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Title: Evidence of functional deficits at the single muscle fiber level in experimentally-induced renal insufficiency

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ABSTRACT

Chronic kidney disease patients present with metabolic and functional muscle abnormalities, called uremic myopathy, whose mechanisms have not yet been fully elucidated. We investigated whether chronic renal insufficiency (CRI) affects skeletal muscle contractile properties at the cellular level. CRI was induced surgically in New Zealand rabbits (UREM), with sham-operation for controls (CON), and samples were collected at 3 months post-surgery, following euthanasia. All protocols had University Ethics approval following national and European guidelines. Sample treatments and evaluations were blinded. Maximal isometric force was assessed in 382 permeabilized psoas fibers (CON, n=142, UREM, n=240) initially at pH7, 10^{o}C ('standard' conditions), in subsets of fibers in acidic conditions (pH6.2, 10^{o}C) but also at near physiological temperature (pH7, 30^{o}C and pH6.2, 30^{o}C). CRI resulted in significant smaller average CSA (~11%) for UREM muscle fibers (vs CON, $P<0.01$). At standard conditions, UREM fibers produced lower absolute and specific forces (i.e. normalized force per fiber CSA) (vs CON, $P<0.01$); force increased in 30^{o}C for both groups ($P<0.01$), but the disparity between UREM and CON remained significant. Acidosis significantly reduced force (vs pH7, 10^{o}C $P<0.01$), similarly in both groups (in UREM by -48% and in CON by -43%, $P>0.05$). For the first time, we give evidence that CRI can induce significant impairments in single psoas muscle fibers force generation, only partially explained by fiber atrophy, thus affecting muscle mechanics at the cellular level.

Introduction

Chronic renal insufficiency (CRI) is a major global health problem expected to affect 40-50% of EU and USA populations (Grams et al., 2013; Zoccali et al., 2010) as well as millions in Asia (Abraham et al., 2016) and Latin America (Cusumano and González Bedat, 2008). Skeletal muscle is heavily compromised by CRI (Kaltsatou et al., 2015; Sakkas et al., 2003a, 2003b) with patients presenting with muscle atrophy (Kouidi et al., 1998), muscle weakness, limited endurance and fatigue intolerance (Campistol, 2002), only partly explained by inactivity (Sakkas et al., 2003b). Collectively described as *uremic myopathy* (Campistol, 2002), this muscular dysfunction spectrum contributes to patients' high cardiovascular and metabolic morbidity and mortality (Johansen et al., 2007; Pereira et al., 2015) and inhibits the efficacy of rehabilitative interventions.

Muscle atrophy occurs in various clinical conditions but also in health under sedentary/unloading or weightless conditions; its functional manifestations and the evidence of underlying mechanisms are influenced by the study model and the timeframe of sampling (Malavaki et al., 2015; Riley, 2005). In rat hindlimb suspension, within 1-4 days, a rapid loss of myofibrillar proteins (Munoz et al., 1993) is associated with force deterioration. However, after the acute phase, especially when disease is implicated, it is difficult to delineate whether force reduction may be solely due to a reduced fraction of contractile proteins within muscle fibers, or because the available contractile proteins are compromised, or both [for a discussion on 'muscle quality' see (Fragala et al., 2015)]. Moreover, apart from a differential time-course of signaling and phenotypic changes as the atrophy-inducing conditions continue (Malavaki et al., 2015), some ultrastructural and functional changes observed early on

[e.g. reduction in myofilament packing (Riley, 2005)], may not be evident at a later stage [e.g. in human space flight study (Fitts et al., 2010)].

Additionally, disease mechanisms may impact function and metabolic properties without obvious muscle atrophy. In a progressive renal failure animal model, fast and slow muscle dysfunction occurred without global changes in muscle mass or physiological cross-sectional area; still, individual fast fiber atrophy was observed (Organ et al., 2016). However, in another, surgical, CRI animal model no fiber atrophy was observed but oxidative capacity was affected (Acevedo et al., 2015). Additional factors such as neuropathy, mitochondrial dysfunction and substrate availability have been implicated in uremic myopathy [for a review refer to (Adams and Vaziri, 2006)], indicating that atrophy alone may not fully account for the observed muscle dysfunction in CRI.

Fatigue intolerance in CRI has been also attributed to the development of rapid acidosis (low intracellular pH) (Johansen et al., 2005; Moore et al., 1993). Low intracellular pH inhibits muscle contraction at the sarcomeric protein level due to an effect of H^+ both on the interaction between the motor proteins and on calcium handling [e.g. (Allen et al., 2008; Fabiato and Fabiato, 1978; Karatzaferi et al., 2008; Nelson and Fitts, 2014)]. However, the force depressing effect of acidosis declines in magnitude with increasing temperature towards physiological levels [e.g. (Karatzaferi et al., 2008; Pate et al., 1995; Westerblad et al., 1997)]. Thus far it is not known if the uremic muscle's response to acidic conditions is similar to that of a control muscle.

Overall, the mechanisms underlying muscle dysfunction in CRI constitute a difficult aspect to precisely evaluate and are not yet clear. Various interventions implemented so far, while beneficial (Gordon et al., 2007; Johansen et al., 2006; Sakkas et al., 2003b) have not fully corrected the functional deficits. Research so far

has been mostly performed at the end-stage renal disease and key issues related to CRI progression and contractile mechanisms are still unanswered. Moreover, it is not clear whether basic contractile properties are affected, whether whole muscle and/or individual fiber atrophy is present or not.

To answer such questions and to avoid confounding factors encountered in human patient studies (e.g. comorbidities, dialysis years, pharmaceuticals etc.), we employed a surgically induced animal model of CRI, maintained with a special diet (Gotloib et al., 1982). We used permeabilized (skinned) single muscle fibers to assess the contractile machinery *per se* independently of metabolic and neural factors *in vitro* (Cooke and Bialek, 1979), under variable conditions of pH and temperature (Karatzaferi et al., 2008, 2004). We studied fibers from psoas muscle which is characterized by its homogeneity in fast IIX (IId) fibers (Aigner et al., 1993; Hämäläinen and Pette, 1993) because the fast/glycolytic fibers, especially the most powerful ones expressing the IIX myosin heavy chain isoform, are more prone to atrophy in renal disease (Sakkas et al., 2003a; Sawant et al., 2011).

We aimed to evaluate, for the first time, the effects of CRI on the contractile properties of isolated single muscle fibers, focusing on the function of muscle fibers *per se* independently of possible acute neurological and metabolic abnormalities. We examined, in a blind design, whether CRI affected the cell's ability to produce maximal isometric force and whether the contractile 'response' to acute acidosis was altered, at an earlier stage of renal insufficiency than that of the available patient data. Moreover, considering the importance of temperature in translating our *in vitro* findings to *in vivo* function, we examined contractile properties both at the commonly employed temperature of 10^{o}C and at the closer to physiological temperature of 30^{o}C.

Methods

Animal care and experimentation procedures were approved by the Ethics Committee of the University of Thessaly (decision 2-1/10-10-2012) and the Scientific Committee of the University Hospital of Larissa, Greece (decision 1/4-1-2012). Animals were under veterinary care, according to national and EU directives (Directive 2010/63/EU).

Animal model

CRI was induced surgically in 6 (N=6) new Zealand young adult female rabbits (UREM group) using a surgical protocol modified from Gotloib et al. (Gotloib et al., 1982). Three age-matched animals underwent sham operation (CON group). To exclude the possibility that the special diet *per se* would affect muscle properties, both control and uremic animals consumed the special rabbit chow (see Supplement). Twelve weeks after surgery, animals were sacrificed by injection of sodium pentobarbital solution (50 mg/ml applied in a dosage of 100 mg/Kg BW) followed by bilateral thoracotomy. Immediately after cardiac arrest, blood samples were collected for subsequent determination of serum urea and creatinine using standard photometric protocols.

Muscle Samples

Psoas muscle samples from UREM and CON animals were rapidly excised and permeabilized as previously described (Karatzaferi et al., 2008) (see Supplement).

Experimental setup for single fiber mechanics

Single fibers were dissected from the muscle bundle under a stereomicroscope on a cold stage and the fiber ends were attached between two tissue mounts of a customized micro-dynamometer (SI Heidelberg/WPI). Data were continuously recorded and later exported for further analysis.

The micro-dynamometer system (see Supplement) allowed for rapid temperature-jumps (t-jumps). The advantage of the t-jump is that by initially fully activating a fiber at low temperatures (here, 10^{o}C), the sarcomere arrangement was stabilized before being briefly exposed to a higher, near physiological temperature (here, 30^{o}C), generating maximal isometric force with less possible damage to the sarcomere arrangement (Karatzaferi et al., 2004) (for an indicative experiment see Figure 1).

Experimental solutions

Basic rigor buffer contained: 120mM KAc, 5mM $MgAc_2$, 1mM EGTA and 50mM MOPS (pH7) or 50mM MES (pH6.2). Relaxing solution: with addition of 5mM ATP. Maximal calcium activation: with addition of 1.1mM $CaCl_2$ (see Supplement). The ionic strength of the solutions was ~0.2M.

Maximum Isometric Force measurements

All assessments and initial data reductions were done in a blind fashion. Average diameter was determined for subsequent cross-sectional area (CSA) calculations assuming a cylindrical shape.

Maximum isometric force (Po) was first evaluated at standard resting conditions (pH7, 10^{o}C), at resting sarcomere lengths (2.2-2.4 μm). A number of fibers were subsequently activated at pH7 and 30^{o}C. A subset of fibers underwent an

assessment at 10^{o}C, in both resting (pH7) and acidic (pH6.2) conditions while fewer fibers were also assessed at pH6.2, 30^{o}C. To avoid an order effect, fibers were randomly assigned to be first activated in pH7 and then in pH6.2 and *vice versa*. Lastly, fibers were re-assessed in initial conditions (to fulfill criteria of stability, i.e. ≤10% initial force decline). We also assessed velocity of contraction using the load-clamp method in a subset of fibers at 10 ^{o}C (see Supplement).

Statistical analysis

Force data distribution was tested using Kolmogorov-Smirnov test of normality. Due to the normal data distribution, statistical analysis was performed using parametric tests. Descriptive (Mean±SD) and Inferential (SEM and exact 95% CIs) statistics of absolute and specific forces, as well as percentage force values are reported. A General Linear Model (GLM) analysis was performed to examine main effects of independent variables and their interaction and also provided pairwise comparisons. To examine possible differences in the response to either temperature or pH changes, force change was calculated in percentages of initial standard conditions and the differences between groups were tested using t-test for independent samples. All statistical analyses were performed using a commercially available statistical package (SPSS 15.0). The significance level was set at $P<0.05$.

Results

Surgery procedures were well-tolerated and animals had a normal after-surgery recovery. Twelve weeks post-surgery, BW ranged between 1,970-4,585 and 3,500-4,965 gr for UREM and CON animals respectively ($P>0.05$), with higher serum

creatinine (2.67±1.15 *vs* 1.38±0.09 mg/dl , P<0.05) and urea levels (67.33±32.02 *vs* 40.67±4.62 mg/dl, P>0.05) in UREM vs CON.

Cross –sectional area

Calculated cross sectional areas (CSAs) of UREM fibers (n=240, 5,040±1,189 μm^2) were significantly lower compared to CONs (n=142, 5,671±1,259 μm^2), P<0.001. Thus, the results were analyzed for both absolute and specific force values (i.e. force values normalized for fiber CSA to appraise force data independently of fiber atrophy).

Contractile properties in resting conditions (pH7)

Single psoas fibers (CON n=142, UREM n=240) were maximally calcium-activated at 10^{o}C, pH7 ('standard conditions'). Some fibers were also assessed at 30^{o}C, pH7 (CON n=41, UREM n=73) using the t-jump method. Descriptive and inferential statistics are presented in Table 1, Figure 2 and in the text.

Whether on absolute or specific force values, GLM analysis indicated a statistically significant main effect of *group* [F(1,492)=83.6, P<0.001 and F(1,492)=33.1, P<0.001 respectively], *temperature* [F(1,492)=114.6, P<0.001 and F(1,492)=108.2, P<0.001 respectively] as well as a significant interaction *group x temperature* [F(1,492)=19.9, P<0.001 and F(1,492)=10.5, P=0.001] for absolute and specific forces respectively.

The pairwise comparisons revealed consistent functional deficits in UREM fibers compared to CONs. At the standard conditions, absolute isometric force (Figure 2A) of UREM fibers was significantly lower (vs CON, P<0.001). After normalizing

force values for fiber CSA, UREM fibers were also found to produce on average significantly lower specific force (vs CON, P<0.01) (Figure 2B).

The t-jump caused an expected significant force rise in both groups (P<0.001). Still, UREM fibers produced significantly lower forces (P<0.001) than CON, at 30^{o}C, pH 7, for both absolute and specific forces, (Figure 2). After expressing the force response to the t-jump as a percentage of a fiber's own baseline force at pH7, 10^{o}C, UREM fibers appeared to gain more, as their average temperature-induced force increase was 2.2 fold that of CONs (+167±170% *vs* +76±42%, P<0.005) without however remedying the significant force disparity between groups.

Contractile properties in acidic conditions (pH6.2)

Lowering the pH, from 7 to 6.2, caused an expected significant force reduction in both groups (P<0.005, in 25 CON and 48 UREM fibers). Specifically, absolute and specific forces at pH6.2, 10^{o}C, were for UREM fibers, 211±125 μN and 46±26 mN/mm^2 & for CON fibers, 316±116 μN and 55±20 mN/mm^2, respectively. Whether on absolute or specific force, GLM analysis indicated a statistically significant main effect of *pH* [F(1,451)=22.9, P<0.001, and F(1,451)=21.7, P<0.001 respectively], with the main effect of *group* being again significant [F(1,451)=18.6, P<0.001 and F(1,451)=5.1, P<0.05 respectively], but the interaction of *group x pH* was non-significant [F(1,451)=0.003, P>0.05, and F(1,451)=0.028, P>0.05 for absolute or specific force respectively].

The pairwise comparisons (using Bonferroni adjustments) showed that at 10^{o}C, pH6.2, the absolute isometric force (Figure 3A) of UREM fibers was significantly lower (vs CON, P<0.05) roughly by approx. -33%. UREM fibers

produced somewhat lower normalized isometric forces, roughly by approx. -17% (Figure 3B) but non-significantly (vs CON, P>0.05).

After expressing the force response to the change of pH as a percentage of a fiber's own Po at standard conditions (pH7, 10^{o}C) the average % force decline due to the pH change tended to be larger for UREM fibers, albeit non-significantly (-48±14% *vs* -43±9%, P=0.06).

The temperature effect at pH6.2 was also assessed in a subset of fibers (UREM= 21, CON=13). Because it was not possible to lower the pH while at 30^{o}C, these data were not included in the global statistical assessments mentioned above. The absolute and specific force values at pH6.2, 30^{o}C, were for UREM 550±248 μN and 114±52 mN/mm^2 & for CON fibers 700±238 μN and 117±33 mN/mm^2, respectively. The effect of the t-jump at pH6.2 was also expressed as percentage of force achieved at pH6.2, 10^{o}C. The average temperature-induced force increase for UREM (+219±160%) and CON fibers (+143±62%) did not differ significantly (P>0.05).

Velocity of contraction

When examining the force-velocity relationship at 10^{o}C (see Supplement), UREM muscle fibers (n=32) produced slower velocities compared to CON (n=15) in both resting and acidic conditions. As a result, Vmax of UREM fibers at either pH7 or pH6.2, was ~50% that of CON respectively.

Discussion

To the best of our knowledge this is the first study to examine the effects of renal insufficiency on the contractile properties of single skeletal muscle fibers, under

resting and acidic (fatigue) conditions. We used methodology that is not acutely confounded by pervasive neural or metabolic abnormalities but instead focuses on fiber function *per se*, independently from the extent of whole muscle atrophy. We found significantly impaired, absolute and specific, isometric force at the single fiber level, in muscle fibers from an animal model mimicking CRI. This functional deficit was thus only partially explained by fiber atrophy and was persistent under 'resting' and 'fatigue' conditions, under near physiological temperature conditions indicating a lower 'muscle quality' in UREM fibers. We also observed some evidence of slower velocities of contraction in UREM fibers (see Supplement) albeit in a small number of fibers. We thus provide compelling evidence of CRI-induced effects on single fiber mechanical properties.

We observed significantly smaller CSAs in UREM psoas fibers compared to CON (by ~11%) in agreement to human studies. In end-stage disease, muscle atrophy is ~ 27% (Sakkas et al., 2003b) affecting mostly the fast type IIA and IIX muscle fibers (Sakkas et al., 2003a; Sawant et al., 2011). Recently Acevedo et al., (Acevedo et al., 2015) reported no evident atrophy in the tibialis cranialis (a mixed fast hindlimb muscle) of surgically-induced uremic rats. In contrast, Organ et al., (Organ et al., 2016) reported atrophy in all fiber types of the extensor digitorum longus (EDL, another, mixed, fast muscle) of Cy/+ uremic rats while the overall mass and physiological CSA of the whole muscles remained unchanged. Together these and our results [in a larger species and a muscle expressing >95% IIX(IId) myosin, (Aigner et al., 1993; Hämäläinen and Pette, 1993)] could indicate a muscle type specificity of fiber atrophy in the early stages of CRI. Our findings are consistent with human studies reporting muscle atrophy in advanced kidney patients (Johansen et al., 2003; Sakkas et al., 2003a; Sawant et al., 2011) and suggest that atrophy could appreciably

affect fast muscles which are normally tasked to provide high levels of muscle power, earlier during the disease progress.

Atrophy is expected to result in lower absolute muscle force as less available cross-bridges per fiber will be generating tension at any given time. That doesn't mean that muscle quality, which is the "physiological functional capacity of muscle tissue" (Fragala et al., 2015) would be necessarily affected. The force-generating capacity relative to the muscle's CSA may or may not remain the same (Fragala et al., 2015), depending on the extent of exposure to the atrophic stimulus and the ability of the organism to adapt on prevailing conditions (Malavaki et al., 2015). In a 2-week suspension and fixed muscle length animal model, a near 60% atrophy in individual soleus muscle fibers was accompanied by a 17% specific force reduction; the concomitant increase in shortening velocity was attributed to an altered thick and thin filaments' packing (Riley, 2005). The latter mechanism however, appeared to be a transient response during the early phase of disuse atrophy; in a recent human space-flight study, soleus atrophied by 20%, shortening velocity decreased, and myofilament packing density increased (Fitts et al., 2010). In the present study we hadn't assessed filament packing and the literature so far doesn't report whether disease-induced atrophy may be linked to alterations in filament packing. Additionally, ageing and/or disease mechanisms may impact function and metabolic properties without a direct link to atrophy, thus affecting overall muscle quality (Fragala et al., 2015).

We found that at resting conditions, UREM fibers produced significantly lower absolute and specific forces compared to CONs, by 25% and 14%, respectively at 10^{o}C (a standard *in vitro* testing temperature), and by 40% and 28% respectively, at 30^{o}C (a closer to physiological temperature). Thus in our study the modest degree of atrophy of uremic fibers only partially accounted for the force deficit. Organ et al.,

(Organ et al., 2016) in a 35-week Cy/+ rat model, reported a 21% reduction in absolute ankle dorsiflexion torque despite unchanged whole muscle dimensions. However, they noted individual fiber atrophy (roughly 20%) in the studied EDL mixed fast muscle. Our finding of an 11% atrophy in psoas muscle fibers is similar. However, since no single fiber mechanics were assessed in that study, how the individual fiber's force-generating capacity may have been affected in their model, is unknown. Notably, no fiber type shift was observed in the Organ et al study, further highlighting the complexity of mechanisms underlying reduced muscle quality in disease.

Muscle contraction is temperature sensitive (Ranatunga, 2010) and in agreement to previous reports (Coupland et al., 2001; Karatzaferi et al., 2008, 2004; Pate et al., 1995) both UREM and CON fibers substantially increased force generation in response to a t-jump from 10^{o}C to 30^{o}C. At pH7, force rise in CON fibers was ~76% in agreement to others using rabbit psoas skinned fibers (Coupland et al., 2001; Pate et al., 1995). Although the temperature-induced force increase was percent-wise higher in UREM, force at 30^{o}C remained significantly lower in UREM vs CON fibers. Thus, absolute and specific force deficits of the UREM fibers observed at standard *in vitro* conditions held also true at a near physiological temperature.

The deficit in UREM fibers' specific force could indicate a reduced capacity to generate force per myosin cross-bridge or a lower number of active cross-bridges (Fitts et al., 1991; Karatzaferi et al., 2004) and the slower velocities could indicate a slower cross-bridge cycle (see Supplement). Possible disturbances on force transmission across the sarcomeric arrangements may also be implicated, such as changes in viscoelastic properties [e.g. in human chronic heart failure (Miller et al., 2010)] or filament packing [e.g. in human space flight (Fitts et al., 2010)]. Other data

from our group show increased protein carbonylation and other redox disturbances (Poulianiti et al., 2015), which could foreseeably cause structural modifications affecting the actomyosin interaction. One way would be via glycation, as oxidative stress could also promote the formation of advanced glycation end-products, AGEs (Miyata et al., 1997), which in studies of reversible glycation (Ramamurthy et al., 2003, 2001), have been indicated to cause glycation-related structural alterations in myosin affecting the in vitro motility speed.

Fatigue intolerance in kidney disease is associated with the rapid development of acidosis (Johansen et al., 2005; Moore et al., 1993). Acidosis alone or in combination with other 'fatigue' metabolites, contributes to force reduction (Allen et al., 2008; Karatzaferi et al., 2008; Nelson and Fitts, 2014). Here, the drop of pH reduced force by approx. 45% for both groups, in agreement to others (Cooke et al., 1988; Karatzaferi et al., 2003; Pate et al., 1995). The acidosis effect may be less pronounced at near physiological temperatures in single fibers (Karatzaferi et al., 2008; Pate et al., 1995), but still significant. However, the functional consequence of an acidosis-induced force reduction, coupled with a slower contractile velocity (see Supplement), could prove worse for uremic muscles *in vivo*. One can fathom that in acidosis, even during rest or with low exercise intensities (Johansen et al., 2005), uremic muscles could be at a severe functional disadvantage.

Our study had some limitations. Despite implementing the same surgical approach, a large variability in uremic psoas fibers' contractile properties was observed. Also, UREM fibers protein extracts could not be resolved in SDS-PAGE (data not shown). Moreover, in retrospect, UREM fibers were more difficult to dissect and handle; due to the blind design, a 'positive' bias was probably inadvertently introduced; given standard criteria for force data quality [e.g. (Karatzaferi et al., 2003;

Liang et al., 2008)], it later transpired that relatively more UREM fiber data were excluded from statistical analysis than CON. Such discarded UREM fibers could be described as 'mussy' and 'sticky'. Based on the above, possible changes in passive elastic properties may warrant further study [e.g. changes in titin or nebulin may be implicated (Horowits et al., 1986)], as skeletal muscle viscoelastic properties changes, such as reported in heart failure patients (Miller et al., 2010; Toth et al., 2012) could be possible in our model. Advanced glycosylation has also been associated with glycation of type IV collagen of endothelial cells in ESRD (Thornalley and Rabbani, 2009) and further changes in overall muscle elastic properties cannot be excluded.

Main strengths of our study included: the use of the single fiber technique, which allows the assessment of fiber function isolating factors such as muscle atrophy, energetics or excitation-contraction coupling issues; the blind design; the use of sham-operated controls; the t-jump approach (which provides physiological relevance). Moreover, our model developed CRI for 3 months, i.e. a sufficient period considering a rabbit's lifespan, making our results more relevant to human chronic disease. In the future, the stretch-release force response, possible changes in viscoelastic properties, or post-translational modifications of key sarcomeric proteins, such as myosin, titin and nebulin, should be assessed and associated with further functional assessments.

In conclusion, experimentally-induced renal insufficiency led to significant functional impairments in single psoas fibers' mechanics, only partly explained by fiber atrophy. Our observations, if verified in human tissue, could help explain key aspects of functional problems observed in patients.

Conflict of interest statement

The authors declare no conflict of interest.

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REFERENCES

Abraham, G., Varughese, S., Thandavan, T., Iyengar, A., Fernando, E., Naqvi, S.A.J., Sheriff, R., Ur-Rashid, H., Gopalakrishnan, N., Kafle, R.K., 2016. Chronic kidney disease hotspots in developing countries in South Asia. Clin Kidney J 9, 135–141.

Acevedo, L.M., Peralta-Ramírez, A., López, I., Chamizo, V.E., Pineda, C., Rodríguez-Ortiz, M.E., Rodríguez, M., Aguilera-Tejero, E., Rivero, J.-L.L., 2015. Slow- and fast-twitch hindlimb skeletal muscle phenotypes 12 wk after ⅝ nephrectomy in Wistar rats of both sexes. Am J Physiol Renal Physiol 309, F638–F647.

Adams, G.R., Vaziri, N.D., 2006. Skeletal muscle dysfunction in chronic renal failure : effects of exercise. Am J Physiol Renal Physiol 290, F753–F761.

Aigner, S., Gohlsch, B., Hamalainen, N., Staron, R.S., Uber, A., Wehrle, U., Pette, D., 1993. Fast myosin heavy chain diversity in skeletal muscles of the rabbit: heavy chain IId, not IIb predominates. Eur J Biochem 211, 367–372.

Allen, D.G., Lamb, G.D., Westerblad, H., 2008. Skeletal muscle fatigue: cellular mechanisms. Physiol Rev 88, 287–332.

Campistol, J.M., 2002. Uremic myopathy. Kidney Int 62, 1901–1913.

Cooke, R., Bialek, W., 1979. Contraction of glycerinated muscle fibers as a function of the ATP concentration. Biophys J 28, 241–258.

Cooke, R., Franks, K., Luciani, G.B., Pate, E., 1988. The inhibition of rabbit skeletal muscle contraction by hydrogen ions and phosphate. J Physiol 395, 77–97.

Coupland, M.E., Puchert, E., Ranatunga, K.W., 2001. Temperature dependence of active tension in mammalian (rabbit psoas) muscle fibres: effect of inorganic

phosphate. J Physiol 536, 879–891.

Cusumano, A.M., González Bedat, M.C., 2008. Chronic kidney disease in Latin America: time to improve screening and detection. Clin J Am Soc Nephrol 3, 594–600.

Fabiato, A., Fabiato, F., 1978. Effects of pH on the myofilaments and the sarcoplasmic reticulum of skinned cells from cardiace and skeletal muscles. J Physiol 276, 233–255.

Fitts, R.H., McDonald, K.S., Schluter, J.M., 1991. The determinants of skeletal muscle force and power: Their adaptability with changes in activity pattern. J Biomech 24, 111–122.

Fitts, R.H., Trappe, S.W., Costill, D.L., Gallagher, P.M., Creer, A.C., Colloton, P.A., Peters, J.R., Romatowski, J.G., Bain, J.L., Riley, D.A., 2010. Prolonged space flight-induced alterations in the structure and function of human skeletal muscle fibres. J Physiol 588, 3567–3592.

Fragala, M.S., Kenny, A.M., Kuchel, G.A., 2015. Muscle Quality in Aging: a Multi-Dimensional Approach to Muscle Functioning with Applications for Treatment. Sport Med 45, 641–658.

Gordon, P.L., Sakkas, G.K., Doyle, J.W., Shubert, T., Johansen, K.L., 2007. Relationship between vitamin D and muscle size and strength in patients on hemodialysis. J Ren Nutr 17, 397–407.

Gotloib, L., Crassweller, P., Rodella, H., Oreopoulos, D.G., Zellerman, G., Ogilvie, R., Husdan, H., Brandes, L., Vas, S., 1982. Experimental model for studies of continuous peritoneal'dialysis in uremic rabbits. Nephron 31, 254–259.

Grams, M.E., Chow, E.K.H., Segev, D.L., Coresh, J., 2013. Lifetime Incidence of

CKD Stages 3–5 in the United States. Am J Kidney Dis 62, 245–252.

Hämäläinen, N., Pette, D., 1993. The histochemical profiles of fast fiber types IIB, IID, and IIA in skeletal muscles of mouse, rat, and rabbit. J Histochem Cytochem 41, 733–743.

Horowits, R., Kempner, E.S., Bisher, M.E., Podolsky, R.J., 1986. A physiological role for titin and nebulin in skeletal muscle. Nature 323, 160–4.

Johansen, K.L., Chertow, G.M., Jin, C., Kutner, N.G., 2007. Significance of frailty among dialysis patients. J Am Soc Nephrol 18, 2960–2967.

Johansen, K.L., Doyle, J., Sakkas, G.K., Kent-Braun, J.A., 2005. Neural and metabolic mechanisms of excessive muscle fatigue in maintenance hemodialysis patients. Am J Physiol Regul Integr Comp Physiol 289, R805–R813.

Johansen, K.L., Painter, P.L., Sakkas, G.K., Gordon, P., Doyle, J., Shubert, T., 2006. Effects of resistance exercise training and nandrolone decanoate on body composition and muscle function among patients who receive hemodialysis: A randomized, controlled trial. J Am Soc Nephrol 17, 2307–14.

Johansen, K.L., Shubert, T., Doyle, J., Soher, B., Sakkas, G.K., Kent-Braun, J.A., 2003. Muscle atrophy in patients receiving hemodialysis: effects on muscle strength, muscle quality, and physical function. Kidney Int 63, 291–297.

Kaltsatou, A., Sakkas, G.K., Poulianiti, K.P., Koutedakis, Y., Tepetes, K., Christodoulidis, G., Stefanidis, I., Karatzaferi, C., 2015. Uremic myopathy: is oxidative stress implicated in muscle dysfunction in uremia? Front Physiol 6, 1–7.

Karatzaferi, C., Chinn, M.K., Cooke, R., 2004. The force exerted by a muscle cross-bridge depends directly on the strength of the actomyosin bond. Biophys J 87,

2532–2544.

Karatzaferi, C., Franks-Skiba, K., Cooke, R., 2008. Inhibition of shortening velocity of skinned skeletal muscle fibers in conditions that mimic fatigue. Am J Physiol Regul Integr Comp Physiol 294, R948–R955.

Karatzaferi, C., Myburgh, K.H., Chinn, M.K., Franks-Skiba, K., Cooke, R., 2003. Effect of an ADP analog on isometric force and ATPase activity of active muscle fibers. Am J Physiol Cell Physiol 284, C816–C825.

Kouidi, E., Albani, M., Natsis, K., Megalopoulos, A., Gigis, P., Guiba-tziampiri, O., Tourkantonis, A., Deligiannis, A., 1998. The effects of exercise training on muscle atrophy in haemodialysis patients. Nephrol Dial Transplant 13, 685–699.

Liang, B., Chung, F., Qu, Y., Pavlov, D., Gillis, T.E., Tikunova, S.B., Davis, J.P., Tibbits, G.F., 2008. Familial hypertrophic cardiomyopathy-related cardiac troponin C mutation L29Q affects Ca2+ binding and myofilament contractility. Physiol Genomics 33, 257–266.

Malavaki, C.J., Sakkas, G.K., Mitrou, G.I., Kalyva, A., Stefanidis, I., Myburgh, K.H., Karatzaferi, C., 2015. Skeletal muscle atrophy: disease-induced mechanisms may mask disuse atrophy. J Muscle Res Cell Motil 36, 405–21.

Miller, M.S., VanBuren, P., LeWinter, M.M., Braddock, J.M., Ades, P.A., Maughan, D.W., Palmer, B.M., Toth, M.J., 2010. Chronic heart failure decreases cross-bridge kinetics in single skeletal muscle fibres from humans. J Physiol 588, 4039–4053.

Miyata, T., Wada, Y., Cai, Z., Iida, Y., Horie, K., Yasuda, Y., Maeda, K., Kurokawa, K., van Ypersele de Strihou, C., 1997. Implication of an increased oxidative stress in the formation of advanced glycation end products in patients with end-

stage renal failure. Kidney Int 51, 1170–1181.

Moore, G.E., Bertocci, L.A., Painter, P.L., 1993. 31P-magnetic resonance spectroscopy assessment of subnormal oxidative metabolism in skeletal muscle of renal failure patients. J Clin Invest 91, 420–424.

Munoz, K.A., Satarug, S., Tischler, M.E., 1993. Time course of the response of myofibrillar and sarcoplasmic protein metabolism to unweighting of the soleus muscle. Metabolism 42, 1006–1012.

Nelson, C.R., Fitts, R.H., 2014. Effects of low cell pH and elevated inorganic phosphate on the pCa-force relationship in single muscle fibers at near-physiological temperatures. Am J Physiol Cell Physiol 306, C670–C678.

Organ, J.M., Srisuwananukorn, A., Price, P., Joll, J.E., Biro, K.C., Rupert, J.E., Chen, N.X., Avin, K.G., Moe, S.M., Allen, M.R., 2016. Reduced skeletal muscle function is associated with decreased fiber cross-sectional area in the Cy/+ rat model of progressive kidney disease. Nephrol Dial Transplant 31, 223–230.

Pate, E., Bhimani, M., Franks-Skiba, K., Cooke, R., 1995. Reduced effect of pH on skinned rabbit psoas muscle mechanics at high temperatures: implications for fatigue. J Physiol 486, 689–694.

Pereira, R.A., Cordeiro, A.C., Avesani, C.M., Carrero, J.J., Lindholm, B., Amparo, F.C., Amodeo, C., Cuppari, L., Kamimura, M.A., 2015. Sarcopenia in chronic kidney disease on conservative therapy: prevalence and association with mortality. Nephrol Dial Transplant 30, 1718–1725.

Poulianiti, K.P., Kaltsatou, A., Karioti, A., Jamurtas, A., Stefanidis, I., Tepetes, K., Christodoulidis, G., Koutedakis, Y., Sakkas, G.K., Karatzaferi, C., 2015. Systemic and Muscle Specific Redox and Biochemical Status in Chronic Kidney

Disease [Abstract]. J Muscle Res Cell Motil 36, 558.

Ramamurthy, B., Höök, P., Jones, a D., Larsson, L., 2001. Changes in myosin structure and function in response to glycation. FASEB J 15, 2415–2422.

Ramamurthy, B., Jones, a D., Larsson, L., 2003. Glutathione reverses early effects of glycation on myosin function. Am J Physiol Cell Physiol 285, C419–C424.

Ranatunga, K.W., 2010. Force and power generating mechanism(s) in active muscle as revealed from temperature perturbation studies. J Physiol 588, 3657–3670.

Riley, D.A., 2005. Skeletal muscle fiber atrophy: altered thin filament density changes slow fiber force and shortening velocity. Am J Physiol Cell Physiol 288, C360–C365.

Sakkas, G.K., Ball, D., Mercer, T.H., Sargeant, A.J., Tolfrey, K., Naish, P.F., 2003a. Atrophy of non-locomotor muscle in patients with end-stage renal failure. Nephrol Dial Transplant 18, 2074–2081.

Sakkas, G.K., Sargeant, A.J., Mercer, T.H., Ball, D., Koufaki, P., Karatzaferi, C., Naish, P.F., 2003b. Changes in muscle morphology in dialysis patients after 6 months of aerobic exercise training. Nephrol Dial Transplant 18, 1854–1861.

Sawant, A., Garland, S.J., House, A.A., Overend, T.J., 2011. Morphological, electrophysiological, and metabolic characteristics of skeletal muscle in people with end-stage renal disease: A critical review. Physiother Canada 63, 355–376.

Thornalley, P.J., Rabbani, N., 2009. Highlights and hotspots of protein glycation in end-stage renal disease. Semin Dial 22, 400–404.

Toth, M.J., Miller, M.S., VanBuren, P., Bedrin, N.G., LeWinter, M.M., Ades, P.A., Palmer, B.M., 2012. Resistance training alters skeletal muscle structure and function in human heart failure: effects at the tissue, cellular and molecular

levels. J Physiol 590, 1243–1259.

Westerblad, H., Bruton, J.D., Lännergren, J., 1997. The effect of intracellular pH on contractile function of intact, single fibres of mouse muscle declines with increasing temperature. J Physiol 500, 193–204.

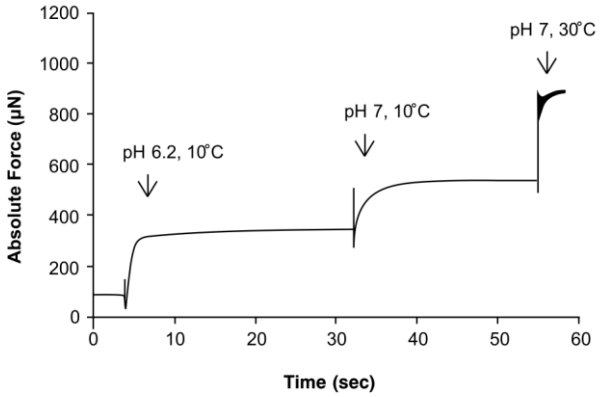
Zoccali, C., Kramer, A., Jager, K.J., 2010. Epidemiology of CKD in Europe: An uncertain scenario. Nephrol Dial Transplant 25, 1731–1733.

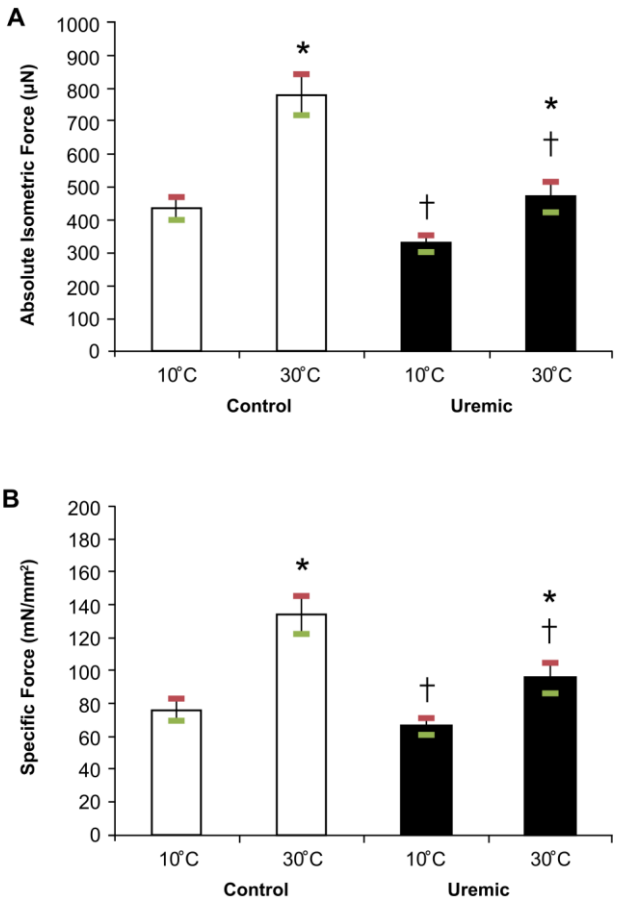
Figure 1. Indicative example of single fiber force recording. A fiber initially immersed in a relaxing solution was transferred (first arrow) in an activating solution (pH6.2, 10^{o}C); once a steady state force was reached, the fiber was transferred (second arrow) in another activating solution (pH7, 10^{o}C) and was allowed again to reach a steady state force. Following the low temperature activation, the fiber was briefly transferred (third arrow) in a pH7 activating solution at 30^{o}C (t-jump). The fiber was returned to a relaxing solution of the pH of interest and reactivated to verify stability (not shown). The order of exposure to different conditions was random. Up to five maximal activations were possible.

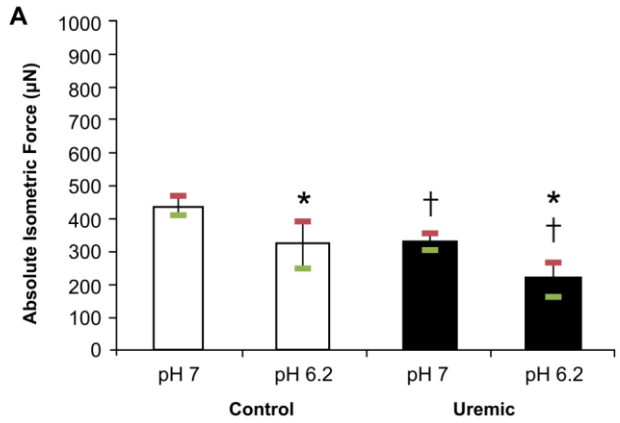
Figure 2. Isometric force for CON (open bars) and UREM (filled bars) psoas muscle fibers. Data collected at 10^{o}C and 30^{o}C pH7 are presented as Mean values with exact Upper and Lower 95% CIs for: A. absolute and for B. specific isometric forces. At pH7, UREM fibers produced lower isometric forces compared to CON in all conditions even after correcting for muscle atrophy * Denotes significant difference from corresponding 10^{o}C value ($P<0.001$); † Denotes significant differences from corresponding value of CON fibers ($P<0.01$).

Figure 3. Effect of pH on isometric force for CON (open bars) and UREM (filled bars) psoas muscle fibers. Data collected at resting (pH7) and acidic (pH6.2) pH, at 10^{o}C, are presented as Mean values with exact Upper and Lower 95% CIs for: A. absolute and for B. specific isometric forces. Lowering of pH caused significant reductions in absolute and specific forces in both fiber groups; At pH 6.2 UREM fibers produced significantly lower absolute forces compared to CON but the difference between groups was non-significant after correcting for muscle atrophy *

Denotes significant difference from corresponding pH7 value ($P<0.005$); † Denotes significant difference from corresponding value of CON fibers ($P<0.05$).







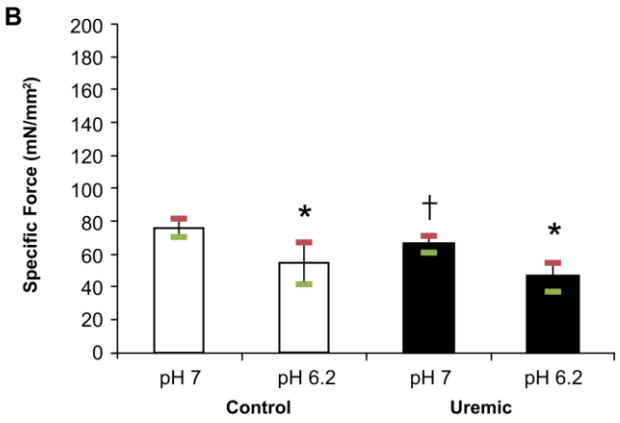


Table 1. Contractile properties of UREM and CON single psoas muscle fibers at 10°C and 30°C, pH7. Descriptive (Mean ± SD) and Inferential statistics (SEM and exact 95% CIs) are reported for the collected isometric force data.

Dependent Variable	Group	Temperature (°C)	*n*	Mean	SD	SEM	95% Confidence Interval	
							Lower Bound	Upper Bound
Force (µN)	CONTROL	10	142	434	171	17	400	468
		30	*41*	780	216	32	717	844
	UREMIC	10	240	327	203	13	301	353
		30	*73*	470	263	24	423	517
Specific Force (mN/mm²)	CONTROL	10	142	76	25	3	70	83
		30	*41*	134	40	6	123	146
	UREMIC	10	240	66	38	2	61	71
		30	*73*	96	55	4	87	105

The value for *n* represents fibers assessed. GLM analysis indicated a significant main effect of either group, temperature and their interaction (group * temperature) (P≤0.001).