently, its applicability to CKD that is characterized as insulin resistance and IGF-1 deficiency remains to be determined.

We have examined how ursolic acid influences muscle metabolism in a mouse model of CKD that exhibits BUN values ~80 mg/dL and actuates muscle wasting via the UPS. Unlike starvation or denervation, CKD-induced muscle wasting is usually associated with an up-regulation of myostatin, increased proinflammatory cytokines, IGF-1 deficiency and insulin resistance. Because glucocorticoid excess can also stimulate myostatin expression associated with insulin resistance, we studied whether ursolic acid would blunt dexamethasone (Dex)-induced muscle atrophy in mice.

Methods

Animal models and treatments

All animal procedures were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee. Male C57BL/6 mice (8 week old) underwent two-stage nephrectomy to create CKD as previous described.[16] Briefly, ~75% of the right kidney was removed, and bleeding was stopped by Vetbond tissue adhesive (3 M, St. Paul, MN). One week later the left kidney was removed, and mice were pair-fed with sham-operated control mice until muscle samples were collected. Aortic blood obtained from anaesthetized mice was used to measure BUN and serum creatinine (Cayman Chemical, Ann Arbor, MI) to confirm CKD. For ursolic acid treatment, ursolic Acid (Sigma-Aldrich, St. Louis, MO) were dissolved with sesame oil and was given *t* at 100 mg/kg by oral gavage for 21 days. The pair-fed mouse was received with an equal amount of sesame oil. For Dex treatment, 8-week-old male mice were treated with 50 mg/L Dex (water-soluble Dex; Sigma, St. Louis, MO) provided in the drinking water for 3 weeks. Based on daily water consumption, mice were given a dose of 5 mg/kg/day of Dex.[17] Grip strength was measured by Force Measurement (Chatillon instrument, Columbus, OH). At the end of treatment, muscle samples were collected from mice under anaesthesia. Tibialis anterior (TA) muscles were fixed at resting length for histologic analysis. Protein synthesis and degradation were measured in isolated soleus and EDL muscles. Gastrocnemius muscles were frozen in liquid nitrogen and stored at—80°C for mRNA and protein analyses.

Cell culture

C2C12 cells were cultured in DMEM supplemented with 10% FBS (HyClone, Logan, UT), penicillin (200 units/mL) and streptomycin (50 μg/mL) (Life Technologies). At 90% confluence, the media was changed to DMEM plus 2% horse serum (American Type Culture Collection) to induce myotube formation. Three days following the medium switch, the cells were treated with 5 μM Ursolic Acid (dissolved in DMSO) for 48 h to measure protein synthesis or were treated with Dex (2 μM Dex) for 36 h following ursolic acid treatment for 24 h.

RNA Preparation and real-time qPCR

Total RNA from tibialis anterior muscles or cultured C2C12 myotubes was extracted by TRIzol (Sigma-Aldrich, St. Louis, MO) and precipitated in isopropanol. Approximately, 0.5 μg RNA was reverse transcribed using iScript cDNA Synthesis Kit (Quanta, Gaithersburg, MD) to cDNA. SYBR Green real-time quantitative PCR was performed and analysed with Bio-Rad CFX96 Touch™ Real-time PCR Detection System (Bio-Rad). The primer sequences for mouse were showed as Supporting Information Table S2 (Atrogin-1, MuRF-1, Myostatin, MUSA1, IL-6 and GAPDH).

RT^2 profiler PCR array analysis

Total RNA was transcribed to cDNA with the first strand cDNA kits (Qiagen Sciences, Germantown, MD). RT^2 Profiler™ PCR

Array including Myogenesis & Myopathy array (PAMM-099Z), Signal Transduction PathwayFinder (PAMM-014Z) and Mouse Common Cytokines (PAMM-021Z) were performed as manufactory instruction (Qiagen Sciences). Transcript levels were measured by Bio-Rad CFX96 real-rime PCR system. The threshold cycle (Ct) is defined as the number of cycles required for the fluorescence signal to exceed the detection threshold. mRNA expression was standardized to five house-keeping genes as manufacturer suggested. Results were presented in heatmap, cluster-grammes and volcano plots.

Protein synthesis assay using The Click-iT® HPG Alexa Fluor® 488 Protein Synthesis Assay Kit

C2C12 myotubes were incubated in methionine free DMEM containing 50 μM HPG (Homo-propargylglycine) for 30 min. Cells were fixed with 3.7% formaldehyde in PBS for 15 min, permeabilized with 0.5% Triton X-100 in PBS for 20 min, washed twice with 3% bovine serum albumin in PBS and incubated in Click-IT reaction cocktail (1X Click-iT HPG reaction buffer, 2 mM $CuSO_4$, 3 μM Alexa Fluor azide, 1X Click-iT HPG buffer additive) for 30 min at room temperature protected from light. Cells were washed with Click-iT reaction rinse buffer then incubate with 1X HCS Nuclear-Mask Blue Stain working solution for 30 min at room temperature. Images and fluorescence intensity measurements were taken using NIS-Elements system (Nikon USA).

Protein synthesis and degradation using isotopic

C2C12 myotubes were incubated in media containing L-^{3}H-tyrosine (5 μCi/mL; PerkinElmer, USA) for 24 h. After washed with DMEM media for three times, the cells were incubated with chase buffer (containing 2 mM unlabeled tyrosine) for 2 h to remove unincorporated L-^{3}H-tyrosine and that contained in short-lived proteins. After adding fresh media, samples (100 μL) of the media were taken at specified times for quantitation of L-^{3}H-tyrosine release. At the end of sampling, cells were collected and proteins were precipitated by adding 10% trichloroacetic acid. The precipitations were solubilized by sonication in lysis solution (1% Triton X-100 and 0.5 N NaOH). The residual L-^{3}H-tyrosine in cell proteins was measured using liquid scintillation counter (Beckman, USA) and protein concentration was measured using Pierce BCA Protein Assay (Thermo Scientific). L-^{3}H-tyrosine in cells and aliquots were used to calculate total radioactivity. Protein breakdown was estimated by radioactivity in each aliquot divided by the total radioactivity (expressed as percentage). The linear slope of between 24 and 36 h was calculated as the rate of protein degradation. To measure protein synthesis and degradation in muscles, soleus and EDL muscles were incubated for 30 min in 3 mL Krebs–Henseleit bicarbonate buffer containing 0.5 mmol/L L-phenylalanine, 10 mmol/L glucose, plus 0.05 μCi of L-^{14}C-phenylalanine (MP Biomedicals, Solon, OH). After gassing with 95% O2/5% CO_2, muscles were incubated for 30 min and then incubated for 2 h in fresh buffer gassed with 95% O_2/5% CO_2. The rate of protein synthesis was measured as the incorporation of L-^{14}C- phenylalanine into muscle protein. The rate of protein degradation was measured as the rate of release of tyrosine into the media during the 2 h of incubation.[18]

Serum myostatin quantitation and cytokines array

Blood was centrifuged for 20 min at 2000 g and serum was used for immunoassay using Quantikine ELISA GDF-8/ Myostatin Immunoassay or Mouse XL Cytokine Array following manufacture's protocol (R&D Systems, Minneapolis, MN).

Western blot analysis

Protein from gastrocnemius muscles or cultured C2C12 myotubes was isolated using RIPA buffer (20 mM Tris, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% NP40, 0.5% Na-deoxycholate, 0.025% SDS, 1 mM Na-orthovanadate, 10 mM NaF and 25 mM β-glycerophosphate) with protease and phosphatase inhibitor (Thermo Fisher Scientific, Houston, TX). The protein concentration was measured using Pierce BCA Protein Assay (Thermo Scientific). The protein was subjected to western blot as previously described.[16] The following antibodies were used in western blots: p-Akt (Ser 437), Akt, p-STAT3 (Tyr 705), STAT3, p-NF-κB (p65, Ser 536), NF-κB, p-p38 (T180), p38, C/EBP-δ, p-FoxO1 (Ser 256), FoxO1, Myostatin and GAPDH purchased from Cell Signaling Technology (Beverly, MA) , Abcam Biotechnology (Cambridge, MA) or Santa Cruz Biotechnology (Santa Cruz, CA).

Immunofluorescence staining and myofibers size measurement

The frozen tibialis anterior muscles sections (5 μm) were fixed with 4% formaldehyde. After washing, the slide was blocked by protein blocking (Dako North America, Carpinteria, CA) for 20 min. The anti-dystrophin antibody (Santa Cruz Biotechnology) was diluted with antibody diluent (Dako) with 1:200 dilution and 4°C overnight incubation. The secondary antibody (Life technologies) was used with 1:600 dilutions and incubated 30 min at room temperature protected from light. The areas of myofibers were measured using NIS-Elements software (Nikon, USA), and at least 1000 myofibers per TA muscle were examined.

Luciferase assay

To construct myostatin promoter luciferase reporter plasmid (Luc-MSTN), we PCR-amplified a mouse myostatin promoter (−1177/−1 relative to the translation initiation site) using a forward primer (5′-TAGTGGACGCGTCCCTAGACTCTAG CCCAGATC-3′) and a reverse primer (5′-TAGTGGCTCGA GAACCTGCCACGCCAGGGAGTC-3′). The promoter fragment was subcloned into MluI and an XhoI site of PGL3 plasmid (Promega, Madison, WI) as previous described.[19] To evaluate the regulation of myostatin transcriptional activity by Dex, ursolic acid or C/EBP-δ, we co-transfected C2C12 cells with Luc-MSTN plus an equal amount of pcDNA vector that encodes C/EBP-δ. After 24 h, luciferase activities were measured using the Dual Luciferase Assay System (Promega) according to manufacturer's protocol.

ChIP assay

The chromatin immunoprecipitation (ChIP) assay was performed with the use of a SimpleChIP Plus Enzymatic Chromatin IP Kit (Cell Signaling Technology) following the manufacturer's protocol. C2C12 cells were treated with UA and/or Dex for 24 h and then fixed with 1% formaldehyde for 10 min to cross-link proteins to DNA. Nuclei were treated with nuclease for 20 min at 37°C to digest the chromatin into 150–900 bp DNA/protein fragments. Anti-C/EBP-δ antibody or normal Rabbit IgG and ChIP Grade Protein G Magnetic Bead were used to immunoprecipitate C/EBP-δ/MSTN bound DNA fragments. Normal Rabbit IgG was used as a negative control. After chromatin was eluted from the beads, the cross links were reversed by NaCl and Proteinase K through incubating for 2 h at 65°C. DNA was purified with spin column and analysed by quantitative PCR using primer set: forward 5′-TGTGACAGACAGGGTTTTA ACC-3′ and reverse 5′-ATATTCCAACTTAGAGGAGAC-3′.

Statistical analysis

Results are presented as mean ± SEM. For experiments comparing two groups, we analysed results by two-tail, Student's *t*-test. When more than two groups were compared, two-way ANOVA followed by Student–Newman–Keul's test was used to analyse difference between groups. Differences were considered statistically significant at $P < 0.05$ (*).

Results

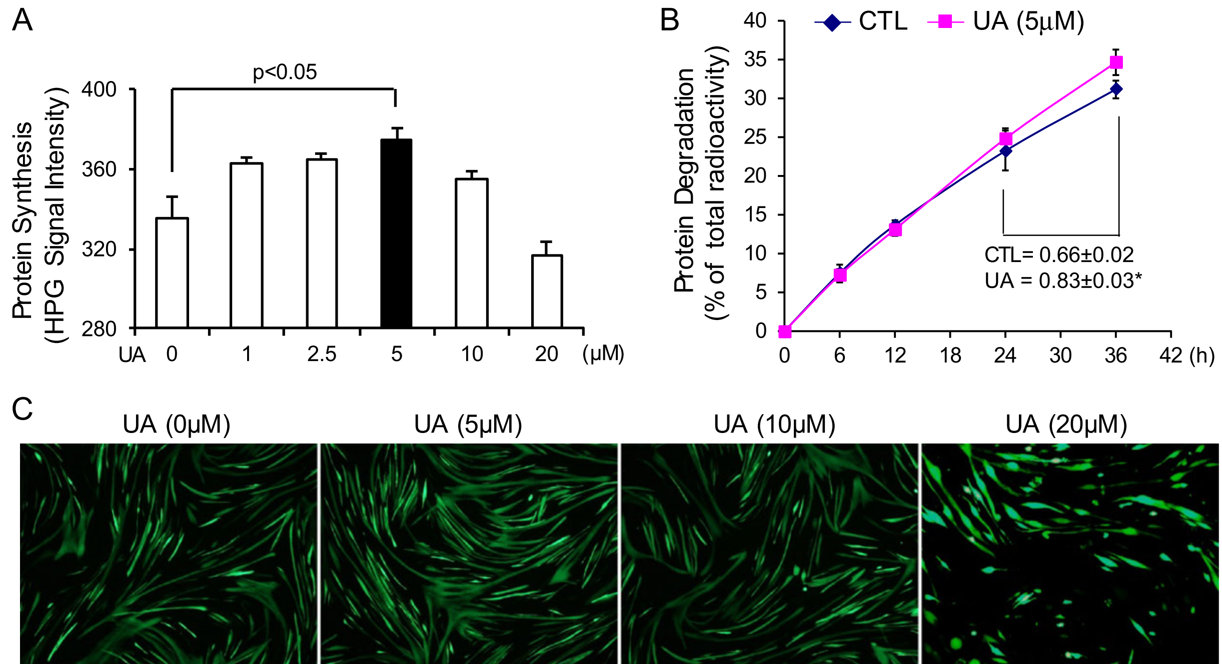
The influence of ursolic acid on protein synthesis and degradation in C2C12 myotubes

Although ursolic acid reportedly benefits muscle protein metabolism, the compound may exert cytotoxic effects in cultured cells.[20] To identify cytotoxicity, we examined how different doses of ursolic acid influenced protein synthesis by taking advantage of the bioorthogonal non-canonical amino-acid tagging (BONCAT) technique.[21] We cultured C2C12 myotubes in DMEM containing 2% horse serum and added various concentrations of ursolic acid (1, 2.5, 5, 10 and 20 μM) to the media and examined protein synthesis in mature C2C12 myotubes. After treatment for 48 h, we found that low concentrations ursolic acid (1 to 5 μM) increased the rate of protein synthesis in myotubes in a dose-dependent fashion. At higher concentrations (>10 μM), ursolic acid blocked protein synthesis (*Figure* 1A). We then examined how ursolic acid affects protein degradation by measuring release of L-^{3}H-tyrosine from pre-labelled C2C12 myotube proteins. After 36 h of exposure to ursolic acid (5 μM), we detected a significant increase in the rate of protein degradation compared with results obtained in myotubes unexposed to ursolic acid (*Figure* 1B). Concomitant with the increase in protein synthesis and degradation, the sizes of myotubes treated with 5 μM of ursolic acid were not different from those untreated myotubes. There was, however, a marked decrease in the sizes of myotubes after 48 h treatment with a high-concentration ursolic acid (*Figure* 1C). Thus, our results indicate that 5 μM ursolic acid can increase muscle protein synthesis, but at a high concentration (>10 μM), it exerts cytotoxicity with increased proteolysis and decreased protein synthesis in muscle cells.

Identification of ursolic acid as a suppressor of myostatin expression

To understand why ursolic acid simultaneously stimulates muscle protein synthesis and degradation, we performed a PCR-based mRNA array (Myogenesis & Myopathy PCR Array) using C2C12 myotubes that had been treated with or without ursolic acid (5 μM in DMEM with 2% horse serum). The Myogenesis & Myopathy PCR Array profiles genes involved in skeletal muscle growth, differentiation, function plus disease-related processes. As shown in *Figure* 2A and B, ursolic acid stimulated 12% of the tested genes including those involved in skeletal myogenesis, hypertrophy and atrophy-related genes. Ursolic acid suppressed 18% of the tested genes including those affecting muscle autocrine signalling and atrophy-related genes. Among these genes, we found that ursolic acid significantly suppressed myostatin (decreased 2.14-fold vs. CTL), while IL-6 was decreased 2.0-fold and IGF-1 binding protein-3 (IGFBP3) 2.42-fold. Ursolic acid also stimulated the expressions of the E3 ubiquitin ligase, Atrogin-1 or MuRF-1 (*Figure* 2C). Using qRT-PCR, we confirmed that ursolic acid suppresses myostatin and IL-6 expression and increases Atrogin-1, MuRF-1 expression in C2C12 myotubes (see Supporting Information Figure S1). Of

Figure 1 In low serum culture media, ursolic acid stimulates both protein synthesis and proteolysis in C2C12 myotubes. (*A*) Protein synthesis was assessed by the amount of homopropargylglycine (HPG) incorporated into proteins. After ligating with Alexa Fluor® 488 azide, protein synthesis (HPG signaling intensity) was measured with Nikon NIS-Elements system (mean ± SEM; *n*=6 in each group). (*B*) Protein degradation was measured by the L-^{3}H-Tyr released to cultural media and plotted as a percentage of total L-^{3}H-tyrosine incorporated into cell proteins. The rates of proteolysis was calculated from the linear slopes between 24 and 36 h (mean ± SEM; *n*=6 in each group; *$P<0.05$, UA vs. CTL). UA and CTL represent ursolic acid and control, respectively. (*C*) Myotubes were visualized by infecting adenovirus coding green fluorescent protein (GFP) and were treated with various dose of ursolic acid (UA).



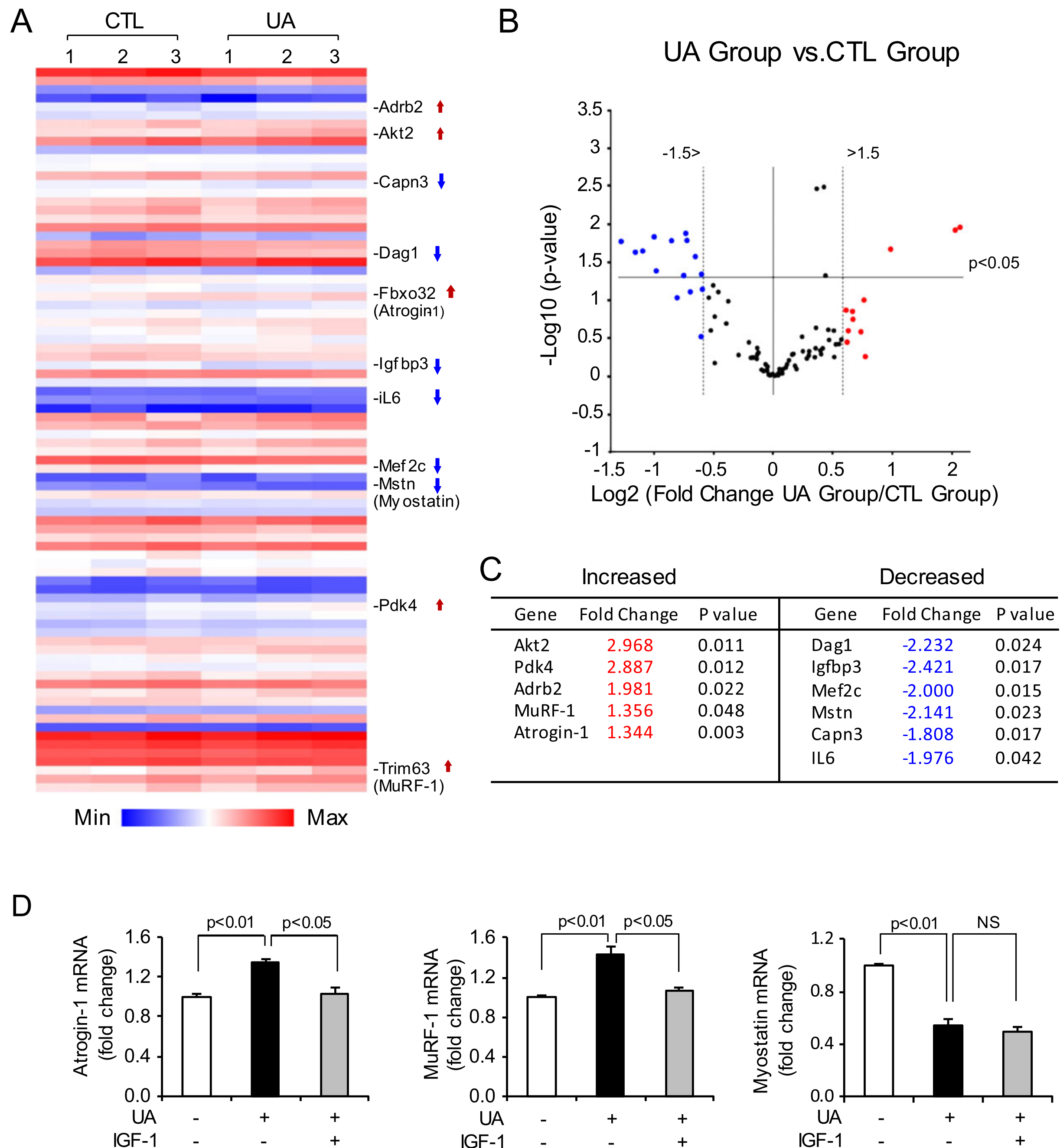
note, addition of IGF-1 (2 ng/mL) eliminated the ursolic acid-stimulated induction of Atrogin-1 and MuRF-1; there was no significant change in myostatin (*Figure* 2D). It is possible, therefore, that ursolic acid increases muscle proteolysis by stimulating E3 ubiquitin ligases while suppresses myostatin to activates protein synthesis.

To test if this new property of ursolic acid is relevant *in vivo*, we examined how ursolic acid affects muscle protein metabolism. Mice treated with ursolic acid (100 mg/Kg/day by gavage) for 7 days, and fasted for 6 h to reduce changes in insulin/Akt signalling caused by food taking. Ursolic acid significantly increased protein synthesis in both soleus and extensor digitorum longus (EDL) muscles compared to values obtained from vehicle-treated, control mice (*Figure* 3A). In contrast with *in vitro* results, we detected that there was a trend to a decrease in protein degradation in soleus or EDL muscles of mice treated with ursolic acid (*Figure* 3B). In agreement with results of measured protein synthesis and degradation, the mRNA expressions of myostatin, Atrogin-1 and MuRF-1 were suppressed by exposed to ursolic acid (*Figure* 3C). We conclude that ursolic acid acts to suppress myostatin expression providing an improvement in muscle protein metabolism.

Ursolic acid suppresses the expression of myostatin induced by glucocorticoids

To test if ursolic acid also suppresses myostatin in catabolic condition, we examined the expression of myostatin in response to glucocorticoids in C2C12 myotubes. Physiologic concentration of glucocorticoids can cause a decrease in protein synthesis and an increase in protein degradation; these effects of glucocorticoids contribute to muscle wasting in many catabolic conditions.[22,23] Since glucocorticoids reportedly increase myostatin expression in muscle cells, we examined whether ursolic acid would suppress the expression of myostatin that is induced by dexamethasone (Dex). We exposed C2C12 myotubes to 2 μm Dex added to the DMEM containing 2% horse serum for 24 h. There was a significantly increase in myostatin expression but when C2C12 myotubes were incubated with Dex plus ursolic acid, myostatin expression was blocked (*Figure* 4A). Note that Dex stimulates the expressions of Atrogin-1/MAFbx, MuRF-1 and MUSA1 (a newly identified muscle ubiquitin E3 ligase) while ursolic acid did not alter the induction of these ubiquitin E3 ligases that stimulated by Dex (*Figure* 4B–D). Ursolic acid also prevented the decline of protein synthesis that occurs in

Figure 2 Ursolic acid suppresses Myostatin but stimulates Atrogin-1 and MuRF-1 in C2C12 myotubes cultured in low serum media. (*A*) Heatmap displays of Myogenesis & Myopathy RT-PCR Array which compared gene expression in control (CTL) and cells treated with ursolic acid (UA). The change in expression of interested gene was remarked (red indicates an increase in expression while blue indicates a decrease in expression). (*B*)Volcano plots comparing gene expressions between CTL and UA groups. A cut-off of ≥|1.5| fold cut-off and $P \leq 0.05$ was used for the analyses. (*C*)Selected genes from Myogenesis & Myopathy RT-PCR Array with significant increase and decrease in expressions. (*D*) The increase in Atrogin-1 and MuRF-1 stimulated by UA (5 μM) were largely suppressed by adding IGF-1 (2 ng/mL) into low serum media. Note that IGF-1 did not influence myostatin expression in response to UA (mean ± SEM; *n*=3).



Increased			Decreased		
Gene	Fold Change	P value	Gene	Fold Change	P value
Akt2	2.968	0.011	Dag1	-2.232	0.024
Pdk4	2.887	0.012	Igfbp3	-2.421	0.017
Adrb2	1.981	0.022	Mef2c	-2.000	0.015
MuRF-1	1.356	0.048	Mstn	-2.141	0.023
Atrogin-1	1.344	0.003	Capn3	-1.808	0.017
			IL6	-1.976	0.042

myotubes that are treated with Dex (*Figure* 4E). These results suggested that in low serum condition, ursolic acid can directly act to suppress myostatin transcription rather than via pathways such as IGF/insulin/Akt signaling. In agreement with this speculation, we found that ursolic acid did not change the levels of p-Akt (Ser 473) or p-FoxO$_1$ (Ser 256) in C2C12 myotubes that were treated with Dex (*Figure* 4F). Taken together, these results indicate that ursolic acid

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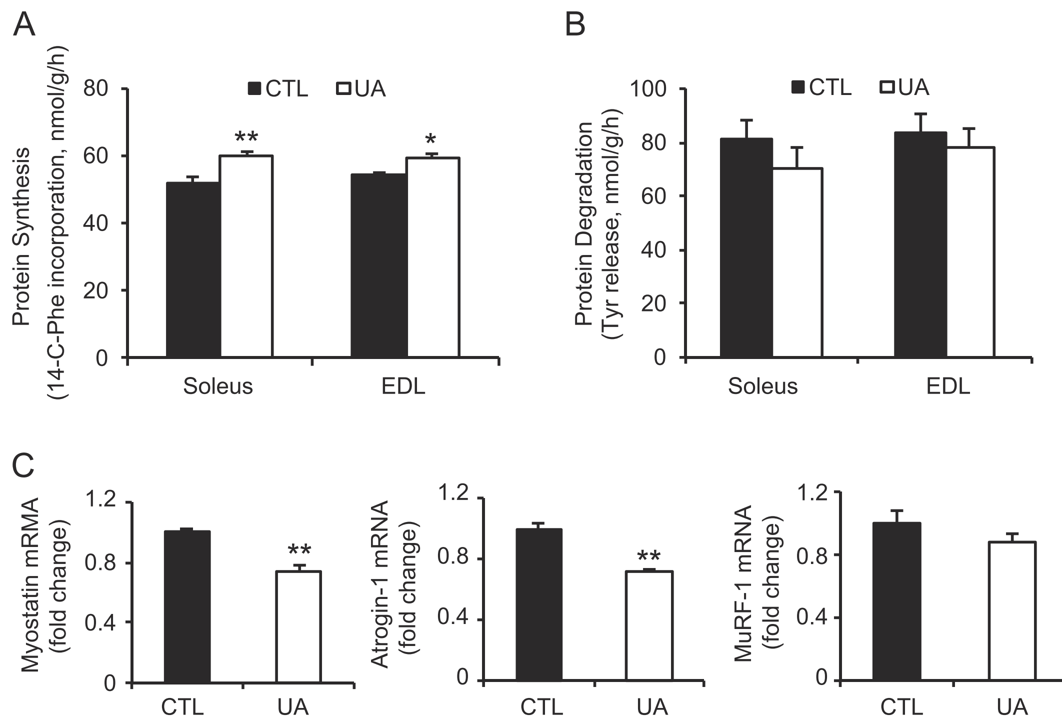
Figure 3 Ursolic acid stimulates protein synthesis and suppresses protein degradation in muscles of mice treated with ursolic acid. (*A*) Protein synthesis was measured with ^{14}C-Phe incorporation in Soleus and extensor digitorum longus (EDL) muscles from mice treated with vehicle (CTL) or UA (mean ± SEM; *n*=5; **P*<0.05, ***P*<0.01, UA vs. CTL). (*B*) The rates of protein degradation was measured by the tyrosine release from Soleus and EDL muscles of mice treated with ursolic acid (mean ± SEM; *n*=5). (*C*) The mRNA levels of myostatin, Atrogin-1 and MuRF-1 in TA muscles from mice treated with or without UA (mean ± SEM; ***P*<0.01 vs. CTL mice, *n*=5).



prevents Dex-induced catabolic effects by suppressing myostatin expression.

Ursolic acid blocks C/EBP-δ mediated activation of myostatin promoter

To identify how ursolic acid suppresses myostatin transcription, we examined myostatin promoter activity and potential signaling pathways that might lead to myostatin expression. First, we transfected a luciferase reporter plasmid containing the mouse myostatin promoter (Luc-MSTN) into C2C12 myoblasts following Dex treatment with or without ursolic acid. As expected, Dex strongly activates the myostatin promoter and this response was completely blocked by ursolic acid (*Figure* 5A). To understand whether ursolic acid interacts with transcription factors such as STAT3, AP-1 and C/EBPs to interfere with myostatin expression, we performed a signaling pathway profile PCR array in C2C12 myotubes to examine candidate factors that are blocked by ursolic acid. Dex treatment upregulated several genes that could participate in different signaling pathways (*Figure* 5B). Among these, ursolic acid significantly reduced C/EBP-δ genes despite being induced by Dex (*Figure* 5C). This finding is supported by previous report that there are two C/EBP-δ binding sites in promoter region of myostatin in many species.[24] To verify that C/EBP-δ is targeted by ursolic acid, we co-transfected the Luc-MSTN plasmid and a plasmid encoding mouse C/EBP-δ into C2C12 cells. As shown in *Figure* 5D, ursolic acid is able to block Dex-induced myostatin promoter activation. Overexpression of C/EBP-δ, however significantly diminished the ability of ursolic acid to suppress activation of the myostatin promoter. We also performed ChIP assays and confirmed that Dex stimulates C/EBP-δ binding to the myostatin promoter; this response was eliminated by ursolic acid (*Figure* 5E). Our results demonstrate that ursolic acid can suppress myostatin expression via inhibition of C/EBP-δ (Supplemental *Figure* 2).

Ursolic acid ameliorates muscle atrophy induced by CKD or Dex in mice

To assess whether the inhibitory effect of ursolic acid on myostatin also can improve muscle protein metabolism in pathophysiologic conditions, we examined the loss of muscle proteins that is stimulated by CKD or Dex. In mice with CKD following subtotal nephrectomy, the BUN and serum creatinine levels were 4.2-times higher than results from sham-operated, control mice, confirming renal insufficiency (Supplemental

Figure 4 Ursolic acid inhibits the catabolic effects of dexamethasone in myotubes cultured with low serum media. (*A*) The expression of myostatin examined by RT-PCR in C2C12 myotubes treated with dexamethasone (Dex) or/and ursolic acid (mean ± SEM; *n*=3). (*B–D*) The expression of Atrogin-1, MuRF-1 and MUAS1 has examined by RT-PCR in C2C12 myotubes. Note that ursolic acid did change the expressions of these genes stimulated by Dex (mean ± SEM; *n*=3). (*E*) Dex suppressed the protein synthesis in myotubes, while ursolic acid blocked this response (mean ± SEM; *n*=5). (*F*) Western blot of phosphorylation of Akt and FoxO1 in C2C12 myotubes treated with Dex with or without ursolic acid. Note that ursolic acid did not change the levels of p-Akt and p-FoxO1 in myotubes incubated with low serum media (mean ± SEM; *n*=6).

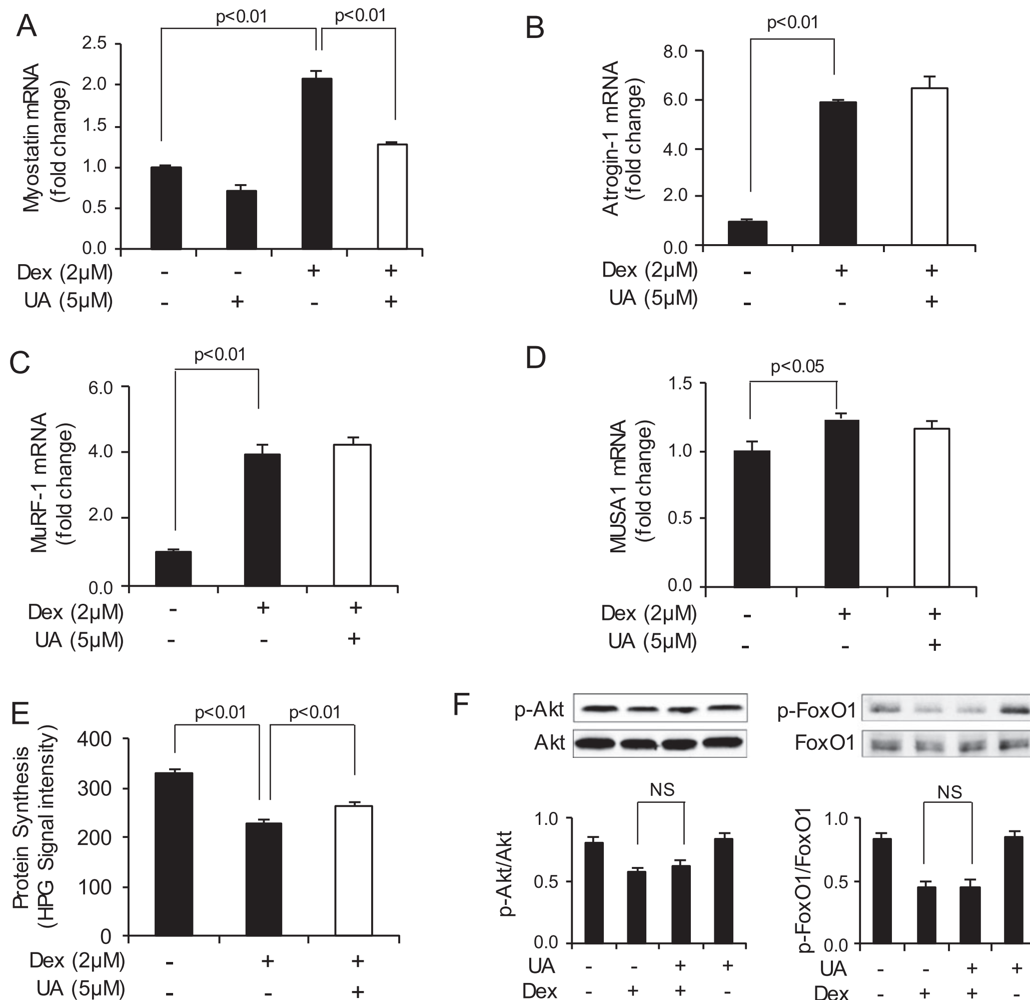
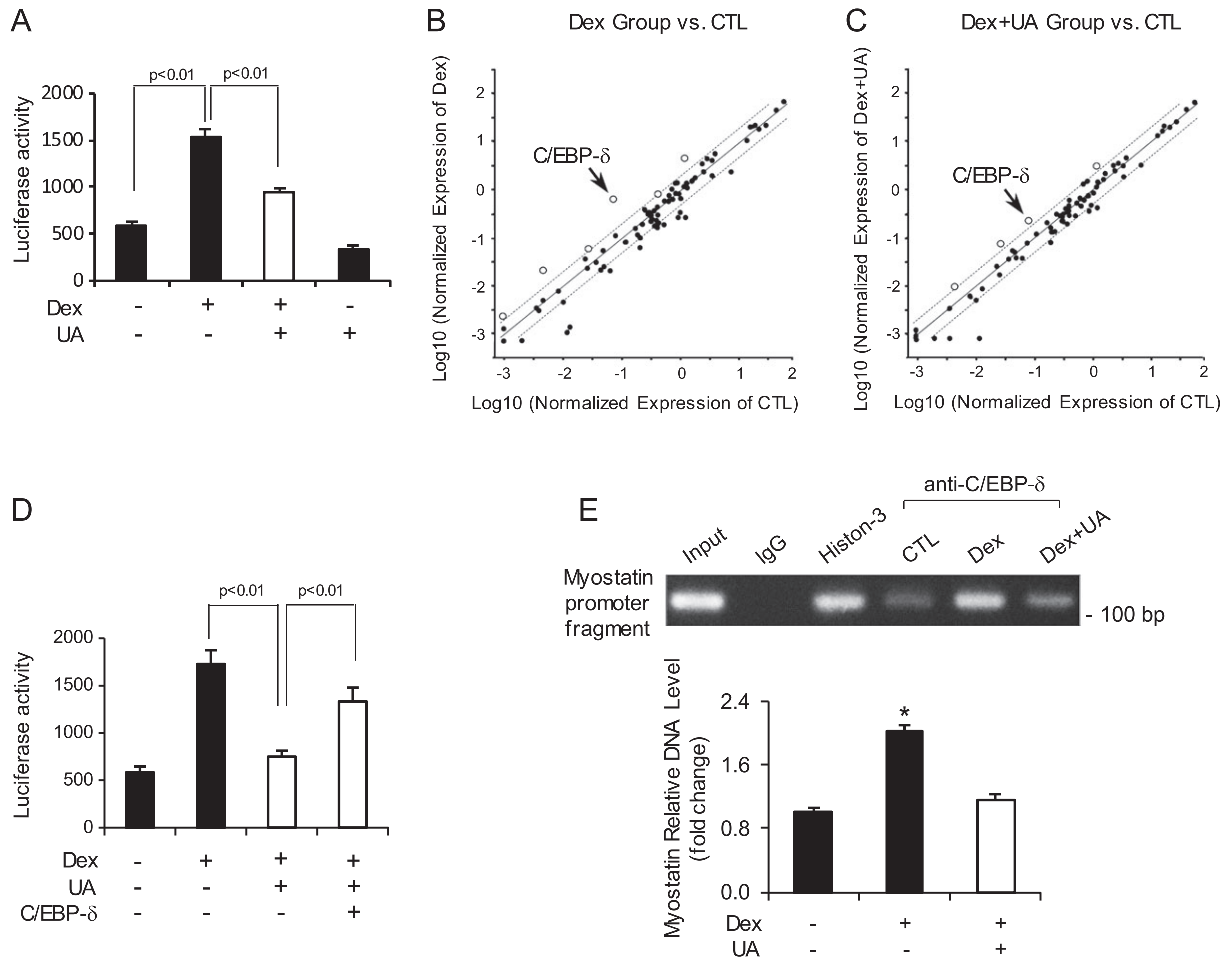


Table 1). When mice with CKD were given ursolic acid (100 mg /kg/day by gavage) for 3 weeks, there was a significant increase in the body weight mice compared to results from pair-fed, mice with CKD that did not receive ursolic acid (*Figure* 6A). The increase in bodyweight from adding ursolic acid included an increase in weights of tibialis anterior (TA), soleus and EDL muscles that were normalized to tibia lengths in CKD mice treated with ursolic acid (*Figure* 6B). The improved muscle mass in CKD mice treated with ursolic acid was confirmed by a rightward shift in the distribution of myofiber sizes (*Figure* 6C) plus an increase in average cross-sectional area of myofibers in TA muscles from CKD mice (*Figure* 6D). Concomitant with the increase in muscle mass, muscle function, assessed by grip strength, was significantly improved in CKD mice treated with ursolic acid (*Figure* 6E). In the model of Dex-induced muscle protein loss, we also found that ursolic acid administration improved Dex-induced loss of muscle mass (see Supporting Information *Figure* 3A). Again, the increase in muscle mass was accompanied by an improvement in grip strength and a rightward shift in the distribution of myofiber sizes in muscles of mice treated with Dex plus ursolic acid (see Supporting Information *Figure* 3B and C).

Ursolic acid protects against muscle protein catabolism via suppressing myostatin in CKD mice

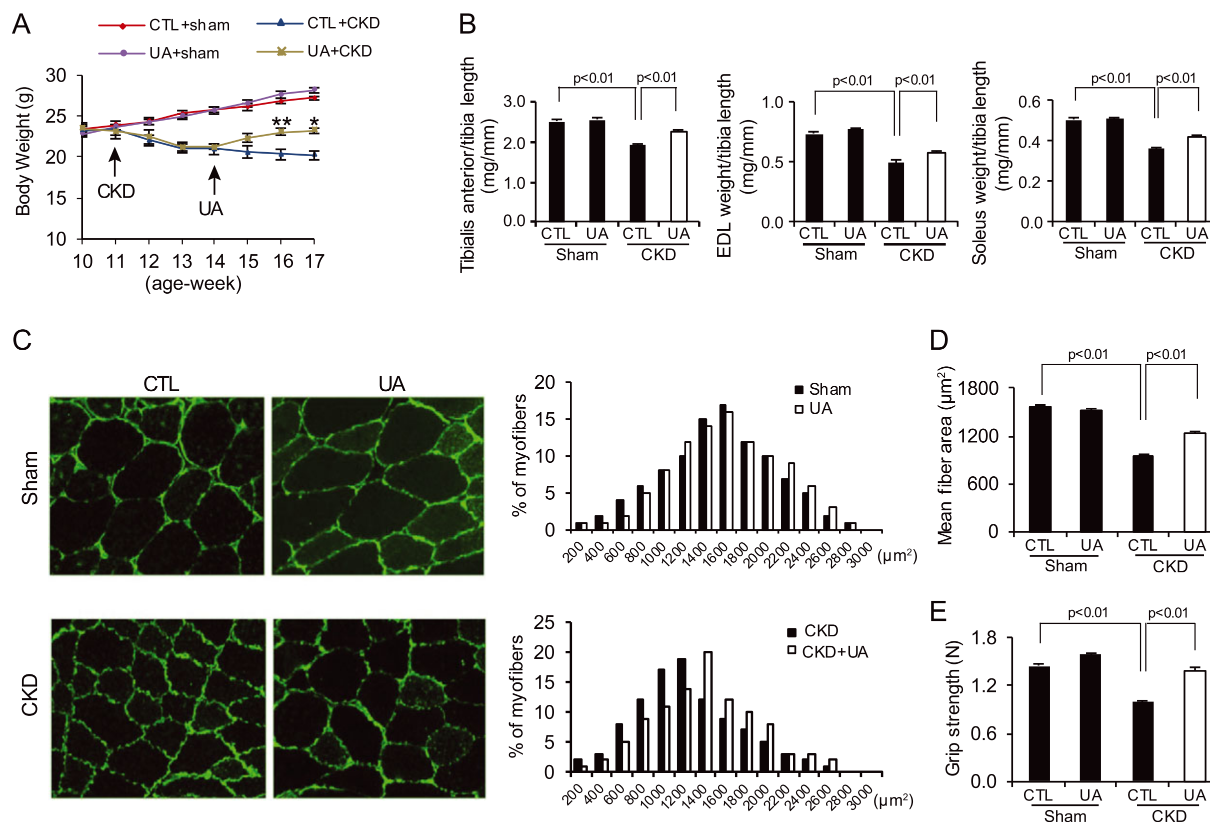
Chronic kidney disease induces muscle wasting by impairing protein synthesis and stimulating proteolysis.[25] To understand how ursolic acid ameliorates muscle atrophy, we

Figure 5 Ursolic acid blocks myostatin transcriptional activity via suppressing C/EBP-δ. (*A*) Luciferase reporter assay revealed that Dex activated myostatin promoter but ursolic acid blocked the response (mean ± SEM; *n*=6). (*B* and *C*) Scatter plot compared genes changes in Signal Transduction Pathway Finder RT-PCR Array in myotubes treated Dex and/or ursolic acid. Note that UA suppressed the induction of C/EBP-δ. (*D*) Luciferase reporter assay showed that ursolic acid blocked Dex-induced activation of myostatin promotor but overexpression of C/EBP-δ blocked this response (mean ± SEM; *n*=6). (*E*) ChIP assay showed that Dex stimulated C/EBP-δ binding to myostatin promoter and ursolic acid diminished the interaction (mean ± SEM; *n*=3).



measured protein synthesis and degradation in muscles of CKD mice treated with or without ursolic acid. Mice with CKD that received ursolic acid for 3 weeks had a significant increase in muscle protein synthesis and a significant decrease in protein degradation compared with results obtained from CKD mice that did not receive ursolic acid (*Figure* 7A). Because CKD stimulates the expression of myostatin contributing to CKD-induced muscle wasting,[26] we measured circulating myostatin and found that there was a significant decrease in serum level of myostatin in mice with CKD treated with ursolic acid (*Figure* 7B, left panel). This decrease was accompanied by suppression of myostatin mRNA in muscle of mice with CKD (*Figure* 7B, middle panel). In addition to suppressing myostatin, ursolic acid significantly suppressed the expressions of IL-6 (*Figure* 7B, right panel). In contrast with *in vitro* results obtained from myotubes cultured in DMEM containing 2% horse serum, we detected that ursolic acid was suppressing the expression of ubiquitin E3 ligases including Atrogin-1 and MuRF-1. Interestingly, we found that MUSA1 (muscle ubiquitin ligase of the SCF complex in atrophy-1), a newly identified muscle ubiquitin E3 ligase,[27] was up-regulated in muscle of mice with CKD and ursolic acid significantly suppressed this response (*Figure* 7C). Concomitant with these changes in mRNA, ursolic acid administration significantly suppressed the protein level of myostatin in muscles of CKD mice, this response was associated with a significant decrease in C/EBP-δ and an increase in p-Akt or p-FoxO1 (*Figure* 7D). Consistent with the decrease in myostatin, the phosphorylation of p-Smad3 were significantly impeded in muscles of CKD mice treated with ursolic acid (*Figure* 7D). The phosphorylated Smad1/5, a negative up-stream regulator of MUSA1, was increased in muscle of CKD mice treated with

Figure 6 Ursolic acid ameliorates the loss of muscle mass in mice with chronic kidney disease (CKD). (*A*) Body weight changes sham-operated and CKD mice treated with or without ursolic acid (UA). Mean ± SEM; **P*<0.05, ***P*<0.01 vs. CTL + CKD mice, *n*=7. (*B*) Weights of TA, extensor digitorum longus (EDL) and Soleus muscles normalized by tibia length (mean ± SEM; *n*=7). (*C*) The distribution of the myofiber cross-sectional area (CSA) in TA muscles from Sham-operated and CKD mice treated with or without UA (*n*=5, ~300 myofibers in each mouse were measured). (*D*) Mean fibre areas of TA muscles (mean ± SEM; *n*=5, ~300 myofibers in each mouse were measured). (*E*) Muscle grip strength in Sham-operated and CKD mice treated with or without UA (mean ± SEM; *n*=7).



ursolic acid (*Figure* 7D). We conclude that ursolic acid stimulates muscle protein synthesis and suppresses muscle protein degradation in muscles of CKD mice. The underlying mechanisms include inhibition of myostatin and ubiquitin E3 ligases in muscles of CKD mice.

Ursolic acid inhibits inflammatory mediators in muscles of mice with CKD

Because our *in vitro* results indicated that ursolic acid suppresses the expression of several inflammatory cytokines expressions (e.g. IL-6 and TNF-α), we investigated whether ursolic acid exerts an anti-systemic inflammatory response in mice with CKD. A serum cytokine array revealed that circulating levels of many cytokines (TNF-α, IL6, and IFN-γ) were increased in CKD mice and reduced in CKD mice treated with ursolic acid (see *Figure* 8A and Supporting Information *Figure* S4). We also evaluated whether ursolic acid would suppress inflammatory cytokines in muscles of CKD mice. As expected from the reduction in cytokines when myostatin is blocked, ursolic acid suppresses CKD-induced generation of inflammatory cytokines in muscles of CKD mice (*Figure* 8B). We also found that levels of phosphorylation of NF-kB (p65) and STAT3 and p38 were significantly suppressed by ursolic acid (*Figure* 8C). These results indicate that there is an anti-inflammatory property of ursolic acid and this could improve the cachexia occurred in mice with CKD.[28] Based on previous reports and our results, we summarized that ursolic acid can exert its anti-cachexia effect via three properties: suppression of myostatin, inhibition of inflammatory responses and enhancement of IGF-1/insulin/Akt signaling (*Figure* 8D).

Discussion

Cachexia is a serious complication of chronic illnesses such as CKD, diabetes, cancer and heart failure. It is characterized by progressive loss of muscle proteins that substantially reduces

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Figure 7 Ursolic acid improves muscle protein metabolism associated with suppression of myostatin in mice with chronic kidney disease (CKD). (*A*) The rates of protein synthesis (left panel) and protein degradation (right panel) in Soleus and extensor digitorum longus (EDL) muscles of CKD mice treated with or without ursolic acid (UA) (mean ± SEM; *$P<0.05$, *n*=7). (*B*) Serum myostatin levels in sham-operated and CKD mice treated with or without UA (left panel). UA treatment decreased myostatin level in serum of CKD mice that were accompanied by a reduction of myostatin and IL-6 mRNA expression in muscles (middle and right panel). Mean ± SEM; *n*=5–8. (*C*) The mRNA levels of Atrogin-1, MuRF-1 and MUSA1 in muscles from sham-operated and CKD mice treated with or without UA (mean ± SEM; *n*=5). (*D*) Western blot analyses of myostatin, C/EBP-δ proteins as well as the levels of p-Akt or p-FoxO1 in muscles from sham-operated and CKD mice treated with or without UA. There was also an increase in p-SMAD1/5 and a decrease in p-SMAD3 in muscle of CKD mice treated with UA. Data are presented as mean ± SEM; *n*=6.

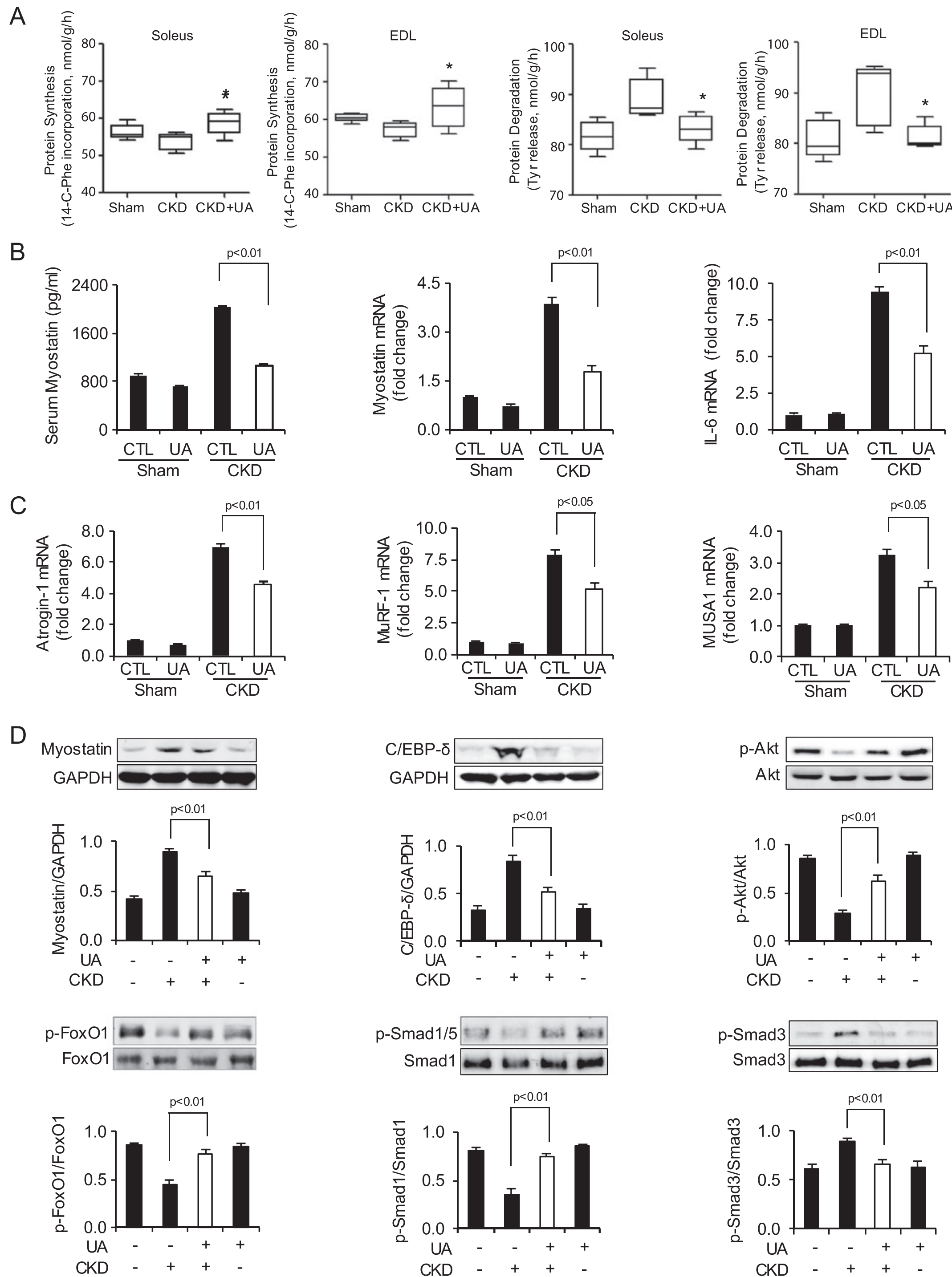
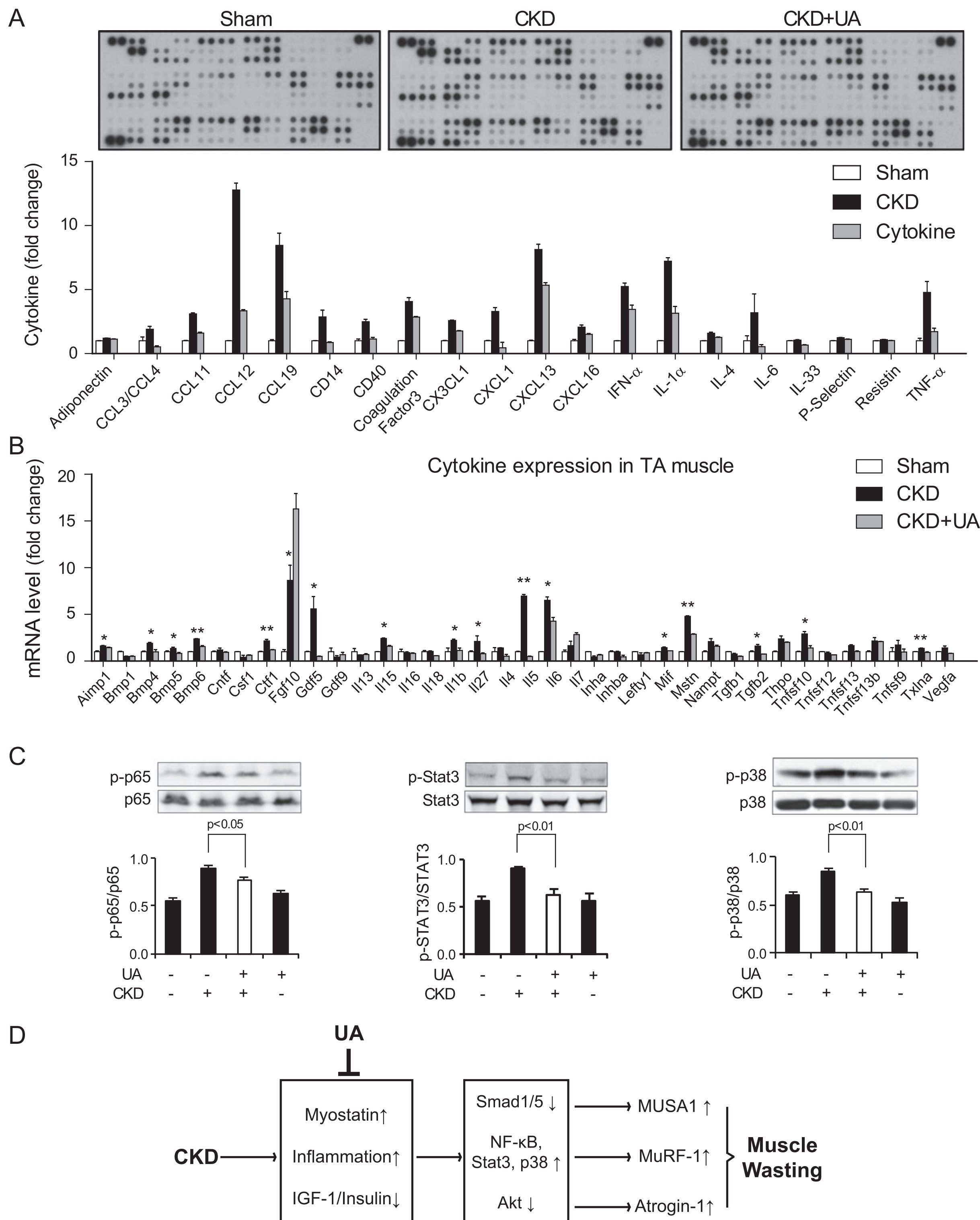


Figure 8 Ursolic acid (UA) suppresses the inflammatory cytokines in mice with chronic kidney disease (CKD) (*A*) Serum cytokine array showed that many inflammatory cytokines were increased in mice with CKD and the level of those cytokines were decreased after UA treatment. (*B*) The mRNA levels of selected cytokines from the results of Common Cytokines RT-PCR Array in muscles of CKD mice treated with or without UA (mean ± SEM; *$P<0.05$, **$P<0.01$, n=3). (*C*) Phosphorylation of NF-κB (p65), STAT3 and p38 were measured by western blot. UA suppressed the levels of p-p65, p-STAT3 and p-p38 in muscles of CKD mice (mean ± SEM; n=7). (*D*) A summative diagram of UA acting as a suppressor of muscle wasting in CKD.



the quality of life and increases morbidity and mortality. In the case of CKD, there are established factors, including excess glucocorticoids, increased myostatin and impaired insulin/IGF-1 signalling that have been shown to stimulate muscle proteolysis and suppress protein synthesis leading to loss of muscle mass.[29] Recent studies reveal that inflammatory cytokines (IL-6, STAT3, TNF-α, tweak, etc.) and related signaling pathways are activated (NF-kB, Stat3 and C/EBP signalling) were increased during cachexia,[30] indicating that systemic inflammation can play a causal role in CKD-induced muscle wasting.

As a plant compound, ursolic acid has emerged as a potential anabolic agent to treat muscle wasting and increase muscle mass. Kunkel *et al*. reported that ursolic acid treatment of mice with muscle wasting following denervation stimulates skeletal muscle insulin/IGF-I signalling leading to reduced muscle atrophy.[15] In addition, ursolic acid also reportedly reverses HFD-induced myopathy and insulin resistance.[14] The mechanism involved ursolic acid enhancement of the phosphorylation of IRS-1, a downstream effector of the IGF-I receptor or insulin receptor. This process requires certain level of IGF-I or insulin.[15] It is also raises the question does ursolic acid exert an anabolic effect in conditions that are associated with insulin and /or IGF-1 deficiency. In the present experiments, we demonstrated that ursolic acid can block CKD-induced muscle atrophy by suppressing myostatin expression associated with inhibition of C/EBP-δ. In addition, we identified that ursolic acid can reduce inflammatory responses associated with activation of STAT3 and NF-κB activations in muscles with progressive protein loss. Thus, we discovered two new properties of ursolic acid to extend ursolic acid responses that Kunkel's reported.[15]

How does ursolic acid block muscle wasting in CKD? One mechanism is that ursolic acid suppresses myostatin expression because myostatin causes muscle loss by restraining muscle growth and activates proteolysis by UPS. Indeed, myostatin infusion or over expression of myostatin can cause cachexia.[31] On the other hand, inhibition of myostatin leads muscle hypertrophy.[9] In an earlier study, we found that blocking myostatin with a neutralizing pepbody leads to suppression of SMAD3 activity and reverses muscle atrophy in mice with CKD.[25] Because the myostatin/SMAD3 pathway might act as a negative regulator muscle growth by suppressing protein synthesis,[32] one may speculate that ursolic acid stimulates protein synthesis rather than suppresses protein degradation. We measured the rate of protein synthesis and degradation in C2C12 myotubes cultured in DMEM with low concentration of serum (2% horse serum), we found that ursolic acid (5 μm) stimulates protein synthesis and suppresses myostatin expression. We also observed an increase in ubiquitin E3 ligases (atrogin-1/MuRF-1) expression plus increased proteolysis. As a net result, the increase in protein synthesis was cancelled out by the increase in protein degradation; therefore, myotube sizes were unchanged (*Figure* 1). However, when IGF-1 was added into media containing 2% horse serum, the expressions of ubiquitin E3 ligases that were stimulated by ursolic acid were eliminated (*Figure* 2D). These results are consistent with reports that the anabolic effect of ursolic acid dependents on higher level of growth factors (e.g. a physiological level of IGF-1).[15] These result provided the mechanistic interpretation for the findings reported by Figueiredo *et al*.[20] Their results indicated that ursolic acid does not increase the protein content of C2C12 myotubes cultured in a media with low concentration of serum. In CKD mice treated with ursolic acid, we found that there were stimulation of protein synthesis and suppression of protein degradation, and these response was associated with a decrease in atrogin-1/MuRF-1 expression plus an increase in p-Akt signalling (*Figure* 7C and D) Thus, administration of ursolic acid *in vivo* does not stimulate atrogin-1/MuRF-1 nor cause muscle proteolysis, presumably because of circulating IGF-1 or other growth hormones, which can activates PI3K/IRS-1 signalling.

Another mechanism that ursolic acid blocks muscle wasting is suppression of inflammation in CKD. Indeed, there are increased circulating cytokines and inflammatory response associated with the loss of protein stores in CKD patients.[33] This is relevant because we found that ursolic acid directly impedes expression of cytokines (e.g. IL-6) in muscles of mice with CKD. Evidence that ursolic acid can suppress systemic inflammation is found in the decrease in circulating cytokines in mice with CKD that were treated with ursolic acid. These results are consistent with previous observations; for example, there are reports that ursolic acid suppresses inflammatory regulatory genes including NF-κB, NF-AT and STAT3 in inflammatory cells[34–36]; Xu *et al*. found that ursolic acid increases interleukin-17 (IL-17) production but blocks the inflammatory effects of IL-6.[37] The decrease in circulating inflammatory cytokines can be linked to myostatin inhibition, because we have found that myostatin increased the production of inflammatory cytokines in muscle cells.[12,25] By ascertaining potential mechanisms for these linkages, we identified that ursolic acid can suppress several inflammation-related transcriptional factors including NF-κB. Inhibition of NF-κB by ursolic acid blocks the expression of IL-6 that is stimulated by CKD and possibly other cytokines; sequentially, this reaction leads to decrease in p-STAT3 and suppress its ability to activate C/EBP-δ transcription.

In summary, our results have identified new properties of ursolic acid, namely, the ability of ursolic acid to suppress myostatin and systemic inflammation. These properties of ursolic acid exert major pleiotropic effects to reverse the muscle protein losses in mice with CKD. The results also suggest a strategy that could prove to be beneficial for treating patients with CKD, including those with IGF-1 deficiency, increased glucocorticoids and chronic inflammation. If ursolic acid has similar effects in mice and humans, then it and/or structural analogues might be used to develop therapeutic

agents that prevent muscle wasting in CKD or the cachexia arising from other diseases that associated with increase in inflammatory cytokines, defects in insulin/IGF-1 signaling and muscle protein losses.

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Disclaimer

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Online supplementary material

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Ursolic acid suppresses myostatin but stimulates Atrogin-1 and MuRF-1 in C2C12 myotubes cultured in DMEM containing 2% horse serum. The changes of myostatin, IL-6, Atrogin-1 and MuRF-1 indicated in Myogenesis & Myopathy RT-PCR Array were confirmed using qRT-PCR. Ursolic acid (UA) suppressed myostatin and IL-6, while increased Atrogin-1 and MuRF-1 (mean ± SEM; $^{**}P < 0.01$ vs. CTL group; n = 3).

Figure S2: Ursolic acid blocks the promoter of myostatin via suppressing C/EBP-δ. Schematic diagram shows that ursolic acid (UA) blocks dexamethasone-induced myostatin expression via abolishing C/EBP-δ binding to the promoter of myostatin.

Figure S3: Ursolic acid ameliorates Dex-induced muscle atrophy in mice. (**a**) Weights of TA muscle, EDL muscle and Soleus were normalized by tibia length (mean ± SEM; n = 5). (b) Grip strength measured in Sham and CKD mice with/without ursolic acid (mean ± SEM; n = 5). (c) The distribution of the myofiber cross sectional area (CSA, μm2) (n = 5, ~300 myofibers in each mouse were measured).

Figure S4. Serum inflammatory cytokine array analysis of sham-operated (Sham) mice and CKD mice treated with/without ursolic acid (UA). The changes of interested cytokines (circled) were showed in Figure 8b. The low panel listed the coordinate position, protein name and gene ID of selected cytokines.

Table S1. Blood urea nitrogen (BUN) and serum creatinine (Cre) levels in sham-operated mice (Sham) and CKD mice following subtotal nephrectomy.

Conflict of interest

None declared.

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