Toll-like receptor 4 signalling mediates inflammation in skeletal muscle of patients with chronic kidney disease

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Introduction

Loss of protein content from skeletal muscle is related to a reduced survival in aging and in several pathological condition, including cancer and cardiac disease. (1-3)

Muscle depletion is common in advanced stages of chronic kidney disease (CKD). In this condition, sarcopenia is related to an increase in morbidity and mortality (4), especially in advanced stage of disease and in dialysis.

For long time, malnutrition was identified as a predominant cause of CKD induced sarcopenia (5) along with hypoalbuminemia and reduced protein intake (6). Malnutrition is often multifactorial and the possible causes include metabolic acidosis, intestinal dysbiosis, systemic inflammation with activation of complements, endothelin-1 and renin-angiotensin-aldosterone (RAAS) axis, anabolic hormone resistance, energy expenditure elevation and uremic toxin accumulation (7).

Several studies addressed the question if malnutrition is the exclusively cause of sarcopenia. Ikizler and al analysed the effect of dialysis on protein catabolism studying patients with stage 5 CKD before and after a dialysis session (8). In this study, authors demonstrated that the dialysis stimulates protein degradation and reduces protein synthesis with an effect that last for hours after dialysis end; moreover, increasing dietary protein intake did not completely resolved the catabolic effects of haemodialysis.

Moreover, modifying caloric intake did not demonstrated an improvement in sarcopenia, strongly suggesting that other factors need to be involved in this process (9).

Similar results have been obtained introducing parenteral nutrition during haemodialysis session, along with oral nutritional supplement. Even in this condition, 2-year mortality, body mass index, laboratory markers of nutritional status and the rate of hospitalization were the same as the control group of patients who were given only the oral supplement (10). Collectively, these data indicate that malnutrition cannot be the only factor that cause cachexia and muscle wasting in advanced stage of CKD.

In recent years, several studies allowed to better understand the pathophysiology of muscle dystrophy in CKD. Muscular proteins that compose the whole muscle are constantly renewed with a process of formation and degradation; to be efficient and to avoid muscle loss, as seen in cancer, cardiac cachexia and CKD, this process must be extremely tightly regulated. Three mechanisms are involved in loss of muscle protein stores: impaired growth of new muscle fibres, suppression of protein synthesis or stimulation of protein degradation.

Muscle is composed of muscle fibres surrounded by basal lamina which covers myofibrils and satellite cells (also called muscle precursor cells) (11). These cells are able to react to muscular injury and metabolic stimuli like growing hormones as IGF-1; the result is the production of anabolic factor myogenic regulator factor 5 (Myf-5) and myoblast determination protein (MyoD) that cause satellite cell proliferation and differentiation in myoblasts and myocytes (12).

In CKD, satellite cells have an impaired function, probably due to a reduced IGF-1 activity, that could lead to muscle atrophy, at least in murine models (11,13). A

resistance in the action of insulin and GH/IGF-1 pathway could also be secondary to CKD proinflammatory cytokines (14,15)

CKD also induces suppression of protein synthesis. Patients with CKD showed a reduced rate of synthesis of mixed muscle protein and synthesis of myosin (16) but a similar rate of mitochondrial proteins. Using the measurements of turnover of a labelled amino acid, leucine oxidation and nonoxidative leucine disposal (an in vivo index of protein synthesis) were significantly reduced in patients with CKD compared with control values (17). Reduced protein synthesis has been observed in haemodialysis patients, along with an increased protein degradation (18); both responses were favourited by acidosis and blocked by infusion of sodium bicarbonate.

Another regulatory mechanism of muscle protein impaired in CKD is protein degradation. Protein degradation is executed by two principal mechanisms: ubiquitine proteasome system (UPS) and caspase 3 pathway. Both systems cooperate to provide an extremely efficient and tightly regulated degradation of proteins, including muscle proteins. This specificity is necessary due to the variety of proteins involved in muscle (and cell) formation, each with a different half-life. Errors in protein degradation could potentially lead to cell damage and death.

UPS degrade a large variety of protein. To be specific, UPS acts marking proteins to degradate with ubiquitine, a member of heat shock protein family (19). The bind of ubiquitin is promoted by E1 ubiquitin derived enzyme along with one of the 20 ubiquitin carrier (E2) that can interact with an E3 ubiquitine ligase enzyme. Process of ligation and activation is ATP-dependent. There are more than 1000 E3 ubiquitin ligases that

recognize a specific protein (or a class of proteins) and mark them for destruction. After the creation of a chain of 5 ubiquitinated proteins, the complex is recognized by the 26s proteasome and subsequently degraded. The role of UPS in kidney disease is well known for various kidney disease like Liddle syndrome (20) or in Von Hippel Lindau Disease (21).

Caspase 3 is also involved in muscle wasting process. Caspase 3 provides degradation substrate for UPS in catabolic conditions cleaving the complex structure of muscle proteins (22,23). For his intrinsical characteristics, UPS degrades slowly actomyosin and myofibrils while is more efficient in degrading monomeric actin and myosin. Caspase 3 increase the efficiency of muscle protens degradation process by UPS specifically stimulating proteolytic activity of the proteasome and increasing the size of unfolded protein able to enter 20S subunits of proteasome (the proteolytic site of proteasome). This effect is obtained by interacting with 19S subunits and modifying their morphology (24).

Recent evidences suggested a role of innate immunity in causing muscle wasting in CKD patients (25-27). Transforming growth factor β (TGF β) and TGF β family members Activin A and myostatin are associated with muscle loss in catabolic condition (28). TGF β family members act activating SMAD2/SMAD3 pathway that ultimately increase proteolysis and muscle atrophy (29,30). Myostatin, in particular, suppress growth of skeletal muscle. Animals that lack of myostatin show a very large increase in

muscle mass and strength (31,32). Binding of Myostatin to his receptor activates Smad2/Smad3 pathway and the phosphorylation of Akt in muscle. An increase in the levels of phosphorylated (p)Akt reduce phosphorylation of the Forkhead box O (FoxO) family of transcription factors (33); dephosphorylated FoxO increase the expression of two E3 ubiquitin ligase: TRIM63 (also known as MuRF1) and F-box only protein 32 (MAFbx, also known as atrogin-1) that promote UPS mediated degradation of muscle proteins.

Apart from TGF β family, other proinflammatory cytokines are supposed to have a considerable effect on muscle loss. Blood levels of C-reactive protein (CRP), activin A and proinflammatory cytokine like Interleuchin-6 (IL-6) and tumoral necrosis factor a (TNFa) are elevated in patients with CKD and directly related to muscle atrophy (34-36). An up-regulation of several genes associated with inflammation in muscle has been demonstrated to occur both in rodent models (37) and in humans with CKD (38-40).

A similar result has been observed in other studies. A reduced protein synthesis and an increased protein degradation has been observed along with high level of IL-6 and TNF-α in human and animal model (41-43). In mice, injection of IL-6 caused an increase in muscle wasting but the mechanism was not identified (44). Il-6 has been linked to muscle wasting with two different mechanisms. The first mechanisms, highlighted in a mice model of Angiotensin-II induced inflammation model (45), involves an increased expression of suppressor of cytokine signalling 3 (SOCS-3) in muscle. A high level of SOCS-3 reduces IRS-1 levels and suppresses intracellular insulin signalling, leading to

activation of caspase-3 and the UPS (46). A second mechanism links Il-6 to signal transducer and activator of transcription 3 (Stat3). Stat3 is upregulated in kidney disease (37) and related to muscle atrophy. Il-6 directly activates Stat3 to increase the expression of CCAAT/enhancer-binding protein δ (C/ EBPδ) that in turn enhances the expression of myostatin.i

More recently, the importance of insulin and GH/IGF1 pathway has been highlighted. Activation of GH/IGF1 pathway causes downstream activation of insulin receptor substrate 1 (IRS-1) and PI3K that in turn activate the final effector Akt. Activation of Akt stimulates many metabolic responses, including promotion of protein synthesis and inhibition of protein degradation (13). In mouse model of CKD, suppression of Akt lead to muscle atrophy while upregulation of PI3K and Akt prevented muscle atrophy (11, 47-49). Phosphorylation of Akt is the cornerstone of its action. In muscle, pAkt downregulates FoxO function; FoxO1 and FoxO3 regulate the functions of TRIM63 and MAFbx, which are critical for muscle-protein breakdown (50-52). As previously noted, activation of TRIM63 and MAFbx, as occurs in insulin resistance, lead to accelerated protein degradation and muscle atrophy (13). In a previous study, we observed that pAkt is markedly downregulate in muscle of patients with advanced CKD and is associated with apoptotic cell loss, suggesting that insulin resistance is a major player in the regulation of muscle cell survival and catabolism in renal patients (40).

Collectively, all the studies above don't cover all the mechanisms involved in muscle atrophy in CKD patients. Currently, we lack studies that address the cause why uremia upregulates proinflammatory cytokines in skeletal muscle. Skeletal muscle possesses both the afferent and efferent limbs of the innate immune system, including Toll-like receptors (TLRs) and both early and late-phase cytokines (53,54). Toll like receptors are able to sense foreign peptides, like bacterial and viral protein, but also endogenous signals of tissue injury, including debris from apoptotic and necrotic cells, oligosaccharides, heat shock proteins, and nucleic acid fragments. As a result, TLRs activate the transcription factors that regulate the expression of proinflammatory cytokines in several cell types and tissues. In skeletal muscle, TLRs act as a sentinel to monitor for the presence of pathogens and, upon activation, induce a local inflammatory response culminating in the translocation of NF-kB to the nucleus and activation of inflammatory genes, including TNF-α, IL-1ß, and IL-6 (55,56).

In this study we hypothesize that CKD patients have abnormal function of TLRs in muscle and that TLRs may be involved in initiating events associated with the stimulation of muscle pro-inflammatory cytokine transcription. We tested this postulate by different measures. First, we studied TLR gene and protein expression in muscle biopsies of patients with CKD and compared the results to those obtained in subjects with normal renal function. As a second step, to identify specific TLR-linked transcriptional pathways we studied the expression profiles of selected TLR4 downward genes and molecules in uremic muscle. As a third step, we studied the clinical correlations associated with muscle TLR4 expression and we observed that TLR4 protein expression

is inversely related to residual renal function, suggesting that more advanced uremic state activates muscle TLR4. Additionally, we studied the response to uremic serum of C2C12 myotubes and we observed that uremic serum up-regulates TLR4 and TNF-α expression and down-regulates pAkt. Such effects are prevented by TLR4 inhibitors or TLR4 knockdown. Overall, our data demonstrate the activation of TLR4 and its downward inflammatory cascade in muscle of subjects with CKD and suggest that enhanced TLR4 signalling contributes to the up-regulation of native immunity in skeletal muscle in uremia.

Methods

Study participants

We recruited 29 nondiabetic CKD patients (18 M/11 F) scheduled for peritoneal dialysis catheter insertion at the Nephrology Division, Department of Internal Medicine, University of Genoa that meet inclusion criteria for enrolment in this protocol. Characteristics of patients are summarised in table 1. The study was part of a protocol on the effects of peritoneal dialysis on protein turnover approved by the Ethical Committee of the Department of Internal Medicine of the University of Genoa. All subjects were informed about the nature, purposes, procedures, and possible risks of the study, before their informed consents were obtained. The procedures were in accordance with the Helsinki declaration regarding ethics of human research.. The patients were enrolled in the study on a consecutive basis. Exclusion criteria were the following: patients age under 18 an over 85 years old; congestive heart failure defined as New York Heart Association Class III–IV, a recent (<12 months) myocardial infarction, liver cirrhosis, infection, or diabetic nephropathy. Clinical and biochemical characteristics of the subjects are shown in Table 1. The two groups were matched for age and gender. All subjects had a sedentary life style. Their mean age was 67 years (range 43–82 years). Their estimated GFR was 8 ± 1 mL/min 1.73m2 (range 4–14 mL/min). Estimated GFR was calculated using CKD-EPI equation. Causes of renal diseases were hypertensive nephrosclerosis (11 patients), chronic glomerulonephritis (8 patients), polycystic kidney disease (6 patients),

tubulointerstitial nephritis (3 patients), and obstructive uropathy (1 patient). Their mean estimated protein and calorie intake were 0.9 g/kg and 27 kcal/kg, respectively. We used the 7-point Subjective Global Assessment (SGA) to assess malnutritional status (57,58). In this scale, an overall SGA classification of 1–7 is assigned; a score of 7 indicates a normal nutritional status and a score of 1 indicates severe protein-energy wasting. Albumin levels were low (<3.8 g/100 mL) in 14 subjects, while BMI was low (<23 kg/m2) in six subjects. The evidence of an inflammatory response (CRP >5 mg/L) was shown in 11 subjects. Mean muscle fibre cross-sectional area (CSA) was lower in patients with CKD (median = 976 μm2, range 745–1615; controls median = 1422 μm2, range 1100–1974) ($P<0.05$), suggesting muscle atrophy. We used HOMA index (homeostasis model assessment) to determine insulin resistance. CKD subjects were more insulin resistant than control subjects, based on a higher HOMA index and plasma insulin concentration ($P<0.05$). As a control, we used a sample of rectum abdominis muscle from 14 patients without chronic illness and acute inflammatory process, who underwent elective surgery for abdominal wall hernias. Obviously, these patients had normal baseline renal function (eGFR 99 ± 6, range 78–120 mL/min) as well as normal biochemical tests of renal, hepatic, haematological, and metabolic function (thyroid function and fasting plasma glucose).

Muscle biopsies

Muscle biopsies were obtained from rectus abdominis muscle, at the beginning of surgery. Tissue (90 mg) RNA was isolated using the Qiazol Lysis reagent (Qiagen

Sciences, Maryland, USA). Isolated RNA was stored at 80 °C until use. Other sample aliquots were used for immunohistochemical staining, and for protein (western blot) analysis. For the study of fat infiltration, tissue samples were stained with Oil-red-O, which detect lipids, mainly neutral fats and cholesteryl esters (Sigma-Aldrich, Milan, Italy) (31). Digital images of immunohistochemical and Oil-red-O stained sections were obtained using a Leica microscope (Leica Microsystems GmbH Wetzlar, Germany) equipped with a digital camera controlled by Q500MC Software-Qwin (Leica).12

Tissue analyses

mRNA analysis RNA concentration and integrity of each sample were evaluated on a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). In all, 1 mg RNA was used for cDNA synthesis with Improm II reverse transcriptase (Promega, Madison, WI). PCR amplification was performed in a 20 ml volume, including specific PCR primers and a TaqMan FAM dye-labeled MGB probe (Hs00174131_m1 (IL-6); Hs00193363_m1 (myostatin); Hs01547656_m1 (IGF-I); Hs99999903_m1 (b-actin,). Primers and probes were synthesized by Applied Biosystems Applera Italia (Monza, Italia). Assays were run in triplicate with Universal PCR Master Mix on MasterCycler realplex (Eppendorf, Hamburg, Germany) PCR system. To quantify target mRNA abundance, differences in threshold cycles between the gene target and b-actin were calculated, and then relative mRNA abundance was calculated using the 2DDCt method.

Western blot: Tissues were homogenized and lysed in lysis buffer (20 mmol/l HEPES, 150 mmol/l NaCl, 10% (v/v) glycerol, 0.5% (v/v) NP40 (Nonidet-P40), 1 mmol/l EDTA (ethylenediaminetetraacetic acid), 2.5 mmol/l DTT (dithiothreitol), 10 mg/l aprotinin, leupeptin, pepstatin A, 1 mmol/l PMSF (phenylmethanesulfonyl fluoride), and Na3VO4 (Sigma-Aldrich S.r.L, Milan, Italy). Protein concentration was determined by Coomassie protein reagent (Pierce, Rockford, IL) and 50 mg was resolved on SDS-polyacrylamide gels and electrotransferred into a nitrocellulose membrane (GE Healthcare, Buckinghamshire, England). Blots were blocked for 1 h at room temperature in phosphate-buffered saline (5% non-fat dry milk) and then probed using anti-human phospho-Akt 1/2/3 (Thr 308) polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 1C. Membranes were incubated for 1 h in horseradish peroxidase secondary antibodies (Santa Cruz Biotechnology). Immunoblots were developed with Immobilon western chemiluminescent horseradish peroxidase substrate (Millipore, Billerica, MA). The membranes were stripped and reprobed with polyclonal Akt 1/2/3 (Santa Cruz Biotechnology) for normalization of the loading. Band intensities were determined using IAS 2000 software from Delta Sistemi (Latina, Italy).

Histological preparation and immunohistochemical staining: Paraffin sections (5 mm) of 2% paraformaldehyde-fixed muscle were deparaffinized, hydrated, and treated with 3% H2O2 in methanol. Each sample was analyzed for the detection of apoptosis and expression of p-Akt, (monoclonal antibodies, Santa Cruz Biotechnology), IL-6 (polyclonal antibodies, Santa Cruz Biotechnology), myostatin (polyclonal antibody; Novus Biologicals, Littleton, CO), IGF-I (clone Sm 1.2, mouse monoclonal antibody;

Upstate, Lake Placid, NY) (Dako, Glostrup, Denmark). Sections were incubated overnight in primary antibody at room temperature, followed by incubation in biotinylated antibody for 30 min. The expressions of p-Akt, myostatin, and IL-6 were examined by image analysis and expressed as positive areas (Leica Q500 MC Image Analysis System, Leica, Cambridge, UK).

Cell culture and treatments

Mouse skeletal muscle cell line C2C12 was propagated as myoblasts in DMEM (Euroclone, Milan, Italy) containing 2mmol L-glutamine and 100 U/mL penicillin-streptomycin (Euroclone), with 5% FBS and incubated at 37 °C. For differentiation into myotubes, the myoblasts at 90% confluence were incubated with DMEM plus 2% horse serum (Sigma Chemical Co, St. Louis, MO, U.S.A.). The myotubes began to form in 2–4 days, and multinucleated muscle fibre cultures were used at 7 days. For evaluating TLR 4 induction, myotubes were incubated with 10% pooled human normal serum or pooled human uremic serum.

Experimental conditions

C2C12 mouse myotubes were incubated in the presence of uremic serum or normal serum for 2, 4, and 6 h to assess TLR4 and PKC expression. TLRs expression was

studied by western blot. In selected experiments, TLR4 and PKC receptor antagonists were added to the cells 1 h before uremic serum stimulation. C2C12 were blocked for 2 h before uremic serum treatment with 30 μM VIPER (viral inhibitory peptide of TLR4) or CP7 (inert control peptide, designed as negative control for inhibitory assay). Cells were exposed to uremic serum for 5 h and then total RNA was extracted using TRIzol, reverse transcribed, and TNF-α mRNA expression was analysed by real time PCR, as above described.

Uremic serum

Uremic serum was collected from patients with ESRD on hemodialysis recruited randomly from a pool of 150 patients at the Nephrology Division at the Genoa University. Patients were recruited over a 2-month period. Informed consent was obtained, and 3mL of blood was collected before the next subsequent hemodialysis. Healthy age-matched donors were used as controls. Blood urea nitrogen, creatinine, and glucose were assayed in all patients, and control sera were excluded if creatinine was >1.0 mg/dL. Exclusion criteria were presence of inflammatory disease, acute or chronic infection, autoimmune or liver diseases, diabetes, and malignancy. None of the patients or controls smoked. Blood was collected in Vacutainer tubes, and serum was separated by centrifuging clotted blood at 1100 g for 10 min at room temperature to obtain serum. In order to minimize minor differences between patients, all serum samples were pooled for the experiments.

Serum samples were frozen at 20 °C until analysis. In preliminary experiments, 10% was found to be the highest nontoxic concentration of uremic serum.

Systemic inflammatory marker detection in uremic serum

Cytokine levels in uremic serum are shown in Table 3. Serum CRP, osteoprotegerin (OPG), TNF-a, CCL2, adiponectin, leptin, resistin, IL-6, soluble IL-6 receptor (s-IL-6R), and osteopontin (OPN) complex levels were measured by colorimetric enzyme-linked immunosorbent assay (ELISA) from R&D Systems (Minneapolis, Minnesota, USA), following manufacturer's instructions. Mean intra- and inter-assay coefficients of variation (CV) were below 8% for all markers. Serum lipopolysaccharide quantification was performed using a Limulus Amebocyte assay (Cambrex, Verviers, Belgium).

Block of Toll-like receptor 4 by viral inhibitory peptide of Toll-like receptor 4

C2C12 were blocked, for 2 h before uremic serum treatment, with 30 μM VIPER (viral inhibitory peptide of TLR4) or CP7 (inert control peptide, designed as negative control for inhibitory assay) (Imgenex, San Diego, CA). Cells were exposed to uremic serum for 5 h and then total RNA was extracted using TRIzol, reverse transcribed, and TNF-α mRNA expression was analysed by real time PCR, as above described.

RNA interference

C2C12 were transfected with 60 nM TLR4 specific siRNA or negative control siRNA (Ambion, Carlsbad, CA) using Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol and then incubated at 37 °C in a CO2 incubator for 24 h until the cells were ready for assay. The efficacy of knockdown was determined by real-time PCR.

Statistical analysis

All data are presented as the mean ± standard error of the mean or median (range). Specific mRNAs were normalized for the internal control gene (β-actin) and are expressed as transcript/housekeeping gene ratios. The control treated group mean was given a value of 100, and individual values are expressed relative to this value. Statistical analysis was performed by using the SPSS statistical package (version 16; SPSS, Chicago, IL). For statistical analysis of expression variables that did not have a Gaussian distribution, values were logarithmically transformed or analysed by nonparametrical tests. Comparisons between groups were performed by one-way ANOVA with a post hoc Bonferroni correction or by a Kruskal–Wallis nonparametric test when appropriate. A multivariate regression model was created to study the predictors of TLR4 content in muscle. Statistical significance occurred if a computed two-tailed $P<0.05$.

Results

Elevated Toll-like receptor 4 gene expression and protein content in muscle of patients with chronic kidney disease

TLR2, TLR3, and TLR4 mRNA and protein levels in muscle of controls and CKD patients are displayed in Figures 1 and 2. We didn't observed significant differences in TLR2 and TLR3 levels (Figure 1), while dwTLR4 mRNA was significantly overexpressed (by approximately two-fold; $P<0.05$) in muscle of CKD subjects compared with controls (Figure 2A). Consistent with the increases in TLR4 gene expression, CKD subjects had higher TLR4 muscle protein content than control subjects ($P<0.05$–0.001) as shown by immunohistochemistry and western blot (Figure 2B–D).

Toll-like receptor 4-driven signalling in skeletalmuscle of patients with chronic kidney disease

As a next step, we examined the downstream signalling expression of TLR4 in CKD subjects to understand if there are some abnormalities in TLR4 signalling. We first measured the abundance of NF-kB p65 (phosphorylated p65 subunit) (p-p65) and IkB-α (p-IkB-α) in muscle biopsies. Normal muscle showed p-p65 positive nuclear staining in a very small percentage ($1.7 \pm 0.6\%$) of muscle fibres (Figure 3A) while this percentage significantly rose to $17.4 \pm 2.5\%$ in patients with CKD (Figure 3A). Also, p-IkB-α and

TNF-α expressions were increased in samples from CKD patients (Figure 3B and 3C, respectively). P38, a second messenger of TNF-α in uremia, was analysed for RNA expression and localisation. Phosphorylated p38 (p38p) expression was present in the normal muscle at minimal levels. Conversely, p38p was up-regulated in CKD patients (Figure 3D). To understand if different muscle fat infiltration could explain difference in cytokine expression in CKD, we studied Oil-red-O expression (see methods) in muscle biopsies of 10 CKD and 7 control subjects. Muscle fat infiltration was low and similar in CKD patients and control subjects (mean score 0.012 ± 0.01 and 0.014 ± 0.02, P =NS, respectively, in CKD patients and controls). Finally, we studied MuRF-1, a muscle-restricted ubiquitin ligase involved in the accelerated protein degradation during various kinds of muscle atrophy; his role is crucial in the TNF-α/p38 pathway. MuRF-1 was highly upregulated in muscles from CKD subjects. In addition, also atrogin-1, another muscle-specific ubiquitin ligase triggering muscle atrophy, was up-regulated (Figure 3E). Collectively, these results indicate that CKD subjects have increased
TLR4 expression/content and TLR4-driven signalling.

Clinical determinants of Toll-like receptor 4 expression in muscle

To understand the clinical involvement of this findings, we evaluated the correlation between TLR4 expression in muscle and some relevant clinical parameters. The associations between individual clinical data and logTLR4 content in skeletal muscle are showed in Table 4. LogTLR4 content in muscle was inversely related to eGFR, SGA,

and haemoglobin levels, while it was directly related to cholesterol levels. Muscle LogTLR4 content was related to serum CRP only as a trend but not reached statistical significance ($P<0.06$). There was no relationship between muscle logTLR4 and age, weight, fat and fat-free mass, cross-sectional muscle area, nPNA, estimated calorie intake, serum albumin, BUN, glucose, bicarbonate, triglycerides, phosphate, fibrinogen, and iron levels. Using the studied variables for inclusion into multivariate analysis models revealed SGA and eGFR only to contribute individually and significantly to the prediction of TLR4 expression in skeletal muscle ($R2=0.53$, $P<0.001$).

Effects of uremic serum in promoting Toll-like receptor 4 expression, p38, and AKT activation in C2C12 myotubes

To explore the ability of uremic serum to induce TLR4 expression and drive his signalling cascade, we incubated C2C12 myotubes with 10% uremic serum for 0–6 h. Uremic serum induced a four-fold increase in TLR4 mRNA after 2 h, an effect which persisted until 6 h. Also, TLR4 protein was up-regulated at 6 h respect to cells exposed to 10% normal serum (Figure 4A). In addition, uremic serum induced TNF-α mRNA expression (Figure 4B). Then, we investigated the effects of uremic serum on AKT and p38 phosphorylation. When C2C12 were exposed to uremic serum for 24 h, AKT phosphorylation was reduced by 40% with respect to cells treated with normal serum (Figure 4C). Moreover, uremic serum up-regulated p38 MAPK phosphorylation within 10min (Figure 4D), an effect that persisted for 240 min.

P38 mitogen-activated protein kinase/protein kinase C is involved in uremic serum-induced Toll-like receptor 4 expression

To further investigate the role of p38 MAPK phosphorylation on uremic serum-induced TLR4 expression, we used p38 MAPK and PKC inhibitors to examine the role of p38 and its related signal pathway including protein kinase C (PKC). Pre-treatment of C2C12 myotubes with the p38 inhibitor SB203580 (10μM) or the PKC inhibitors chelerythrine (5μM), or staurosporine (0.2–0.4μM), 1 h before serum exposure resulted in a marked decrease in the serum induced TLR4 mRNA overexpression (Figure 5). These findings show the implication of the p38MAPK/PKC pathway in TLR4 activation by uremia.

Toll-like receptor 4 mediates the effect of uremic serum on Akt signalling

To evaluate whether the effects of uremic serum on the activation of TLR4 can be prevented by inhibiting TLR4 signalling, we pre-incubated the myotubes with VIPER, a specific TLR4 inhibitor, which acts by directly targeting the TLR4 adaptors Mal and TRAM, thus inhibiting TLR4-mediated responses. As shown in Figure 6A, preincubation of myotubes with VIPER prevented the ability of uremic serum to up-regulate TNF-α.
As a next step, we employed gene silencing as an independent, albeit complementary method to examine the role of uremic serum on TLR4 regulation in muscle. TLR4 siRNA

decreased TLR4 mRNA and protein in C2C12 myotubes (Figure 6B). TLR4 gene silencing reduced uremic serum-induced TNF-α (Figure 6C) and recovered AKT signalling (Figure 6D).

Discussion

This study addressed several issues of muscle pathophysiology in CKD patients. We examined the role of inflammation in CKD related muscle wasting. In a first phase, we tested the hypothesis that TLRs drive the inflammatory process. We found that TLR4 is overexpressed in muscle of patients with CKD compared to controls and that his downstream effector NF-kB is activated during this process. Of note, TLR4 was the only TLR tested that increased his expression in CKD patients. NF-kB is a well-recognized downward TLR4 transcription factor that directs the production of TNF-α and pro-inflammatory cytokines which are major mediators of protein breakdown and atrophy in the skeletal muscle (59,60). In a previous study, TNF-α gene was found to be upregulated in muscle of patients with CKD (40). TNF-α acts binding two different receptors, TNFR1 and TNFR2 but only the former seems to be implicated in muscle wasting (61). Three different mechanisms are involved: a first pathway stimulates apoptosis via interaction with the TNF-α-receptor complex and the Fas-associated protein with death domain; a second pathway activates Jun-N-terminal kinases (JNK) and the transcription factor AP-1; a third pathway activates NF-κB. We already demonstrated the ability of TNF-α to activate JNK (40), while other studies addressed the implication of TNF-α induced translocation of NF-kB in the nucleus of skeletal muscle cells (62-64). NF-kB could in turn activates TNFα expression, leading to a positive feedback loop which potentiates muscle abnormalities (59). TNF-α activates mitogen-activated protein kinases (MAPKs), including p38 and JNK (60) to increase protein breakdown by atrogin1/MAFbx gene

expression in skeletal muscle (65). In this study we confirmed that p38, a second messenger of TNFα (66,67), is upregulated in muscle of CKD patients, coherently with other observation in several other catabolic conditions, such as ageing (68) type 2 diabetes (69) limb immobilization (70) and neurogenic atrophy (71) suggesting that the TNF-α/p38 MAPK driven signalling takes place as a common mechanism in different catabolic conditions.

A second observation in our study is the downregulation of pAkt in muscle of CKD patients. pAkt acts as an anabolic signal that promotes muscle trophism and growth. Downregulation of pAkt implies a shift toward catabolic process, with the activation of FoxO transcription factors that regulate expression of MurF-1 and Atrogin. During atrophy, MuRF-1 and MAFbx direct the polyubiquitination of proteins to target them for proteolysis by the 26S proteasome, mediating muscle breakdown. Analysing the levels of MurF-1 and MAFbx we found elevated level in muscle of CKD patients, confirming the activation of this pathway.

A further step of this study was correlate muscular finding with clinical data. We found that higher TLR4 levels were independently related to lower SGA score and GFR. SGA is a nutritional assessment scale, ranging from 1 (severe malnutrition) to 7 (normal nutritional status), that has been validated as reliable tool to assess protein-energy wasting in several conditions, including dialysis dependent CKD patients (72) in which he has been associated with morbidity, hospitalization, and risk of short-term mortality. Accordingly to our data, patients with lower residual renal function and with a worse nutritional status expressed a higher level of TLR4 and thereby a higher degree of

inflammation. TLR4 content in muscle rose progressively along with progressive decline of residual renal function, with a two-fold increase in TLR4 as eGFR declined from 13 to 4 mL/min. Of note, fat free mass was not associated with TLR4. This finding suggests that TLR4 up-regulation is a part of the stress response that takes place in overt uremia. While metabolic acidosis exerts its effects in already in stage 4 CKD (73), TLR4 pathway is stimulated in stage 5 CKD, suggesting that these two processes are part of the early and late muscle stress response, respectively.

The further step of this study was to test if uremic serum is able to induce a proinflammatory response directly in muscular cells. We exposed C2C12 mice myotubes to uremic serum and we observed a rapid increase in TLR4 and TNF-α mRNA expression along with a decrease in pAkt levels. Blockade of TLR4, both with a specific inhibitor or silencing his gene, prevented the increase in TNF-α and TLR4 expression and the decrease of pAkt. This means that uremic serum has the ability to induce a proinflammatory response both in vitro and in vivo that potentially could lead to decreased insulin signal (via GH/IGF-1 pathway) and consequently to atrophy.

At the moment, we don't know exactly how uremic serum is able to induce activation of TLR4. TLR4 is activated by many different stimuli, both exogenous (like bacterial and viral peptides) and endogenous (collectively named DAMPs or damage associated molecular patterns). TLR4 is considered a cornerstone in innate immunity to discriminate between self and non-self and to activate self defence mechanisms in response to an injury, even in absence of an external stimuli. More than twenty putative

ligands were proposed, like various extracellular and membrane components (74), heat shock proteins (75), HMGB1 (76) or fibrinogen (77).

In uremia, many potential activators were proposed. One possibility is that alterations in calcium and/or phosphate metabolism, which increase the risk for vascular calcification, may act as a yet another nidus for a local inflammatory response (78,79) but we found no relations between TLR4 expression and PTH, serum phosphate and calcium. Other potential ligands are angiotensin II (80), AGEs, free fatty acid, uric acid (81) that all show the ability to activate innate TLRs and innate immunity. In our study, no diabetic patients were enrolled and thereby we cannot confirm this finding.

More recently, other potential mechanisms have been proposed. One interesting theory involve alteration in gut microbiota and intestinal permeability due to uremia that in turn could allow endotoxin (or LPS) to enter systemic circulation. LPS is part of the outer membrane of Gram-negative bacteria and his entrance in systemic circulation drives an inflammatory response mediated by innate immunity (83). In patients studied here the level of circulating endotoxins was borderline high, suggesting that circulating endotoxins might be responsible for the changes in TLR4 muscle expression and inflammatory changes.

In conclusion, this study provides some evidence that TLR4 is upregulated in skeletal muscle cells of patients with CKD and his activation mediates uremic cachexia toward a downregulation of anabolic signals like and an upregulation of catabolic pathways. We also found that uremic serum could promote innate immunity but we need to further understand the identity of all the ligands involved in this setting. Given his

involvement in promote inflammation and muscular atrophy, TLR4 have the potential to be a therapeutic target in CKD related cachexia.

Table 1

Characteristics of patients

	Controls	CKD subjects
Number of subjects	14	29
Age (years)	64 ± 5	67 ± 2
Gender (M/F)	9/5	18/11
BMI (kg/m^2)	25 ± 1	25 ± 1
FFM (kg)	46 ± 2	43 ± 3
Fat mass (kg)	25 ± 1	26 ± 2
nPNA (g/kg)	1.0 ± 0.1	0.93 ± 0.1
SGA score	7 (6–7)	5 (2–7)
CRP (mg/L)	3 (2–4)	8 (2–27)[a]
Estimated GFR ($mL/min.1.73\ m^2$)	99 ± 6	8 ± 0.7[b]
[HCO_3] (mmol/L)	24 ± 1	23 ± 1
Albumin (g/dL)	4.2 ± 0.1	4.0 ± 0.2
Haemoglobin (g/dL)	13 ± 1	11.3 ± 0.2[a]
BUN (mg/dL)	17 ± 2	90 ± 4[b]
Calcium (mg/dL)	9.5 ± 0.3	8.5 ± 1
Phosphate (mg/dL)	4.1 ± 0.1	5.9 ± 0.4[b]
iPTH (pg/mL)	na	408 ± 22[b]

Data are mean±SEM or median (range). Abbreviations: BMI, body mass index; CRP, C-reactive protein; FFM, fat-free mass; GFR, glomerular filtration rate; na, not available; nPNA, normalized protein nitrogen appearance; SGA, subjective global assessment. Significance of difference vs. control subjects: a=P<0.05; b=P<0.01.

Table 2

Primer

Primers	Forward	Reverse
TLR4 (human)	AGCCACGCATTCACAGGG	CATGGCTGGGATCAGAGT CC
TNF-α (Human)	AGGTTCTCTTCCTCTCACAT	ATCATGTTTCAGTGCTCATG
β-Actin (Human)	CATCCCCCAAAGTTCACAAT	AGTGGGGTGGCTTTTAGGAT
Atrogin1/MAFbx (human)	TTTCCTGGAAGGGCACTGAC	ACGACTGACCTCTCGACCCTTAT
MURF1 (human)	GCCACCTTCCTCTTGACTG	ATTCTTCCTCTTCATCTGTC
TLR4 (mouse)	AGCTTCTCCAATTTTTCAGAACTTC	TGAGAGGTGGTGTAAGCCATGC
TNF-α (mouse)	CACGCTCTTCTGTCT ACTGA	GGACTCCGTGATGTCTAAGT
GAPDH (mouse)	CATGGCCTTCCGTGTTCCTA	GCGGCACGTCAGAT CA

Primer for RT-PCR analysis

Table 3

Cytokines levels

	Control serum	Uremic serum
TNF-α (pg/mL)	9.7	15.6
CRP (mg/L)	0.73	11.9
s-IL6 receptor (ng/mL)	32.1	56.2
IL-6 (pg/mL)	9.0	52.0
Endotoxin EU/mL	0.6	1.0
MCP1 (pg/mL)	52.2	67.3
Adiponectin (microg/mL)	3.1	9.1
Leptin (ng/mL)	10.7	43.1
OPN (ng/mL)	46.2	126.5
Resistin (ng/mL)	15.9	36.3
OPG (pg/mL)	920	5443

Cytokines levels in normal and uremic serum

Abbreviations: CRP, C-reactive protein; IL6, interleukin-6; MCP1, monocyte chemotactic protein 1; OPG, osteoprotegerin; OPN, osteopontin; TNF-α, tumour necrosis factor α.

Table 4

Clinical characteristics

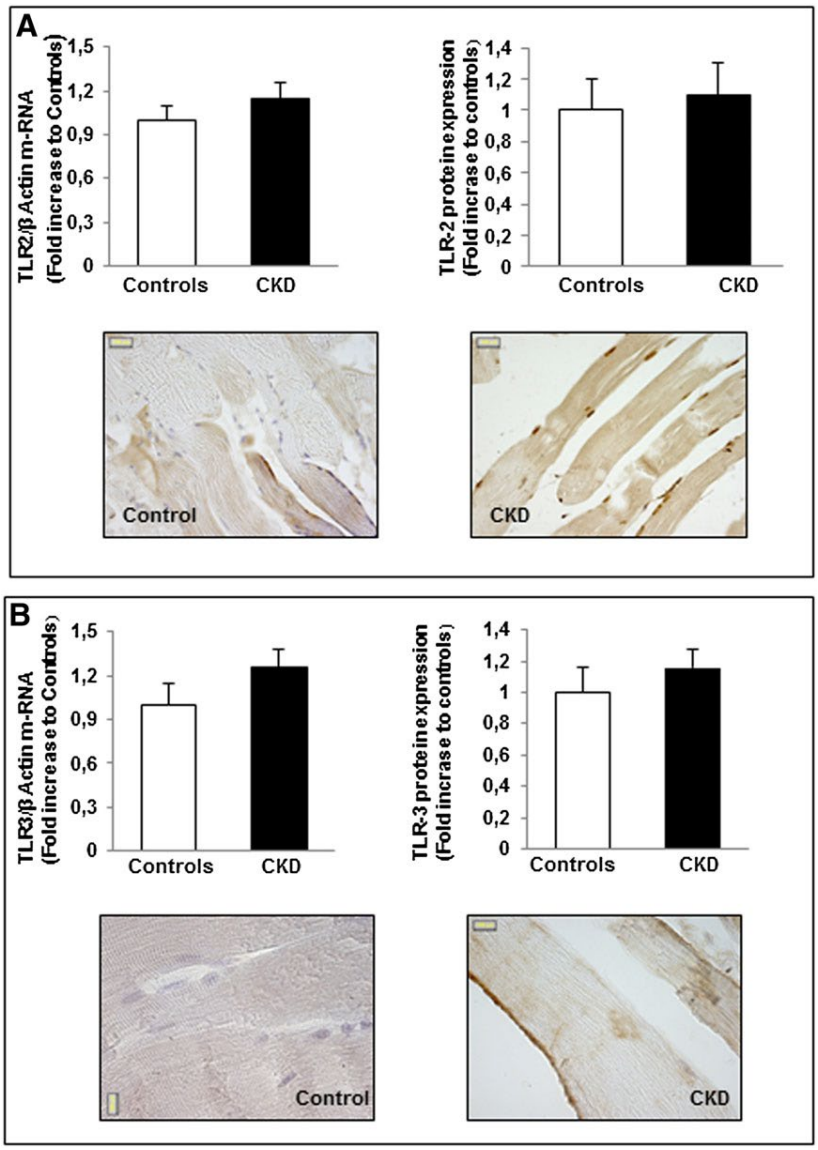
Clinical characteristics	Univariate		Multivariate	
	r	*P*	*t*	*P*
			(model $r^2 = 0.53$; $P = 0.001$)	
Age (years)	−0.043	NS		
Body weight (kg)	−0.310	NS		
Fat mass (kg)	−0.259	NS		
Fat-free mass (kg)	−0.339	NS		
BMI (kg/m^2)	0.022	NS		
SGA	−0.49	0.01	4.205	0.01
Estimated GFR ($mL/min.1.73\ m^2$)	−0.44	0.03	3.902	0.03
BUN (mg/dL)	0.025	NS		
nPNA (g/kg)	−0.232	NS		
Serum albumin (g/dL)	0.220	NS		
LogCRP (mg/L)	0.354	0.06		
Haemoglobin (g/dL)	−0.380	0.05	0.039	NS
Cholesterol (mg/dL)	0.398	0.05	1.073	NS
Triglycerides (mg/dL)	0.102	NS		
Bicarbonate (mmol/L)	−0.181	NS		
Urate (mg/dL)	0.043	NS		
Calcium (mg/dL)	0.125	NS		
Phosphate (mg/dL)	0.037	NS		
PTH (pg/mL)	0.145	NS		
Fibrinogen (mg/dL)	−0.123	NS		
Glucose (mg/dL)	0.180	NS		
Iron (mg/dL)	0.220	NS		

Univariate and multivariate analysis of the correlation between LogTLR4 protein expression and clinical characteristics in patients with CKD (n = 29)

Abbreviations: BMI, body mass index; CRP, C-reactive protein; GFR, glomerular filtration rate; nPNA, normalized nitrogen protein appearance; SGA, subjective global assessment.

Figure 1

Expression of TLR2 and TLR3

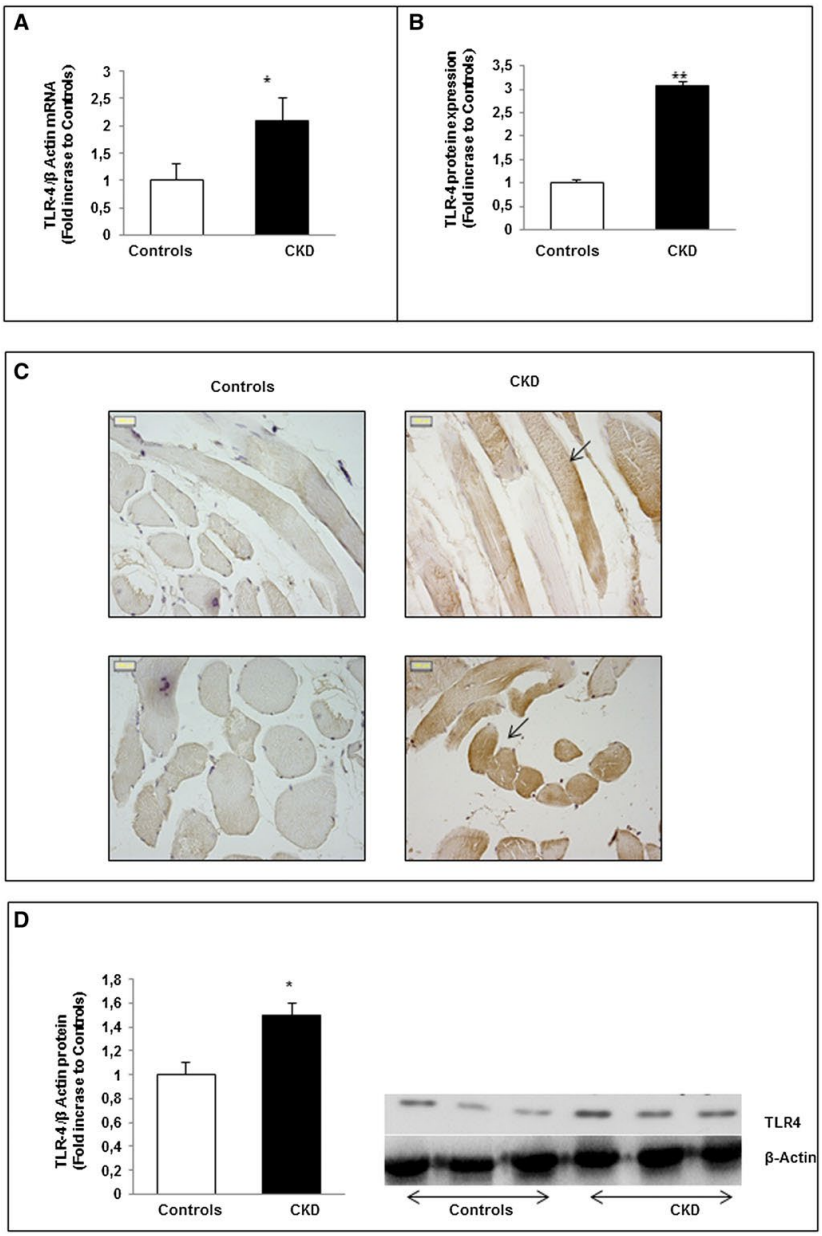


Expression of TLR2 (a) and TLR3 (b) mRNAs and proteins in skeletal muscle of controls (n 14) and patients with chronic kidney disease (CKD) (n = 29). TLR2 and TLR3 mRNA expression was determined by real-time PCR and their protein expression by immunohistochemistry and image analysis. Values are expressed as fold increase ± SEM

to the control muscle. TLR2 and TLR3 mRNAs and proteins were unchanged with respect to control subjects (P = NS). CKD = chronic kidney disease. (Magnification: ×400–1000).

Figure 2

Expression of TLR4

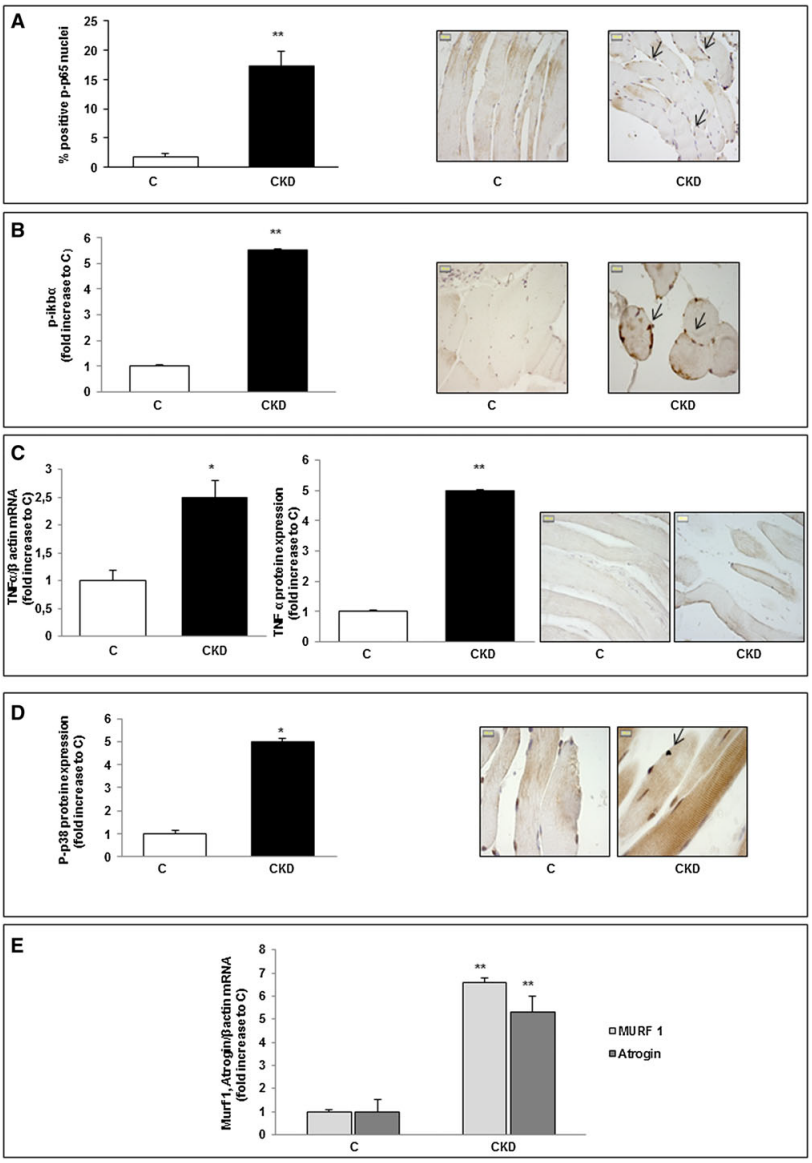


Expression of TLR4 mRNA (a) and protein (b–d) in normal skeletal muscle (n = 14) and in patients chronic kidney disease (CKD) (n = 29). TLR4 mRNA was evaluated by real-

time PCR, and its protein by immunohistochemistry followed by image analysis (b, c) and western blot (d) of muscle lysates. Values are expressed as fold increase ± SEM to the control muscle. TLR4 mRNA was approximately two-folds increased vs. controls. TLR4 protein was absent or very faintly expressed in the normal muscle, while was overexpressed (by 1.5–3-folds) in CKD muscle (panel C). Western blots show upregulated TLR4 in CKD with respect to controls (panel d). Blots were stripped and reprobed with anti β-actin antibody. The gel is representative of 12 CKD and 5 controls. CKD = chronic kidney disease. (Magnification: ×400–1000). The arrows indicate positive cells. *P<0.05, **P<0.001 vs. controls.

Figure 3

Expression of p-p65



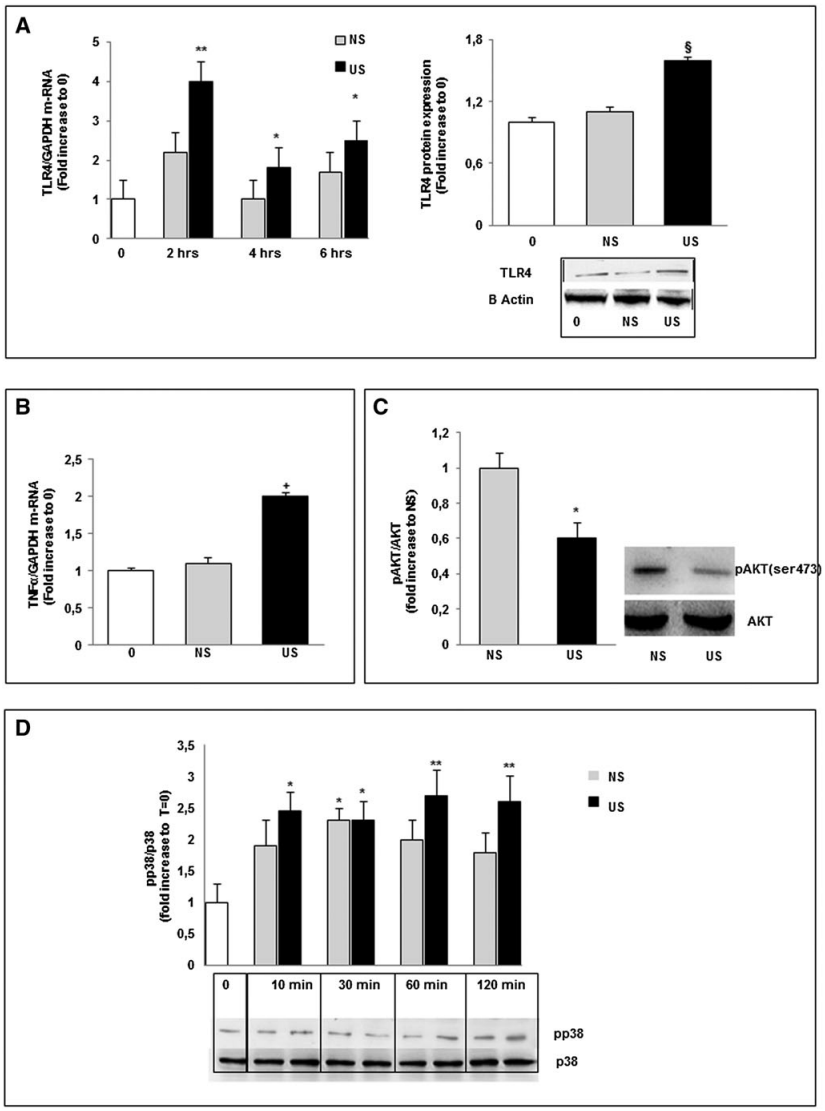
(Panel a). Phospho-p65 (P-p65) expression in the skeletal muscle of CKD patients and controls. Control (n = 11) muscle showed p-p65 positive nuclei in a very small percentage. This percentage increased significantly in patients with CKD (n = 20). The

degree of positive nuclei was estimated by counting the number of p-p65 positive cells for 100 cells examined in average of five high-power fields. (Panel b) Phospho-IkB-α (p-IkB-α) expression in the skeletal muscle of CKD patients and controls. P-IkB-α was highly up-regulated in muscle of patients with CKD.(Panel c) Expression of TNF-α mRNA and protein in CKD (n = 25) and control (n = 11) muscle. TNF-α m-RNA was two-folds overexpressed in CKD samples with respect to the control tissue. The protein expression of TNF-α was minimally detectable in control samples, while it was markedly increased (by approximately five-folds) in muscle

of CKD patients. (Panel d) Immunohistochemistry analysis for p-p38 in normal and CKD subjects. Staining was weakly diffused in normal tissue, but intensely expressed in uremia. (Panel e) Expression of Murf 1 and atrogin mRNA. Murf 1 and atrogin mRNAs expression level was determined by real time PCR. Both genes were over expressed in CKD muscle (n = 12) with respect to controls (n = 10). C = controls; CKD = chronic kidney disease. (Magnification: ×400–1000). The arrows indicate positive areas. Data are expressed as fold increase ± SEM to normal muscle. *P<0.05 vs. C; **P<0.01 vs. C.

Figure 4

Effects of uremic serum



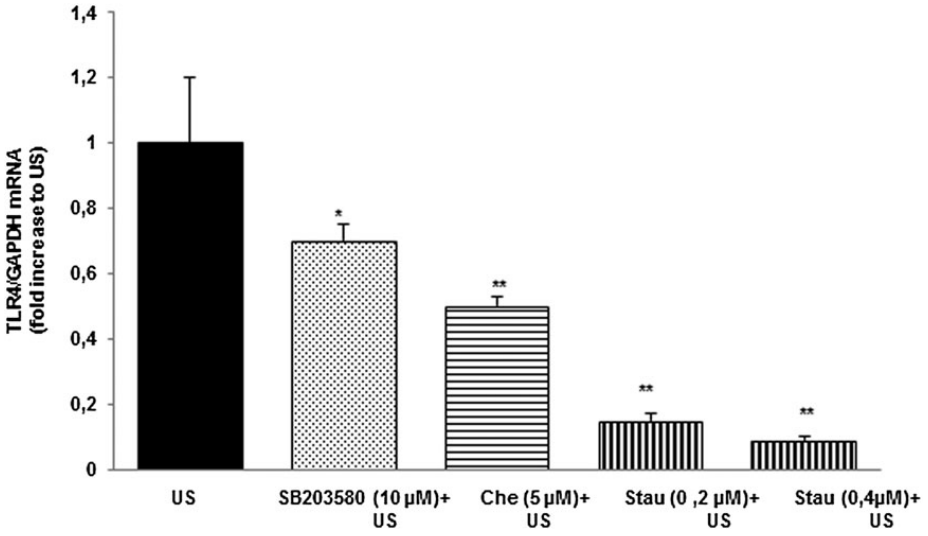
(Panel a). The effect of normal serum (NS) and uremic serum (US) on TLR4 mRNA and protein in C2C12 myotubes. Cells were incubated with 10% serum for 6 h. TLR4 mRNA expression was determined by real time PCR at different times and protein by western blot after six hours. (Panel b) The effect of uremic serum (US) on TNF-α gene expression

in C2C12 myotubes. TNF-α mRNA expression was determined by real time PCR after 5 h treatment. (Panel c) Down-regulation of pAkt in uremic serum (US)-treated cells. pAkt was

evaluated by western blot analysis after 24 h exposition to normal serum (NS) or US. Blots were stripped and reprobed with anti-pAkt antibody.(Panel d) Uremic serum (US) induces p-p38 during time course (0–240 min) in C2C12 myotubes. Blots were stripped and reprobed with anti β actin antibody. All results represent means ± SEM obtained from five independent experiments.*P<0.05 vs. T0; **P<0.01 vs. T0; §P<0.001 vs. T0 and NS; +P<0.01 vs. T0 and NS. NS = normal serum; US = uremic serum; pAkt = phospho-Akt; p-p38 = phospho-p38.

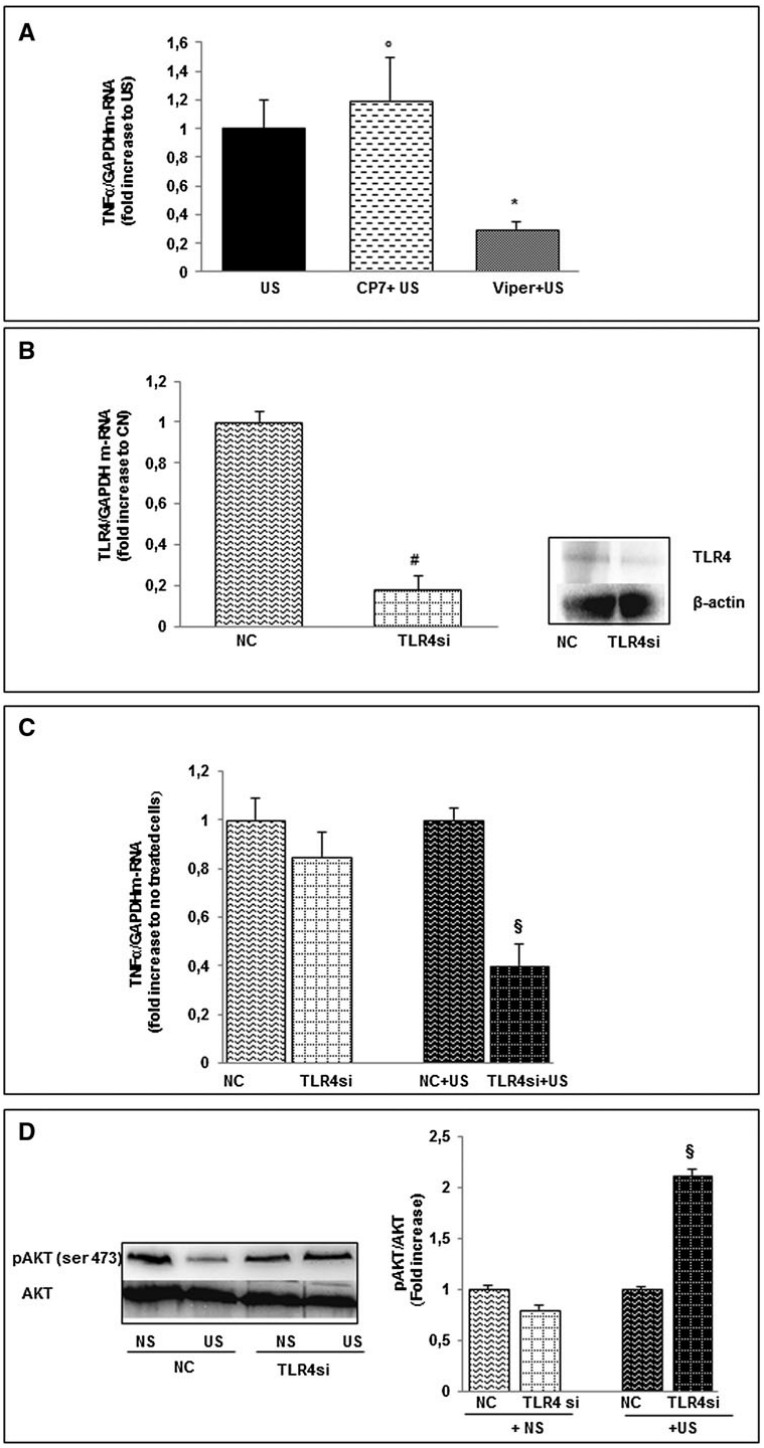
Figure 5

Inhibition of p38 and PKC



Effects of p38 inhibitor (10 µM SB203580), and PKC inhibitors (0.2–4 µM staurosporine or 10 µM chelerythrine) on uremic serum (US)-induced TLR4 mRNA. To further investigate the mechanism of uremic serum-induced TLR4 expression, we examined the role of p38 and its related signal pathway including protein kinase C (PKC). Pretreatment of C2C12 myotubes with the p38 inhibitor SB203580 (10 µM) and the PKC inhibitors chelerythrine (5 µM) and staurosporine (0.2–0.4 µM) 1 h before serum exposure, resulted in a marked decrease in the serum-induced TLR4 mRNA overexpression. All results represent means ± SEM obtained from three independent experiments.*P<0.05 vs. T0;.*P<0.05 vs. US; **P<0.01 vs. US. US = uremic serum; che = chelerythrine; stau = staurosporine.

Figure 6


A
TNFα/GAPDH m-RNA (fold increase to US)
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
°
*
US
CP7+ US
Viper+US
B
TLR4/GAPDH m-RNA (fold increase to CN)
1,2
1
0,8
0,6
0,4
0,2
0
#
NC
TLR4si
TLR4
β-actin
NC
TLR4si
C
TNFα/GAPDH m-RNA (fold increase to no treated cells)
1,2
1
0,8
0,6
0,4
0,2
0
§
NC
TLR4si
NC+US
TLR4si+US
D
pAKT (ser 473)
AKT
NS
US
NS
US
NC
TLR4si
pAKT/AKT (Fold increase)
2,5
2
1,5
1
0,5
0
§
NC
TLR4 si
NC
TLR4si
+ NS
+US

Effect of uremic serum (US) on TNF-α gene expression and pAkt in C2C12 myotubes treated with TLR4 inhibitors or silenced for TLR4. (Panel a) Preincubation of myotubes with VIPER, a specific TLR4 inhibitor, prevented the ability of US to up-regulate TNF-α. (Panel b) As a next step, we employed gene silencing as an independent method to examine the role of US on TLR4 regulation in muscle. C2C12 were transfected with 60 nM siRNA NC and TLR4-specific siRNA and the respective mRNA and protein were evaluated after 24 h. TLR4 siRNA decreased TLR4 mRNA and protein in C2C12 myotubes. (Panel c) Effect of US on TNF-α mRNA in C2C12 silenced for TLR4. TLR4 gene silencing blunted US-regulated TNF-α. (Panel d) Effect of US on Akt phosphorylation. C2C12 with no knockdown and transfected with TLR4 siRNA were exposed for 24 hours to NS or US. pAkt was evaluated

by western blot and membrane was stripped and reprobed with anti Akt antibody. TLR4 knockdown restored pAkt signalling. All results represent means ± SEM obtained from three independent experiments. #P<0.01 vs. NC; §P<0.01 vs. NC + US; °P<0.05 vs. US; *P<0.001 vs. US and CP + US. NS = normal serum; US = uremic serum; pAkt = phospho-Akt; NC = non-specific negative control siRNA; VIPER = viral inhibitory peptide of TLR4; CP7 = inert control peptide.

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