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Work-induced changes in skeletal muscle *IGF-1* and *myostatin* gene expression in uremia

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Resistance to growth hormone (GH)-induced insulin-like growth factor-1 (*IGF-1*) gene expression contributes to uremic muscle wasting. Since exercise stimulates muscle *IGF-1* expression independent of GH, we tested whether work overload (WO) could increase skeletal muscle *IGF-1* expression in uremia and thus bypass the defective GH action. Furthermore, to provide insight into the mechanism of uremic wasting and the response to exercise we examined *myostatin* expression. Unilateral plantaris muscle WO was initiated in uremic and pairfed (PF) normal rats by ablation of a gastrocnemius tendon and adjoining part of this muscle with the contralateral plantaris as a control. Some rats were GH treated for 7 days. WO led to similar gains in plantaris weight in both groups and corrected the uremic muscle atrophy. GH increased plantaris *IGF-1* mRNA >twofold in PF rats but the response in uremia was severely attenuated. WO increased the *IGF-1* mRNA levels significantly in both uremic and PF groups, albeit less brisk in uremia; however, after 7 days *IGF-1* mRNA levels were elevated similarly, >2-fold, in both groups. In the atrophied uremic plantaris muscle basal *myostatin* mRNA levels were increased significantly and normalized after an increase in WO suggesting a myostatin role in the wasting process. In the hypertrophied uremic left ventricle the basal *myostatin* mRNA levels were reduced and likely favor the cardiac hypertrophy. Together the findings provide insight into the mechanisms of skeletal muscle wasting in uremia and the hypertrophic response to exercise, and suggest that alterations in the balance between IGF-1 and myostatin play an important role in these processes.

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Muscle wasting is common in patients with advanced kidney failure and if sustained this has an adverse effect on the clinical course of the patient.[1,2] Multiple factors contribute to this wasting state including reduced protein–calorie intake, acidosis, inflammation, diabetes and acquired resistance to or deficiency of anabolic hormones such as growth hormone (GH), insulin-like growth factor-1 (IGF-1), insulin, and testosterone.[3–6] Although little is known about the role of myostatin in uremia,[7] it is conceivable that this negative regulator of muscle mass[8] also contributes to uremic muscle wasting. IGF-1 is of special interest as it plays a central role in the control of muscle growth, repair and the maintenance of muscle mass,[9–11] and mediates most growth-promoting actions of GH. Indeed, it is now established that alterations in the GH–IGF-1 system especially resistance to IGF-1 action[12,13] and impaired IGF-1 production in response to GH[14–16] contribute to the wasted state in uremia. Resistance to IGF-1 arises because of the accumulation of high-affinity insulin-like binding proteins which trap IGF-1 and reduce its bioavailability[14] and also because of a defect in IGF-1-mediated signaling.[12] Impaired GH-mediated IGF-1 production arises largely because of a signaling defect.[17,18]

IGF-1 is produced in tissues throughout the body largely under the influence of GH with the liver as the main source of circulating hormone.[19] In skeletal muscle IGF-1 production is regulated by GH and by mechanical stimuli induced by exercise in a GH-independent manner,[20,21] and this likely contributes to the beneficial effects of exercise on muscle structure and function.[10,22–24] Our prior studies in uremic rats indicate that resistance to GH arises largely due to a defect in GH-mediated Janus kinase 2-signal transducers and activators of transcription 5 signal transduction,[17,18,25] a pathway essential for GH-stimulated IGF-1 expression.[26] Reduced GH receptor expression,[14] though not a universal finding,[17,18,25] may be another cause of GH resistance. In humans with kidney failure, resistance to GH and IGF-1 not only impairs maintenance of muscle bulk but also inhibits linear growth.[3,14,16] Administration of GH in relatively high doses produces an increase in linear growth and muscle mass in children[27] and a positive anabolic response in adults with advanced renal failure.[3,28]

In view of the resistance to GH-stimulated IGF-1 expression that develops in uremia,[17,18,25] we set out to determine, whether we could bypass this defect by activating a GH-independent stimulus of local IGF-1 expression, namely increased muscle work load,[20,21] and thus stimulate muscle IGF-1 expression and hypertrophy. In addition, to provide further insight into the mechanism of muscle wasting and the response to resistance exercise in uremia, we examined the expression of myostatin, a major negative regulator of muscle mass that limits muscle fiber growth.[8,29] Myostatin also known as growth and differentiation factor-8, is a member of the transforming growth factor-β super family that is expressed in skeletal and cardiac muscle.[8,29,30] Spontaneous mutations of the myostatin gene lead to an increase in muscle mass as recently reported in a child,[31] and accounts for the enormous muscular hypertrophy present in Belgium blue cattle.[29] In contrast when active myostatin is present in excess as described in acquired immunodeficiency syndrome, following glucocorticoid therapy or muscle unloading, muscle mass and function is reduced.[8,32] Resistance exercise usually depresses myostatin expression,[33–36] but this is not a uniform finding.[37]

To achieve our goals we studied rats with surgically induced chronic renal failure (CRF) and unilateral work overload (WO) of the plantaris muscle induced by excision of one gastrocnemius tendon and adjoining part of the muscle. In this model of resistance exercise we show that despite the presence of GH resistance, sustained WO can effectively stimulate IGF-1 expression and muscle hypertrophy. Furthermore, we show that skeletal muscle myostatin expression is increased in uremia and falls following an increase in work load.

RESULTS

Serum biochemistry and anthropometrics

After 21 days of CRF serum creatinine and urea nitrogen levels were increased significantly compared to pairfed (PF) sham-nephrectomized control values (Table 1). In contrast, serum total CO_2 levels in CRF rats were similar to the control values. Body weight and plantaris muscle weight despite pair feeding, were significantly lower in uremic rats while left ventricular weight was increased.

Table 1 | Biochemical and anthropometric characteristics of CRF and pairfed sham-ephrectomized rats

	PF	CRF
Number of rats	11	14
Serum creatinine (mg/dl)	0.35±0.03	1.48±0.13**
Serum urea nitrogen (mg/dl)	17±1	86±9**
Serum bicarbonate (mmol/l)	27±0.86	25±0.65
Body weight (g)	237±4	206±6**
Control plantaris weight (mg)	254±6	202±8**
LV weight (g)	0.54±0.02	0.64±0.01**
LV/BW (%)	0.22±0.01	0.31±0.01**

BW, body weight; CRF, chronic renal failure; LV, left ventricle; PF, pairfed.
**$P<$ 0.01.

Rats with CRF are resistant to GH induced body weight gain and stimulation of *IGF-1* gene expression

Resistance to GH therapy was evident in CRF rats as previously described; treatment with GH for 7 days failed to cause an increase in body weight gain compared to vehicle (V)-treated CRF rats (37.2±2.1 vs 36±4.7 g/7 days, respectively; Figure 1a). In contrast body weight increased significantly in PF controls treated with GH compared to V-treated controls (65.5±3.3 vs 40.4±3.5 g; $P<0.01$). GH had no effect on serum biochemistry in either group (data not show). In respect to *IGF-1* gene expression, resistance to GH was also evident in the CRF group. As shown in Figure 1b, which depicts the findings in the control non-overloaded plantaris muscle, basal *IGF-1* mRNA levels in the PF and CRF rats did not differ significantly even though the average levels were lower in the CRF group (100±16 vs 73±14 relative arbitrary units). Following GH treatment *IGF-1* mRNA levels increased in both groups, but the response was significantly attenuated in the CRF group ($P>0.01$). The relative *IGF-1* mRNA levels rose to 137±25 arbitrary units in the CRF group compared to 223±31 arbitrary units in the PF rats.

WO induces muscle hypertrophy in CRF

WO of the plantaris muscle induced by unilateral ablation of the gastrocnemius tendon and distal muscle was followed by similar increases in plantaris muscle weight in both CRF and PF rats even though the control CRF muscle weights were lower (Figure 2). After 2 days of overload the plantaris muscle weight in the PF and CRF rats increased above the contralateral control values by 22±8 and19±6 mg, respectively, and after 7 days by 86±16 and 100±12 mg, respectively. Thus, while the total weight of the overloaded CRF muscle remained below that of the overloaded muscle of

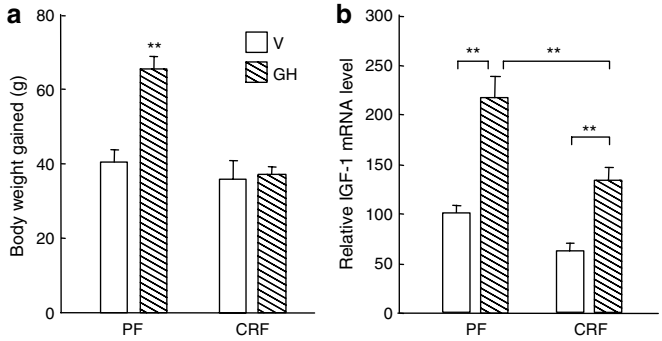

Figure 1 | Growth hormone stimulated body weight gain and *IGF-1* mRNA expression is severely impaired in CRF. (**a**) Body weight. (**b**) Control plantaris muscle *IGF-1* mRNA levels. Rats with CRF or PF rats were treated with bovine growth hormone (GH) or vehicle (V) for 7 days and then killed. *IGF-1* mRNA levels in the plantaris muscle from control intact non-loaded limbs from CRF and sham nephrectomized. PF rats were measured by quantitative real-time PCR and corrected for the internal housekeeping gene *L7* which is unaffected by uremia or GH. Results, mean±s.e.m. of 11–14 rats/group, are expressed relative to the PF V-treated control group and assigned a mean value of 100. **$P<0.01$ vs V treated.

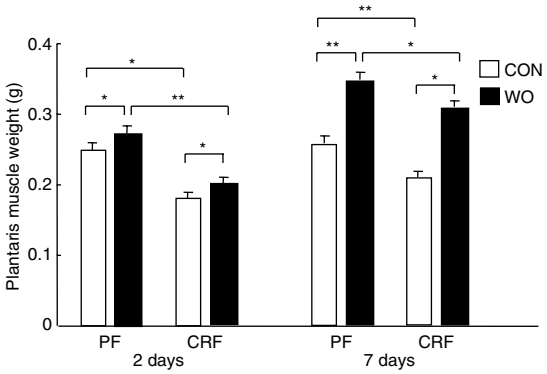

Figure 2 | WO induces a similar increase in skeletal muscle weight in CRF and PF rats even though the weight of control (CON) unloaded muscle is lower in CRF. Rats were studied 2 or 7 days after an unilateral increase in work load induced by excision of one distal gastrocnemius tendon and part of the muscle. Results are mean ± s.e.m. from 5 to 8 rats/group. *$P<0.05$, **$P<0.01$.

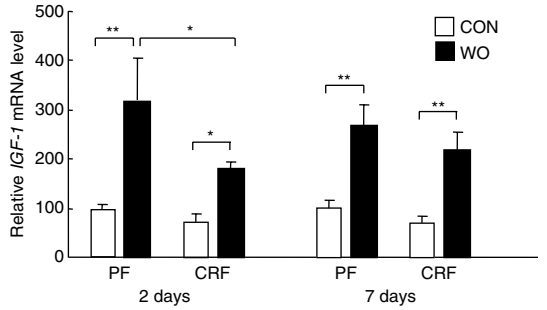

Figure 3 | WO increases plantaris muscle *IGF-1* mRNA levels in CRF and PF rats. Plantaris muscles from both limbs were collected 2 or 7 days after unilateral excision of a gastrocnemius tendon and part of the muscle. *IGF-1* mRNA levels were measured in the WO and contralateral control (CON) plantaris muscle by real-time PCR and corrected for the internal housekeeping gene *L7* which is unaffected by WO. The results, mean ± s.e.m. from 5 to 8 muscles/group, are expressed relative to the values obtained from the non-loaded control plantaris muscle of the PF vehicle (V)-treated group, assigned a mean value of 100. *$P<0.05$. **$P<0.01$.

the PF rats, the CRF values reached levels that did not differ significantly from that of the control PF values. In other words WO appears to correct uremic muscle atrophy.

WO stimulates *IGF-1* gene expression in skeletal muscle of CRF rats

WO of the plantaris muscle (Figure 3) induced a significant increase in local *IGF-1* mRNA levels in both the CRF and PF rats compared to the contralateral non-loaded control values ($P<0.01$). After 2 days of WO the relative *IGF-1* mRNA level increased significantly in both groups of rats though to a lesser level in the CRF rats; relative *IGF-1* mRNA levels increased to 320 ± 83 in the PF group ($P>0.01$) and 180 ± 14 arbitrary units, in the CRF group ($P<0.05$). After 7 days of WO, plantaris muscle *IGF-1* mRNA levels increased further in the CRF group and reached a level similar to that in the PF group (266 ± 44 vs 220 ± 40 arbitrary units, respectively). Thus, exercise is an effective stimulus of *IGF-1* gene

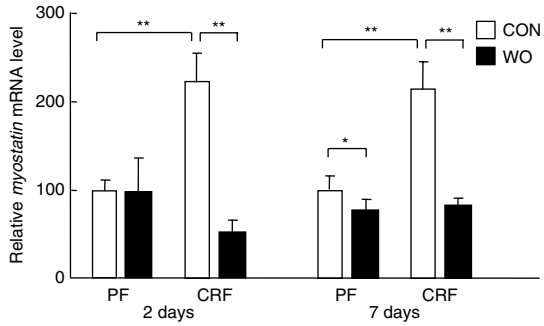

Figure 4 | Plantaris muscle *myostatin* mRNA levels are increased in CRF and fall when work load is increased for 2 or 7 days. Results are from the same animals as in Figure 3. *Myostatin* mRNA levels were measured by real-time PCR and corrected for the internal housekeeping gene *L7*. *$P<0.05$, **$P<0.01$.

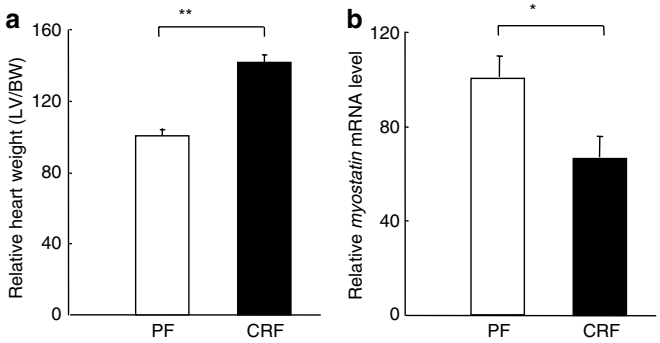

Figure 5 | Cardiac muscle *myostatin* (a) mRNA levels are depressed in the hypertophied left ventricle of (b) CRF rats. *Myostatin* mRNA levels were measured by real-time PCR and corrected for the internal housekeeping gene *L7*. Results are from the rats undergoing tendon ablation 2 days before killing. *$P<0.05$.

expression in uremia, despite resistance to GH-induced IGF-1 expression.

Plantaris muscle myostatin mRNA levels are elevated in CRF and are depressed following WO

Figure 4 illustrates the changes in *myostatin* mRNA that occur in uremia. Of note there was a >2-fold increase in the *myostatin* mRNA levels of the control plantaris muscle of the uremic rats compared to the values in the PF rats ($P<0.01$). WO for 2 or 7 days depressed the elevated *myostatin* mRNA levels significantly in the CRF rats ($P<0.01$), and caused a small but significant decrease in the PF group after 7 days. Taken together this suggests that in uremia over expression of *myostatin* may contribute to the development of muscle wasting and that exercise induced hypertrophy may arise in part through the suppression of *myostatin* gene expression.

Cardiac muscle myostatin expression is depressed in the hypertrophied left ventricle of uremic rats

Following 21 days of CRF there was a significant increase in left ventricular weight and left ventricular weight corrected for body weight compared to the PF values (Table 1 and Figure 5, $P<0.01$). This presumably reflects a response to increased work induced by hypertension and fluid overload

observed in rats following a 5/6 nephrectomy.[38] In the uremic rats as shown in Figure 5, the relative *myostatin* mRNA level in the left ventricle were significantly depressed to a value that was two thirds of the PF control values ($P<0.05$). We suggest that the depression of cardiac myostatin expression may well play a role in the hypertrophy of the left ventricle. Interestingly in contrast to the reduction in *myostatin* expression, relative *IGF-1* mRNA levels in the uremic myocardium did not differ from the PF values (88 ± 4 vs 100 ± 9 arbitrary units), a finding consistent with our previous report.[25]

DISCUSSION

In this study of rats with CRF and muscle wasting we set out to test the thesis that expression of *IGF-1* can be increased through activation of a GH-independent mechanical stimulus, namely increased muscle work load,[20] despite the presence of resistance to GH induced increases in *IGF-1* mRNA levels which arises largely due to a defect in GH-mediated Janus kinase 2-signal transducers and activators of transcription 5 signal transduction.[17,18,25] In addition, in order to provide further insight into the mechanism of uremic wasting and the response to exercise, we examined the expression of *myostatin*. As before[17,18,25] we found that the CRF rats were relatively resistant to the growth-promoting action of GH and to GH stimulation of *IGF-1* mRNA expression. In contrast, unilateral plantaris muscle WO induced by excision of a gastrocnemius tendon and portion of the muscle, caused a significant albeit less brisk increase in local *IGF-1* mRNA levels in the CRF rats that reached levels similar to that achieved in the PF sham-nephrectomized rats. The less brisk *IGF-1* response in the CRF rats could conceivably arise because of reduced physical activity in the uremic state, though in the same rats WO produced an early and effective decrease in elevated myostatin levels and Adams *et al.*[38] reported that voluntary activity was not impaired in 5/6 nephrectomized rats. In both CRF and PF rats the work induced increase in *IGF-1* mRNA levels was accompanied by a significant increase in muscle weight and in the CRF rats this corrected the uremic muscle atrophy. Since IGF-1 is known to cause muscle hypertrophy,[9,39,40] these findings are consistent with a role for local IGF-1 in inducing the observed increase in muscle mass.

Interesting changes in myostatin expression were also noted. In the CRF rats the myostatin mRNA levels in the atrophied non-overloaded plantaris muscle were more than twofold higher than in the corresponding plantaris muscle in PF rats. Furthermore, following an increase in work load the myostatin mRNA levels fell in the CRF rats to values that were similar or on average even lower than the basal levels in PF rats. The situation in the heart of CRF rats differed. In contrast to skeletal muscle atrophy with elevated myostatin mRNA levels, the left ventricular muscle was hypertrophied and the myostatin levels were significantly reduced compared to the PF group. We suggest that this may reflect a response to an increase in cardiac muscle work induced by hypertension and fluid retention in the uremic state.[38]

Circulating IGF-1 is largely derived from hepatic secretion and like IGF-1 produced in other tissues including muscle, is regulated by GH via the Janus kinase 2-signal transducers and activators of transcription 5 signal transduction pathway.[19] Skeletal muscle IGF-1 is also produced in response to mechanical stimuli such as exercise in a GH-independent manner[20,21] and this local IGF-1 plays an important role in muscle growth and repair.[19,40,41] When exercise is prolonged IGF-1 released from muscle contributes to the circulating pool,[42] though circulating IGF-1 appears to be less important than local IGF-1 in promoting muscle growth.[43] The structure of the mature IGF-1 peptide is relatively simple, but its gene structure and pattern of expression is complex and this results in the formation of multiple transcripts.[19,40] In the present study, we measured the total mRNA level of all *IGF-1* transcripts and following WO the level increased in both the CRF and PF groups despite a lack of response to recombinant GH.

The mechanisms whereby mechanical stimuli activated by skeletal muscle work induce hypertrophy are poorly understood.[44] One key mechanism is the induction of *IGF-1* gene expression with increased IGF-1 peptide production.[9,10] This local peptide promotes cell proliferation and differentiation, stimulates protein synthesis and inhibits proteolysis and does so largely through activation of the phosphatidylinositol 3′ kinase/Akt-signaling pathway.[5,10,11,44] Interestingly downstream components of this same pathway are also activated by mechanical stimuli independent of IGF-1 and together with IGF-1 promote an increase in muscle mass.[10,44] Our present findings in a rat model of resistance exercise is consistent with human studies showing that resistance exercise is an effective means of inducing skeletal muscle hypertrophy in kidney failure[23,24] and is compatible with a role for local IGF-1, independent of GH, in this process.

It has been reported that serum IGF-I levels are normal or even elevated in adults receiving maintenance dialysis[7,45] but are reduced in those that are severely malnourished.[15] Children with CRF have normal or low normal IGF-1 levels and there is evidence suggesting that IGF-1 secretion is attenuated despite normal or elevated GH levels, findings consistent with GH resistance.[14] In adults with end-stage renal disease skeletal muscle IGF-1 peptide levels have been reported to be low in subjects with muscle wasting[45] and increased in relatively well-nourished end-stage renal disease subjects.[7] However, as the expression of the major *IGF-1* mRNA transcript, IGF-1Ea, was significantly depressed in the latter study, the authors suggested that the increase in IGF-1 peptide noted may reflect trapping by local IGF binding proteins.[7] In uremic rats *IGF-I* mRNA levels may be reduced in liver, muscle, and long bone growth plate,[14] and to a large extent this may reflect reduced food intake.[15]

Myostatin is another major regulator of skeletal muscle mass.[8,29] This negative regulator is expressed in skeletal and cardiac muscle and impairs skeletal muscle growth by inhibiting myoblast proliferation and differentiation and satellite cell activation.[8] Overexpression of myostatin as a

cause of muscle atrophy has been implicated in several wasting states.[8,29] In contrast, *myostatin* gene mutations can lead to a remarkable increase in muscle mass as described in cattle and in a young child[29,31] and experimental inhibition of myostatin action is under study as a therapeutic for muscle-wasting disorders.[46] Exercise in the form of endurance or resistance training can suppress myostatin expression and induce muscle hypertrophy.[7,29,36,47] In a study of sedentary well-nourished maintenance hemodialysis subjects, Wang *et al.*[7] noted that skeletal muscle *myostatin* mRNA levels were similar to normal control subject values and a preliminary report[22] indicates that myostatin mRNA fall following prolonged endurance exercise training. In the present study of rats with CRF and skeletal muscle atrophy *myostatin* mRNA levels were more than two fold the values in normal PF rat muscle. However following an increase in work load, the elevated *myostati*n mRNA levels fell significantly and reached values that did not differ significantly from PF normal control values. This response was associated with an increase in muscle weight. Accordingly it appears that the work load induced fall in myostatin together with the increase in IGF-1 expression likely contribute to the increase in muscle mass. The difference in the basal *myostatin* mRNA levels noted in the present study in muscle wasted CRF rats and that of Wang *et al.*[7] in humans, may reflect the fact that the hemodialysis subjects were well nourished and had no clinical evidence of muscle wasting. It is also possible that there are species differences.

In contrast to the increase in skeletal muscle *myostatin* mRNA levels present in the atrophied skeletal muscle of uremic rats, the levels were reduced significantly in the hypertrophied left ventricle of these same rats. We suggest that this reduction may reflect the response to a prolonged increase in left ventricular work load caused by hypertension and fluid retention in CRF[38] and may conceivably contribute to the development of left ventricular hypertrophy.[48] These findings differ from that observed in other conditions that cause cardiac hypertrophy. Cook *et al.*[49] reported that *myostatin* is upregulated in the hypertrophied hearts of transgenic mice with cardiac-specific expression of activated Akt, while Shyu *et al.*[30] noted that after the creation of an aorta–caval shunt in rats, there was an early increase in cardiac myostatin peptide levels that returned to baseline levels after 7 days. Both authors suggested that the myostatin increase may reflect a negative feedback response to prevent excessive cardiac hypertrophy. Studying cultured cardiomyocytes Shyu *et al.*[30] also showed that cyclic stretch stimulates an increase in *myostatin* mRNA expression that returned to baseline after 48 h. Thus, the difference in our findings in rats after 21 days of CRF and that of Shyu *et al.*[30] may reflect temporal changes in the expression of *myostatin* in response to mechanical stimuli. It is also possible that the increase in cardiac *myostatin* in CRF occurs through mechanisms peculiar to the uremic state. Further study to clarify the role of myostatin and its regulation in different diseases that affect the heart is required.

In conclusion, we have shown that an increase in skeletal muscle work load is an effective means of stimulating local *IGF-1 mRNA* expression in the uremic state despite severe resistance to GH-stimulated IGF-1 expression. Furthermore, the increase in work load leads to muscle hypertrophy and taken together this is consistent with the anabolic action of local IGF-1. We also found significant changes in the expression of myostatin. *Myostatin* mRNA levels were elevated in the atrophied skeletal muscle of the uremic rats suggesting a role in the wasting process and following an increase in muscle work load these abnormalities were corrected. Taken together these findings add to our understanding of the mechanisms of uremic skeletal muscle wasting and the hypertrophic response to exercise, and appears to be consistent with a change in the balance between local IGF-1 and myostatin expression. Finally, in contrast to the high *myostatin* mRNA levels present in the atrophied skeletal muscle of uremic rats, the levels were low in the hypertrophied left ventricles. Overall these findings are consistent with a role for myostatin in the pathogenesis of skeletal muscle wasting and left ventricular hypertrophy.

MATERIALS AND METHODS

Experimental animals and protocols

Male Sprague–Dawley rats weighing ~125 g were entered into the study. CRF was created by a two-step 5/6 nephrectomy procedure with ketamine (80 mg/kg) and xylazine (10 mg/kg) anesthesia as before.[17] Sham nephrectomy operations were performed on other animals that were PF with the CRF rats to control for the impact of nutrition on IGF-1 expression.[15] After 14 days half the CRF and PF sham-nephrectomized rats underwent a surgical procedure to produce unilateral WO of the plantaris muscle, a model of resistance exercise,[20] with the contralateral plantaris muscle of the same animal serving as a control. The remaining animals underwent the same procedure after 19 days of CRF and all animals were killed after 21 days of CRF. In brief, under ketamine and xylazine anesthesia the distal gastrocnemius tendon of one leg was exposed, the tendon and lower quarter of the muscle was excised and then the skin incision closed with a clip and the rats were allowed to recover from anesthesia. No surgery was performed on the contralateral limb which served as a paired control; in pilot studies we found that skin incision and tendon exposure alone had no impact on plantaris muscle *IGF-1* gene expression. The following groups of 5–10 rats were studied. PF and CRF groups with unilateral WO for 2 days. PF and CRF groups with unilateral WO for 7 days and treated either with V or recombinant bovine GH (gift from Monsanto Corp., St Louis, MO, USA) 5 mg/kg s.c. twice a day. On the last day of the 7 day period, rats received GH or V 5 h apart and were killed 1 h after the last injection to measure GH-stimulated IGF-1 expression. Plantaris muscle was excised, weighed, and stored at −80°C. Serum was collected and creatinine and CO_2 levels were measured with a Beckman LX 20 Analyzer (Beckman Coulter Inc., Fullerton, CA, USA).

Real-time quantitative reverse transcriptase-polymerase chain reaction assay

Real-time quantitative reverse transcriptase-polymerase chain reaction (PCR) with SYBR green dye as the detection agent was performed with the ABI Prism 7900 Sequence Detection System

Table 2 | Primer sequences for quantitative real-time PCR analysis

	Forward (5′–3′)	Reverse (5′–3′)
18S	GGGAATTCCTGCCAGTAGCATATGCTTG	GGAAGCTTAGAGGAGCGAGCGACCAAAG
L-7	GAAAGGCAAGGAGGAAGCTCATT	AATCTCAGTGCGGTACATCGCCT
IGF-1	CCGCTGCAAGCCTACAAAGT	TGAGTCTTGGGCATGTCAGTGT
Myostatin	CAGGACCAGGAGAAGATGGGCT	CGATTCCGTGGAGTGAACATCAC

IGF-1: insulin-like growth factor-1; PCR: polymerase chain reaction.

(Applied Biosystems, Foster City, CA, USA) using the manufactures protocol (Perkin-Elmer Applied Biosystems (1997) User Bulletin 2 updated 2001: ABI Prism 7700 Sequence Detection System, Relative Quantitation of Gene Expression, Applied Biosystems, Foster City, CA, USA). Primers for the quantitation of the *IGF-1*, *myostatin* and internal control genes, ribosomal *18S* and *L7* (Table 2), were designed using the primer design software Primer Express (Applied Biosystems) and from published sequences and were synthesized by Qiagen Inc. (Alameda, CA, USA). For *IGF-1* detection, primers and probes were designed to amplify and detect all splice variants of the *IGF-1* mRNA. Total RNA was extracted from plantaris muscle and used for cDNA synthesis by reverse transcription (Applied Biosystems, Foster City, CA, USA) and the cDNA samples were then subjected to PCR analysis. The results were quantified using the relative standard curve method as described by the supplier. An internal control gene standard curve was also generated and the target gene is normalized for this endogenous control. Each sample was analyzed in triplicate in individual assays performed on two or more occasions.

Data analysis and statistics

Specific mRNAs were normalized for the internal control gene and are expressed as transcript/housekeeping gene ratios. The control vehicle treated PF group mean was given a value of 100, and individual values are expressed relative to this value. Data are given as mean $\pm$ s.e.m. Two-tailed unpaired Student's *t*-tests were applied for comparison of two normally distributed groups, where appropriate paired *t*-tests were applied to compare left and right planatris muscles from the same animal; comparisons between more than two normally distributed groups were made by one-way analysis of variance followed by pairwise multiple comparison with the Holms *t*-test.[50] A P-value <0.05 was considered significant.

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