UNIVERSITY OF THESSALY
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MECHANISMS OF MUSCLE FUNCTION: THE EFFECT OF UREMIA ON MORPHOLOGY, METABOLISM AND FATIGUE

by

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ABSTRACT

Introduction: Chronic kidney disease (CKD) patients present with an increase of waste products in the blood (uremia) which are purported to impact on skeletal muscle causing "uremic myopathy" (incl. abnormalities such as atrophy, especially in fast muscle types, weakness, fat infiltration, peripheral neuropathy, excess acidosis and premature fatigue). A number of patient interventions implemented so far, while greatly beneficial have failed to fully correct for muscular deficits. A host of comorbidities adds complexity to the interpretation of existing results, while most research has examined the end-stage of the disease and it is thus not known *how* and *if* skeletal muscle is affected at earlier stages of disease progression.

This research aimed to examine the effect of chronic renal insufficiency on skeletal muscle's force generation capacity using an animal model of pre-dialysis CKD. Therefore, to reveal if sarcomeric function *per se* may be affected by chronic renal insufficiency, the single muscle fiber's force generation capacity was examined at resting conditions as well as in response to acidosis, its calcium sensitivity and its resilience in the face of redox challenges, by examining:

1. force generation of isolated muscle fiber preparations in conditions mimicking the resting state (Study 1)
2. force generation of isolated muscle fiber preparations in conditions mimicking acidosis (Study 1)
3. force-pCa relationship of isolated muscle fiber preparations in conditions mimicking the resting state (Study 2)
4. force-pCa relationship of isolated muscle fiber preparations in conditions mimicking acidosis (Study 2)

5. stretch activation kinetics relationship of isolated muscle fiber preparations in conditions mimicking the resting state (Study 3)
6. morphology of isolated muscle fiber preparations (Study 1 and Study 3)
7. force generation of isolated muscle fiber preparations in conditions of acute redox imbalance (Study 4)

Materials and Methods: Renal insufficiency was induced surgically in New Zealand rabbits (UREM), with sham-operation for controls (CON), using ethically approved procedures. At 3 months post-surgery, following euthanasia, psoas muscle samples were excised, chemically skinned and stored in 50% glycerol solution at -20°C until mechanical assessment using an SI Heidelberg/WPI micro dynamometer. Fibers' diameters were recorded using the eyepiece of a stereoscope. Sample treatments, dissection and all evaluations were conducted in a blind fashion.

Study 1. Isometric forces (Po) were recorded from maximally calcium activated single fibers (N=142 CON; N=240 UREM) at 'standard' baseline conditions (pH 7, 10°C), and at a near physiological temperature (pH 7, 30°C) in a subset of fibers; the effect of acidosis (pH 6.2) was also evaluated.

Study 2. Isometric force was assessed in single psoas fibers (N=128 CON; N=195 UREM) in various concentrations of calcium, at 10°C, 30°C and at pH 7 and pH 6.2. To facilitate comparison, force data expressed as percentage (%) of Po at 10°C and pH 7 and free calcium expressed in pCa values were fitted in the Hill equation. The value of pCa where 50% relative force was achieved (pCa_{50}), was used as an index of calcium sensitivity. Cooperativity was represented by the n_H value of the fit.

Study 3: Single psoas fibers (N=21 CON; N=42 UREM) were maximally activated under isometric conditions at 22°C, pH7. When force reached a plateau, step-like stretches of 0.3% fiber length were performed to induce isometric force transients.

The time to the peak of stretch-induced delayed force increase (t_3) was evaluated as a measure of cross-bridge kinetics. Resting sarcomere lengths were also determined at a near slack position by laser diffraction.

Study 4. Single psoas fibers' (N=18 CON; N=19 UREM) force response to redox changes [employing Hydrogen Peroxide (H_2O_2) and/or Dithiothreitol (DTT)] was assessed in 2 experimental sets: A) Acute exposure to 10mM H_2O_2 during full activation followed by incubation in 10mM DTT during relaxation and a subsequent activation in standard solutions (N=9 CON; N=9 UREM fibers); B) Exposure to 10mM H_2O_2 during relaxation, preceded and followed by submaximal (pCa_{50}) and maximal activations in standard solutions (N=9 CON; N=10 UREM fibers).

Results: The results of the current thesis are summarized below:

Study 1. Renal insufficiency resulted in significantly smaller average CSA for UREM muscle fibers compared to CON (by ~11%, $P<0.01$). At standard conditions UREM fibers produced lower absolute and specific forces ($P<0.01$); this force disparity remained also when measurements were performed at 30°C ($P<0.01$). For both groups, acidosis significantly reduced force production (vs pH 7, 10°C, $P<0.01$), with a similar percent force decline (UREM -48% vs CON -43%, $P>0.05$).

Study 2. At standard conditions (10°C, pH7), UREM fibers presented with quite similar calcium sensitivity (pCa_{50} UREM 6.12±0.02 vs CON 6.20±0.03) and cooperativity compared to CONs (n_H UREM 2.11±0.14 vs CON 2.36±0.3). Acidosis (pH 6.2) at 10°C caused a loss of calcium sensitivity for both groups, more so for UREM (pCa_{50} UREM 5.32±0.06 vs CON 5.58±0.02). At 30°C pH7, UREM fibers presented with lower sensitivity than CON (pCa_{50} UREM 6.00±0.25 vs CON 6.42±0.19). At 30°C acidosis reduced calcium sensitivity similarly for both groups (pCa_{50} UREM 5.71±0.13 vs CON 5.80±0.05).

Study 3. Fibers of the UREM animals exhibited larger t_3 values (UREM 67±18 ms vs CON 57±16 ms; $P<0.05$). Furthermore, UREM fibers exhibited larger resting sarcomere lengths (UREM 2.25±0.33 μm vs CON 2.05±0.17 μm; $P<0.01$) and smaller mean diameters (UREM 70±19 μm vs CON 79±13 μm; $P<0.05$).

Study 4. A) Acute exposure to H_2O_2 during activation did not affect force generation ($P>0.05$). DTT pre-incubation caused 12% force reduction ($P>0.05$) only in UREM fibers B) H_2O_2 during relaxation reduced subsequent maximal isometric forces in the Pool of fibers (UREM and CON) by 3.5% ($P<0.05$) but not in fiber groups separately (UREM $P>0.05$; CON $P>0.05$).

Discussion: Chronic renal insufficiency induced significant impairments in single psoas muscle fiber force that were only partially explained by atrophy. Further investigation is warranted to pinpoint the contributions of possible changes in sarcomeric protein properties to the evident functional deficit (Study 1). It appears that chronic renal insufficiency may depress calcium sensitivity, the magnitude of this depression being dependent on prevailing experimental conditions. It is important to consider temperature and acidosis parameters when assessing calcium sensitivity in chronic disease (Study 2). Chronic renal insufficiency can induce a slowing of myosin head cross-bridge kinetics and remodelling changes concerning fiber diameters (atrophy) and sarcomere structure. The larger sarcomere lengths in fibers of UREM animals could be due to a decrease of forces restoring the sarcomere length at resting conditions (Study 3). Force generation capacity of CON and UREM fibers is affected by oxidation similarly. However the observation that UREM muscle may have been in a more reduced state at baseline warrants further investigation as it could be linked to disease induced effects (Study 4).

Conclusion: For the first time, contractile properties of uremic muscle were assessed at the single fiber level. Evaluation of maximal isometric force, force-pCa relationship and stretch activation kinetics revealed important functional limitations in uremic fibers which were partly accounted for by atrophy. The elastic elements of the sarcomere could also be affected and explain some results. Extrapolating to the human condition, we suggest that even at a pre-dialysis stage, chronic renal insufficiency can severely disturb a muscle's force generating capacity at the single fiber level. Our findings provide new information to help explain muscle weakness and fatigue in CKD.

ΠΕΡΙΛΗΨΗ

Εισαγωγή: Στην Χρόνια νεφρική νόσο (ΧΝΝ), οι ασθενείς παρουσιάζονται με αυξημένη συγκέντρωση παραπροϊόντων του μεταβολισμού (ουραιμία) τα οποία φαίνεται να επηρεάζουν αρνητικά τον σκελετικό μυ προκαλώντας «ουραιμική μυοπάθεια» (συμπερ. διαταραχές όπως ατροφία κυρίως σε μυϊκό ιστό ταχείας συστολής, αδυναμία, λιπώδης διήθηση, περιφερική νευροπάθεια, αυξημένη οξέωση και πρόωρη κόπωση). Μια σειρά παρεμβάσεων που έχουν εφαρμοστεί στην συγκεκριμένη κλινική ομάδα ενώ έχουν φανεί ευεργετικές, δεν έχουν καταφέρει να διορθώσουν επαρκώς το λειτουργικό έλλειμμα των ασθενών. Δεδομένης της συννοσηρότητας που παρουσιάζεται στην νόσο, η ερμηνεία αυτών των αποτελεσμάτων γίνεται πολύπλοκη ενώ οι περισσότερες μελέτες έχουν πραγματοποιηθεί σε ασθενείς τελικού σταδίου. Ως εκ τούτου δεν είναι γνωστό το *αν* και *με ποιό τρόπο* επηρεάζονται οι σκελετικοί μύες σε προγενέστερα στάδια της εξέλιξης της νόσου.

Σκοπός της συγκεκριμένης έρευνας ήταν να εξεταστεί η επίδραση της χρόνιας νεφρικής ανεπάρκειας στον σκελετικό μυ ως προς την ικανότητα παραγωγής δύναμης, χρησιμοποιώντας ένα ζωικό μοντέλο προ-τελικού σταδίου ΧΝΝ. Με σκοπό λοιπόν να διαπιστωθεί εάν η καθαυτή σαρκομερική λειτουργία μπορεί να επηρεάζεται από την χρόνια νεφρική ανεπάρκεια, εξετάστηκε η ικανότητα απομονωμένων μυϊκών ινών στην παραγωγή δύναμης σε συνθήκες ηρεμίας αλλά και στην ανταπόκριση σε συνθήκες οξέωσης, στην ευαισθησία στο ασβέστιο αλλά και στην ικανότητα λειτουργικής ανταπόκρισης σε οξειδοαναγωγικές μεταβολές, εξετάζοντας:

1. την ικανότητα απομονωμένων μυϊκών ινών στην παραγωγή δύναμης σε συνθήκες ηρεμίας (Μελέτη 1).

2. την ικανότητα απομονωμένων μυϊκών ινών στην παραγωγή δύναμης σε συνθήκες οξέωσης (Μελέτη 1).

3. την σχέση δύναμης-pCa απομονωμένων μυϊκών ινών σε συνθήκες ηρεμίας (Μελέτη 2).

4. την σχέση δύναμης-pCa απομονωμένων μυϊκών ινών σε συνθήκες οξέωσης (Μελέτη 2).

5. την ανταπόκριση απομονωμένων μυϊκών ινών σε αιφνίδιες αλλαγές του μήκους τους σε συνθήκες ηρεμίας (Μελέτη 3).

6. την μορφολογία απομονωμένων μυϊκών ινών (Μελέτες 1 και 3).

7. την ικανότητα λειτουργικής ανταπόκρισης απομονωμένων μυϊκών ινών στην παραγωγή δύναμης σε συνθήκες οξείας πρόκλησης οξειδοαναγωγικών μεταβολών (Μελέτη 4).

Μεθοδολογία: Η νεφρική ανεπάρκεια προκλήθηκε χειρουργικά σε κονίκλους Νέας Ζηλανδίας (UREM) ενώ πραγματοποιήθηκε εικονική χειρουργική επέμβαση για τους κονίκλους της ομάδας ελέγχου (CON). Τρεις μήνες μετά την χειρουργική παρέμβαση και ύστερα από την ευθανασία των ζώων, πραγματοποιήθηκε συλλογή ψοΐτη μυϊκού ιστού. Ακολούθησε χημική απομεμβράνωση του ιστού και στην συνέχεια τα δείγματα αποθηκεύτηκαν σε διάλυμα 50% γλυκερόλης στους -20°C, μέχρι την μηχανική τους αξιολόγηση με την χρήση εξειδικευμένου μικροδυναμομέτρου (SI Heidelberg/WPI). Οι διάμετροι των ινών αξιολογήθηκαν χρησιμοποιώντας τον προσοφθάλμιο φακό ενός στερεοσκοπίου. Η επεξεργασία των δειγμάτων, ο διαχωρισμός και όλες οι αξιολογήσεις διεξήχθησαν σε έναν τυφλό σχεδιασμό.

<u>Μελέτη 1.</u> Πραγματοποιήθηκε αξιολόγηση της μέγιστης ισομετρικής δύναμης (Po) (N=142 CON, N=240 UREM) σε ‘standard’ συνθήκες (pH7, 10°C), και σε

θερμοκρασίες πλησίον των φυσιολογικών (pH7, 30°C) σε μια υποομάδα ινών· αξιολογήθηκε επίσης η επίδραση της οξέωσης (pH 6.2).

Μελέτη 2. Η ισομετρική δύναμη απομονωμένων μυϊκών ινών ψοΐτη (N=128 CON, N=195 UREM) αξιολογήθηκε σε ένα εύρος συγκεντρώσεων ασβεστίου στους 10°C, 30°C αλλά και σε pH 7 και pH 6.2. Για να διευκολυνθεί η σύγκριση, τα δεδομένα της δύναμης εκφράστηκαν ως ποσοστό (%) των τιμών Po στους 10°C και pH 7 και το ελεύθερο ασβέστιο εκφράστηκε σε τιμές pCa. Αυτές οι τιμές κατόπιν προσαρμόστηκαν στην εξίσωση Hill. Η τιμή pCa στην οποία επιτεύχθηκε το 50% της σχετικής δύναμη (pCa_{50}) χρησιμοποιήθηκε ως δείκτης της ευαισθησίας στο ασβέστιο. Η συνεργατικότητα αντανακλάται στις τιμές $n_{H.}$

Μελέτη 3: Οι απομονωμένες ίνες ψοΐτη (N=21 CON, N=42 UREM) ενεργοποιήθηκαν μέγιστα υπό ισομετρικές συνθήκες στους 22°C, pH 7. Όταν η δύναμη σταθεροποιήθηκε, προκλήθηκε αιφνίδια επιμήκυνση των ινών κατά 0,3% του αρχικού τους μήκους ώστε να προκληθούν αλλαγές στην παραγόμενη δύναμη. Ο χρόνος που χρειάστηκε από την έναρξη της επιμήκυνσης μέχρι την κορύφωση της καθυστερημένης αύξησης της δύναμης (t_3), αξιολογήθηκε ως δείκτης της κινητικής των εγκάρσιων γεφυρών. Τα σαρκομερικά μήκη ηρεμίας των ινών προσδιορίστηκαν επίσης σε θέση που οι ίνες βρίσκονταν σε χάλαση, με περίθλαση λέιζερ.

Μελέτη 4. Η λειτουργική ανταπόκριση απομονωμένων ινών ψοΐτη (N=18 CON, N=19 UREM) σε οξειδοαναγωγικές μεταβολές [υπεροξείδιο του υδρογόνου (H_2O_2) και/ή Διθειοθρεϊτόλη (DTT)] αξιολογήθηκε σε 2 πειραματικές ομάδες: Α) Οξεία έκθεση σε 10mM H_2O_2 κατά τη διάρκεια πλήρους ενεργοποίησης που ακολουθείται από επώαση σε 10mM DTT κατά την διάρκεια χάλασης και την επακόλουθη ενεργοποίηση σε “standard” διαλύματα (N=9 CON, N=9 UREM) Β) Έκθεση σε

10mM H_2O_2 κατά τη διάρκεια της χάλασης, πριν και μετά από υπομέγιστες (pCa_{50}) και μέγιστες ενεργοποιήσεις σε “standard” διαλύματα (N=9 CON, N=10 UREM).

Αποτελέσματα: Τα αποτελέσματα της παρούσας διδακτορικής εργασίας συνοψίζονται παρακάτω:

Μελέτη 1. Η νεφρική ανεπάρκεια οδήγησε σε σημαντικά μικρότερη εγκάρσια διατομή (CSA) τις UREM μυϊκές ίνες συγκριτικά με τις CON (κατά ~ 11%, P<0.01). Σε “standard” συνθήκες οι UREM ίνες παρήγαγαν χαμηλότερες απόλυτες και ειδικές δυνάμεις (διορθωμένες ως προς CSA) σε σύγκριση με τις CON (P<0.01). Αυτή η διαφορά στην δύναμη παρέμεινε, επίσης, όταν οι μετρήσεις έγιναν στους 30°C (P<0.01). Και για τις δύο ομάδες, η οξέωση μείωσε σημαντικά την παραγωγή δύναμης (vs pH7, 10^oC P<0.01), με ένα παρόμοιο ποσοστό μείωσης (UREM -48% vs CON -43%, P>0.05).

Μελέτη 2. Σε “standard” συνθήκες, οι UREM ίνες παρουσιάστηκαν με παρόμοια ευαισθησία στο ασβέστιο (pCa_{50} UREM 6.12±0.02 vs CON 6.20±0.03) και συνεργατικότητα (n_H UREM 2.11±0.14 vs CON 2.36±0.3). Η οξέωση (pH6.2), στους 10°C προκάλεσε απώλεια της ευαισθησίας του ασβεστίου και για τις δύο ομάδες, κυρίως όμως για τις UREM (pCa_{50} UREM 5.32±0.06 έναντι CON 5.58±0.02). Στους 30^oC, pH7, οι UREM ίνες παρουσιάστηκαν με χαμηλότερη ευαισθησία από ό,τι οι CON (pCa_{50} UREM 6.00±0.25 έναντι CON 6.42±0.19). Στους 30^oC, η οξέωση μείωσε την ευαισθησία στο ασβέστιο, παρόμοια και για τις δύο ομάδες (pCa_{50} UREM 5.71±0.13 έναντι CON 5.80±0.05).

Μελέτη 3. Οι UREM ίνες παρουσίασαν μεγαλύτερες τιμές t_3 (UREM 67±18 ms έναντι CON 57±16 ms, P<0.05). Επιπλέον, οι UREM ίνες εμφάνισαν μεγαλύτερα σαρκομερικά μήκη ηρεμίας (UREM 2.25±0.33 μm έναντι CON 2.05±0.17 μm,

P<0.01) και μικρότερες διαμέτρους (UREM 70 ± 19 μm έναντι CON 79 ± 13 μm, P<0.05).

Μελέτη 4. Α) Η οξεία έκθεση σε H_2O_2 κατά την ενεργοποίηση δεν επηρέασε την παραγωγή δύναμης (P>0.05). Ωστόσο, το DTT προκάλεσε σημαντική μείωση της δύναμης κατά 12% (P<0.05) μόνο στις UREM ίνες. Β) Η επώαση με H_2O_2 κατά τη διάρκεια της χάλασης μείωσε την μέγιστη ισομετρική δύναμη στο σύνολο των ινών (CON και UREM) κατά 3.5% (P<0.05), αλλά όχι στις 2 ομάδες ινών ξεχωριστά (UREM P>0.05; CON P>0.05).

Συζήτηση: Η χρόνια νεφρική ανεπάρκεια προκάλεσε σημαντικές διαταραχές στην δύναμη απομονωμένων μυϊκών ινών ψοΐτη που μόνο εν μέρει μπορούν να εξηγηθούν από την μυϊκή ατροφία. Περαιτέρω έρευνα είναι αναγκαία για να εντοπίσει την συμβολή πιθανών μεταβολών στις σαρκομερικές πρωτεΐνες στο εμφανές λειτουργικό έλλειμμα (Μελέτη 1). Φαίνεται ότι η χρόνια νεφρική ανεπάρκεια μπορεί να μειώσει σημαντικά την ευαισθησία στο ασβέστιο, και το μέγεθος αυτής της μείωσης εξαρτάται από τις πειραματικές συνθήκες. Είναι σημαντικό να λαμβάνεται υπόψη η σημασία της θερμοκρασίας και της οξέωσης κατά την αξιολόγηση της ευαισθησίας του ασβεστίου σε χρόνιες ασθένειες (Μελέτη 2). Η χρόνια νεφρική ανεπάρκεια μπορεί επίσης να προκαλέσει επιβράδυνση στην κινητική των εγκάρσιων γεφυρών των κεφαλών της μυοσίνης καθώς και μεταβολές όσον αφορά την διάμετρο των ινών (ατροφία) και την δομή του σαρκομερίου. Τα μεγαλύτερα σαρκομερικά μήκη στις ίνες των UREM ζώων θα μπορούσαν να οφείλονται σε μείωση των δυνάμεων αποκατάστασης του σαρκομερικού μήκους σε συνθήκες ηρεμίας (Μελέτη 3). Η ικανότητα παραγωγής δύναμης των CON και UREM ινών επηρεάστηκε από την οξείδωση παρόμοια. Ωστόσο, η παρατήρηση ότι οι UREM ίνες μπορεί να βρίσκονταν σε μια πιο αναγωγική κατάσταση κατά την έναρξη των πειραμάτων χρειάζεται

περαιτέρω διερεύνηση, καθώς αυτό θα μπορούσε να συνδέεται με την επίδραση της ασθένειας (Μελέτη 4).

Συμπεράσματα: Για πρώτη φορά, αξιολογήθηκαν οι συσταλτικές ιδιότητες του ουραιμικού μυός σε επίπεδο απομονωμένων μυϊκών ινών. Η αξιολόγηση της μέγιστης ισομετρικής δύναμης, της σχέσης δύναμης-pCa και της ανταπόκρισης των ινών σε αιφνίδια επιμήκυνση, αποκάλυψε σημαντικούς λειτουργικούς περιορισμούς στις UREM ίνες που εν μέρει εξηγούνται από την ατροφία. Τα ελαστικά στοιχεία του σαρκομερίου θα μπορούσαν επίσης να επηρεαστούν και να εξηγήσουν κάποια αποτελέσματα. Μεταφέροντας τα αποτελέσματα στην ανθρώπινη κατάσταση, υποδεικνύουμε ότι ακόμη και σε ένα προ-τελικό στάδιο, η ΧΝΝ μπορεί να διαταράξει σοβαρά την ικανότητα παραγωγής δύναμης ενός μυός σε επίπεδο μυϊκής ίνας. Τα ευρήματά μας παρέχουν νέες πληροφορίες για την ερμηνεία της μυϊκή αδυναμίας και πρόωρης μυϊκής κόπωσης στη ΧΝΝ.

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LIST OF ABBREVIATIONS

AA: Aminoacids

ACh: Acetylcholine

ADM: Abductor digiti minimi

ADP: Adenosine diphosphate

AFB: Acetate-free biofiltration

AGEs: Advanced glycation end products

ATP: Adenosine triphosphate

bFGF: Basic fibroblast growth factor

BW: Body weight

Ca^{2+}: Free calcium

$CaCl_2$: Calcium chloride

cADPR: Cyclic adenosine diphosphate-ribose

ck: Creatine kinase

cMAP: Motor action potential

CKD: Chronic kidney disease

CON: Sham operated-control animals/fibers

Cr: Creatine

CRP: C-reactive protein

CSA: Cross sectional area

CVD: Cardiovascular disease

DHPR: Dihydropyridine receptors

DTT: Dithiothreitol

EGTA: Ethylene glycol tetraacetic acid

ELC: Essential myosin light chain

ESN: Early supernormality

ESRD: End stage renal disease

F-actin: Filamentous actin

G-actin: Globular actin monomers

GFR: Glomerular filtration rate

GSH: Reduced form of glutathione

HD: Hemodialysis

HO-1: Heme oxygenase-1

H_2O_2: Hydrogen peroxide

IGF-1: Insulin-like growth factor 1

IGF-BP3: Insulin-like growth factor-binding protein 3

IL-6: Interleukin-6

IMAT: Intermuscular adipose tissue

i.v.: Intravenously

KAc: Potassium acetate

LH: Luteinizing hormone

LVH: Left ventricular hypertrophy

$MgAc_2$: Magnesium acetate

MHC: Myosin heavy chain

MLC: Myosin light chain

MLCK: Myosin light chain kinase

MOPS: 3-(N-Morpholino) propanesulfonic acid

MRI: Magnetic resonance imaging

mtDNA: Mitochondrial DNA

n_H: Hill coefficient

NZ: New Zealand

O_2^-: Superoxide

OH^-: Hydroxide

pCa: Negative logarithm of free calcium concentration

pCa_{50}: pCa for half maximal force

PCr: Phosphocreatine

PD: Peritoneal dialysis

PET: Positron emission tomography

PFK: Phosphofructokinase

pH: Negative logarithm of the hydrogen ion concentration

Pi: Inorganic phosphate

Po: Maximal isometric force

REMP: Electrical membrane potential at rest

RLC: Regulatory myosin light chain

ROS: Reactive oxygen species

RRP: Relative refractory period

RRT: Renal replacement therapy

RyR: Ryanodine receptors

SC: Satellite cell

SDH: Succinate dehydrogenase

SN100: Late supernormality

SOCS-2: Suppressors of cytokine signaling

SOD: superoxide dismutase

SR: Sarcoplasmic reticulum

t_2: The time to the lowest force value before the onset of delayed force increase

t_3: The time to the peak of stretch-induced delayed force increase

T-jump: Temperature jump

TGF-β1: transforming growth factor

TM: Tropomyosin

TNC: Troponin C

TNF-a: tumor necrosis factor-a

TNI: Troponin I

TNT: Troponin T

UREM: Uremic animals/fibers

VEGF: vascular endothelial growth factor

VO_2 peak: Peak oxygen uptake

VRC: velocity recovery cycles

XSN100: extra late supernormality

CHAPTER 1: INTRODUCTION

Chronic kidney disease (CKD) is increasingly recognized as a major global health problem affecting 40-50% of EU and USA populations (Grams, Chow, Segev, & Coresh, 2013; Zoccali, Kramer, & Jager, 2010), as well millions in Asia (Abraham et al., 2016), Latin America (Cusumano & González Bedat, 2008) and elsewhere.

CKD patients present with skeletal muscle structural and functional abnormalities (Kaltsatou et al., 2015; Sakkas, Ball, et al., 2003; Sakkas, Sargeant, et al., 2003a) with symptoms of muscle weakness, limited endurance and fatigue intolerance (Campistol, 2002), neuropathy (Adams & Vaziri, 2006) and a host of other striated muscle problems, collectively described as *uremic myopathy* (Campistol, 2002). Uremic myopathy is a common abnormality presented in CKD patients and is associated with muscle abnormalities, which in turn lead to morbidity and mortality and worsen in patients undergoing hemodialysis therapy (Floyd, Ayyar, Barwick, Hudgson, & Weightman, 1974).

It is also known that skeletal muscle structure and function is of major role not only for body movement and control but also for many other vital body functions such as protein and energy metabolism (Frontera & Ochala, 2014). Therefore, many researchers have studied the effects of various interventions (pharmaceutical, non pharmaceutical or combination) in preserving muscle quality and quantity of CKD patients (Balakrishnan et al., 2010; Johansen et al., 2006; Sakkas, Sargeant, et al., 2003b). Despite the improvements, CKD patients cannot restore their muscle structure and function at the levels of age-matched healthy individuals with a sedentary lifestyle (Sakkas, Hadjigeorgiou, et al., 2008; Sakkas, Sargeant, et al., 2003b).

Many factors have been reported to inhibit muscle function in CKD including mitochondrial function, substrate availability and neuropathy (Adams & Vaziri,

2006). In addition, CKD is accompanied by uremia, a condition which is caused by the accumulation of toxic waste products due to impaired kidney function (Richet, 1988). This toxicity has been suggested to be implicated in the abnormal muscle function and fatigue intolerance experienced by CKD patients (Campistol, 2002; Davis, Karl, Goldberg, & Harter, 1983).

While both intramuscular energetics disturbances and central activation failure are implicated (Johansen, Doyle, Sakkas, & Kent-Braun, 2005), the mechanisms underlying peripheral fatigue in CKD have not yet been fully understood. Additionally, along with the individual patient's characteristics, the interaction of disease specific and/or toxicity factors and disuse, as in other chronic conditions, is not easily untangled (Malavaki et al., 2015). All of the above are adding on the complexity of the challenge to *explain* and ultimately *prevent and/or ameliorate* uremic myopathy. Given most research so far has been done at the end-stage, key issues related to disease progression and contractile mechanisms are still unanswered.

Thus, there is an urgent need to generate new knowledge to enhance our understanding of the characteristics and progression of striated muscle pathophysiology in CKD in order to support the design of appropriate interventions to prevent or alleviate its devastating impact.

AIMS – SIGNIFICANCE

The mechanisms underlying muscle dysfunction in CKD have not yet been fully understood and a number of interventions implemented so far, while greatly beneficial have failed to fully correct for muscular deficits. The understanding of the possible underlying mechanisms to explain muscle dysfunction in CKD is of major clinical relevance as skeletal muscle is associated with many aspects of life and health such as safe and adequate mobility and regulation of metabolism. Thus, any abnormality of skeletal muscle status can lead to a series of physical disturbances, as well as low quality of life, and dangerous falls. It is known that neuropathy can contribute to muscle dysfunction in renal disease, especially at the end stage and its effects have made difficult to distinguish excitation-contraction coupling from sarcomeric function problems.

Therefore, the evaluation of contractile properties at the single fiber level using techniques which overcome excitation contraction coupling (skinned fibers) may lead to important conclusions regarding the factors implicated in muscle dysfunction presented in CKD. Using such methods it is also possible to examine contractile properties in conditions that mimic uremic environment such as acidosis and oxidative stress.

Thus in the current PhD thesis we aimed to examine the effect of chronic renal insufficiency on:

1. force generation of isolated muscle fiber preparations from an animal model of uremia in conditions mimicking resting muscle
2. force generation of isolated muscle fiber preparations from an animal model of uremia in conditions mimicking acidic muscle

3. force-pCa relationship of isolated muscle fiber preparations from an animal model of uremia in conditions mimicking resting muscle
4. force-pCa relationship of isolated muscle fiber preparations from an animal model of uremia in conditions mimicking acidic muscle
5. stretch activation kinetics relationship of isolated muscle fiber preparations from an animal model of uremia in conditions mimicking resting muscle
6. force generation of isolated muscle fiber preparations from an animal model of uremia in conditions mimicking oxidized and reduced state of muscle
7. morphology of isolated muscle fiber preparations from an animal model of uremia

CHAPTER 2: LITERATURE REVIEW

Skeletal Muscle - Structure and Function

The human body consists of three types of muscle tissue named cardiac muscle, smooth muscle and skeletal muscle. Cardiac muscle is the main content of the heart and is essential for circulating blood into the body. Smooth muscle is found in the walls of most of our internal organs such as stomach and blood vessels and is responsible for moving materials from, into and within the body. Skeletal muscles, which make up about 40% of our body weight, are attached to our skeleton and they support and control its movement while at the same time contributing to breathing, thermoregulation, protein storage, immune function, blood circulation (venous return) and metabolic health. Cardiac and smooth muscles can be characterized as involuntary muscles as they are not directly under conscious control while skeletal muscles as voluntary muscles. However this is not a very precise classification given that skeletal muscles can be activated both voluntary and involuntary (Silverthorn, 2004; Wilmore & Costill, 1994)**,** the latter for example as in shivering.

Each muscle is surrounded from connective tissue called epimysium and its role is to hold the entire muscle together. If we take away the epimysium we can see many groups of muscle bundles which are separated due to the presence of another connective tissue called perimysium. These groups of muscle bundles (fascicles) are composed of muscle fibers each one of them is also covered from a connective tissue called endomysium (Wilmore & Costill, 1994).

Muscle Fiber

Skeletal muscles are composed of several hundred or thousand muscle cells called muscle fibers depending on the size of a muscle. Each muscle fiber is

surrounded of a cell membrane called sarcolemma (plasmalemma). The sarcolemma is responsible for the connection of each fiber with the tendon of a muscle and for the transmission of the force generation of each fiber and therefore for the movement of the bone (MacIntosh, Gardiner, & McComas, 2006; Silverthorn, 2004; Wilmore & Costill, 1994).

The largest subunit of a muscle cell is the *myofibrils* or *myofilaments* which are responsible for the muscle contraction and relaxation. Between the myofibrils there is the cytosol (the fluid part of the cell) which consists of inorganic ions, sugars, peptides, amino acids and proteins. *The myofibrils* together with the *cytosol* and organelles form the sarcoplasm (cytoplasm) (MacIntosh et al., 2006; Silverthorn, 2004; Wilmore & Costill, 1994)**.**

The sarcoplasm houses also the sarcoplasmic reticulum (SR) and the transverse tubules (T tubules) which are essential for the calcium storage and release and therefore for the muscle contraction. The sarcoplasmic reticulum is an endoplasmatic network which consists of longitudinal tubules which are wrapped across a myofibril like a mesh and of the terminal cisternae and is responsible for the Ca^{2+} storage and release in the cytosol (Silverthorn, 2004). The T tubules are also an extensive network which lies laterally through the fiber. They are an extension of the sarcolemma and their role is to transmit nerve pulse from the sarcolemma to the myofibrils of a fiber. By this way, the action potential (which starts from the neuromuscular junction) can “travel” in a fast way from the cell membrane to the sarcoplasm. In each sarcomere there are two zones of T tubules and together with the terminal cisternae they form a *triad* (MacIntosh et al., 2006; Wilmore & Costill, 1994).

Myofibrils

A muscle fiber contains hundreds or thousands of myofibrils which are the contractile structures of a muscle cell. For example, a fiber of 50 μm diameter consists of ~2000 myofibrils with a diameter of 1-2 μm. The myofibrils are separated through the mitochondria, the sarcoplasmic and tubular system (MacIntosh et al., 2006). Each myofibril consists of a thick and a thin filament; both of them contain the responsible proteins for the muscle contraction and are located along a fiber. The thick filaments consist mainly of the myosin and the thin filaments of the actin, troponin and tropomyosin molecules.

If we look a muscle fiber under the microscope we can see light and dark bands. The light bands correspond to the actin filaments (I-bands-Isotropic) and the dark bands to the myosin filaments (A-bands-anisotropic) (MacIntosh et al., 2006).

Sarcomere

A myofibril contains many sarcomeres which are the basic functional units of a fiber. Each one of them is connected and anchored to its neighbors through the Z discs. Z discs are protein structures which lie vertically to the myofibril and each sarcomere is composed of two Z disks and the thick and thin filaments between them (Frontera & Ochala, 2014; Silverthorn, 2004). The length of a thick filament is almost 1.6 μm while the length of a thin filament ranges between 1.0-1.3 μm depending on the species and muscle type. Within a sarcomere each thick filament is surrounded from six thin filaments which are also surrounded from six thick filaments. By this way each thin filament is surrounded from three thick filaments. According to the above thick and thin filaments form the “double hexagonal array of the myofilament lattice” and the distance between the filaments is a key determinant

for myosin and actin content inside a muscle cell as well as for the force generation. Almost the 70% of the total protein of a muscle cell is myosin and actin (43-50% is myosin and 18-22% is actin) (Greising, Gransee, Mantilla, & Sieck, 2013).

Thick filament

Thick filaments are composed mainly from myosin which interacts with actin in order to generate force. Myosin is a motor protein with two heads, a long tail and a neck region which connect the heads with the tail. Myosin heads are also known as S1 fragments and together with the neck region form a cross bridge (MacIntosh et al., 2006).

Myosin consists of 2 myosin heavy chains (MHCs) with molecular mass ~200 kDa which wrap around each other and they form a double helix which corresponds to the tail region of the myosin molecule (Greising et al., 2013). Two pairs of myosin light chains (MLCs) are also placed at the neck region of the molecule. Specifically two essential or alkali myosin light chains (MLC_{17} or ELCs) with molecular mass of 17 kDa and 2 regulatory or phosphorylatable light chains (MLC_{20} or RLCs) with molecular mass of 20 kDa are also placed at the neck region of the molecule (Greising et al., 2013; Gordon, Homsher, & Regnier, 2000; Schiaffino & Reggiani, 1996).

Each myosin head is the motor domain which holds both adenosine triphosphate (ATP)-binding site and actin binding site and each pair of ELCs and RLCs is close to the motor domain and the tail respectively (Schiaffino & Reggiani, 1996). ELCs play a crucial role in the structure and stabilization of the myosin head while phosphorylation of RLCs lead to higher cross-bridge cycling and ATP

consumption rates and therefore may affect force and calcium sensitivity (Greising et al., 2013).

M-line is an additional protein of thick filament which is placed in the middle of the thick filament and composed from two additional proteins known as M-protein (only in fast fibers) and myomesin all contributing to the stabilization and alignment of the sarcomeres. Additionaly, titin, the largest protein, is also part of the thick filament and is placed between Z-line and M-line. The role of titin is essential in stabilizing the thick filament and maintaining the sarcomere. Lastly, myosin binding protein-C is located along the thick filament and its role is to contribute to the thick filament backbone (Greising et al., 2013).

Thin filament

The main contractile protein of thin filament is actin with a molecular weight of 40kDa and together with myosin make up the 70-80% of the protein content of a muscle fiber. Actin is a globular protein containing the binding sites for myosin in order to form cross bridges. Globular actin monomers (G-actin) are connected forming a double-helical chain named filamentous actin (F-actin).

Another major protein of thin filament is tropomyosin. Tropomyosin dimers are also associated forming a helical filament which lies along the F-actin. A tropomyosin dimer covers 7 actin monomers and each group of seven actin contains also a troponin complex (TnC, TnT, TnI) (Schiaffino & Reggiani, 1996). Troponin and tropomyosin regulate the exposure of actin to myosin heads and therefore are essential for muscle contraction (Greising et al., 2013). Thin filament also contains the giant protein named nebulin with a molecular weight of 0.6 to 0.9 MD. Nebulin

lies along the thin filament and its role is to regulate the length of F-actin (Greising et al., 2013).

Types of muscle fibers

All of our muscle fibers have not the same properties and they have been classified into two major groups according to their speed of contraction and fatigue resistance. Slow-twitch (type I) muscle fibers need two to three-fold more time compared to fast-twitch (type II) fibers in order to contract. On the other hand slow fibers are resistant to fatigue in contrast to fast fibers. In addition, fast fibers can be classified into three main subgroups (Silverthorn, 2004; Wilmore & Costill, 1994).

According to the myosin heavy chain (MHC) isoforms' expression, fast fibers have been categorized into three main subcategories known as IIA, IIX and IIB. Although all three isoforms of MHC are expressed in rodents, IIB is not expressed in humans. However, there are fibers which express more than one MHC isoform termed hybrid fibers. Except type I which is the slowest fiber type, type IIA is the slowest form of fast fibers and it is followed by IIX and IIB which are the fastest (Westerblad, Bruton, & Katz, 2010; Galler, Schmitt, & Pette, 1994).

The classification of fibers according to their MHC isoforms is in consistence with the metabolic profile of the fiber types depending on the ATP consumption rate from cross bridges. However, other proteins which also consume ATP in skeletal muscle are not classified in the same way. For instance Sarcoplasmic Reticulum (SR) calcium pumps have been classified into SERCA 1 and SERCA 2 isoforms. SERCA 1 exists in fast fibers while SERCA 2 in slow fibers. Furthermore, slow fibers have been characterized as oxidative and fast fibers as glycolytic but in some cases oxidative

capacity of IIA fibers has been observed to be better compared to I fibers (e.g. in rats) (Westerblad et al., 2010).

Motor unit and motor impulse

As it has been described above, skeletal muscles consist of muscle cells or muscle fibers which are under the control of motor neurons. A muscle is innervated by various motor neurons, each one of them innervating a group of muscle fibers through its ending (axon terminal). The number of fibers innervated by a single motor neuron can range from a few to thousands of fibers depending on the muscle size and the function to be performed (the finer the motor control required the fewer the fibers). A motor neuron together with the group of fibers (all of fibers are of the same type) which it supplies is called motor unit.

A motor unit is the smallest unit of motor system and is responsible for the activation of the given group of fibers (Westerblad et al., 2010; MacIntosh et al., 2006; Silverthorn, 2004; Wilmore & Costill, 1994). Motor neurons are located almost at the middle of a single fiber and axon terminals near sarcolemma. Upon nerve impulse arriving, a neurotransmitter substance named acetylcholine (ACh) is released from axon terminal and it is binded to acetylcholine receptors which are placed on sarcolemma. Therefore an electrical charge is transmitted along the muscle fiber known as action potential generation and this is the beginning for the muscle contraction (Wilmore & Costill, 1994).

Sliding filament theory and Cross bridge cycle

In relaxing conditions Ca^{2+} concentration is almost 50 nM. After action potential, the electrical charge travels through the SR and T tubules into the muscle

cell leading to release of stored calcium ions from SR to the sarcoplasm. The mechanism by which this happens is part of a mechanism known as excitation-contraction coupling. The first step of this mechanism is the depolarization of T tubules and the activation of dihydropyridine receptors (DHPR). The second step is the Ca^{2+} release from SR in the sarcoplasm through specific channels known as ryanodine receptors (RyR) elevating cytosolic calcium concentration to almost 100-fold higher levels (MacIntosh et al., 2006; Berchtold, Brinkmeier, & Muntener, 2000).

After Ca^{2+} release, it binds to troponin causing a position change of tropomyosin which holds the active sites of actin for binding from myosin heads (forming a cross bridge). In relaxing conditions tropomyosin hides the active sites from myosin heads but due to Ca^{2+} release and its binding to troponin, tropomyosin uncovers the active sites of actin on the thin filament. By this way, myosin heads are able to attach to thin filament (Wilmore & Costill, 1994).

After the attachment of thick to thin filament, myosin heads tilt causing the movement of myosin and actin filaments in the opposite direction (power stroke). After the tilt of the myosin head, it detaches from the specific active site of actin, it goes back to its original position and it attaches in a new active site (sliding filament theory). This is repeated until myosin head meets the Z disk of the sarcomere. Muscle action ends when Ca^{2+} is depleted in the sarcoplasm and it goes back to the SR (Wilmore & Costill, 1994).

This theory was based on the findings of 2 independent researchers, Andrew Huxley and Hugh Huxley, in the early 1950's. Andrew Huxley using an interference microscope observed a shortening of light I band and a constancy of length in dark A band during muscle contraction. At the same period Hugh Huxley using an electron microscopy observed that the composition of a myofibril was of many myofilaments

of two types. These two types of myofilaments referred to thick filaments with 1.6-μm length in the A-band and thin filaments with 1-μm length stretching from Z-disk to H-zone. Both A. and H. Huxley also associated thick filaments with myosin and thin filaments with actin. All these observations gave rise to the theory that sliding force is generated between thick and thin filaments due to the action of cross-bridges as they were the only point to connect the thin filaments (Ebashi & Ohtsuki, 2007; MacIntosh et al., 2006; Szent-Györgyi, 2004). Until then it was believed that muscle contraction occurs due to the lengthening of the filaments and the idea of sliding filaments had not been yet considered (Szent-Györgyi, 2004).

However the molecular mechanism underlying this theory was not yet known. In 1957, A. Huxley gave the first explanation based on his structural observations combined with force-velocity measurements. Since then, the molecular mechanism underlying muscle contraction is known as the cross bridge cycle and it refers to the mechanical and energetic events during muscle contraction. He also divided the cycle in two main states: attachment and detachment. However many observations such as fast force transients after a quick stretch could not be explained by the two-states model. Later, in 1971, A. Huxley proposed a model consisting of 1 detached and many attached states but this idea made the understanding of cross bridge cycle more complex (Herzog, 2000).

Finally, in 1971 R. W. Lymn and E. W. Taylor (Lymn & Taylor, 1971) proposed the cross bridge cycle model of 4 states based on two main findings. The first one was the hydrolysis of ATP in the detached state (no bound of myosin to actin) and the second one was the burst of ATP hydrolysis due to the addition of ATP to myosin when active sites were unoccupied. The latter gave evidence that the limiting reaction of the cross-bridge cycle was the adenosine diphosphate (ADP)

dissociation. Thus, they presented the following 4 states: First, ATP binds to the actomyosin complex, leading to the rapid detachment of actin from myosin cross bridge before the hydrolysis of ATP (state 1). The cross bridge undergoes conformational changes due to the hydrolysis of ATP (state 2). The cross bridge reattaches to actin after product release (ADP·Pi) (state 3). The cross bridge moves causing the drive stroke due to the displacement of products (state 4).

However, from the beginning to the end of muscle contraction, energy supply is required and the energy source is ATP. In anaerobic conditions ATP is re-synthesized using phosphocreatine (PCr) (ATP-Pc system) and in aerobic and anaerobic conditions lasting longer than a few seconds energy ATP is maintained using muscle glycogen (glycolytic system). ATP is placed in one of the two binding sites of myosin heads. The first one is for the attachment of myosin head to actin and the second one is the binding site for ATP. In addition to ATP, in the myosin molecule there is an enzyme called myosin ATPase which is necessary for the breakdown of ATP to ADP, Pi and energy. If ATP is not available or if it cannot been hydrolyzed, thin and thick filaments remain “locked” together and this is known as rigor state (as rigor mortis which occurs after death) (Wilmore & Costill, 1994).

Muscle Fatigue

Definition and classification

Muscle fatigue is the inability to generate or sustain the expected power (Edwards, 1981) and it has been classified into central and peripheral fatigue. Central fatigue may occur due to disturbances in mechanisms of central nervous system and especially due to the impaired activation of motor neurons (Westerblad et al., 2010). On the other hand, peripheral fatigue may occur due to disturbances in any mechanism between neuromuscular junction and the contractile machinery (Silverthorn, 2004). It has been reported that central fatigue is more pronounced in prolonged low intensity activities where metabolic changes are limited compared to high intensity activities (Westerblad et al., 2010). However, it has been generally accepted that muscle fatigue mainly arises at the muscle tissue level (Allen, Lamb, & Westerblad, 2008).

Causes of fatigue

Energy depletion theory

Muscle fatigue may occur at the muscle tissue level through the depletion of energy sources and particularly PCr and glycogen which lead to insufficient levels of ATP inside the muscle. Regarding PCr, which is the main source of ATP in short lasting and high intensity activities, it has been shown that PCr is depleted leading to low muscle performance. PCr is depleted before ATP while ATP is being produced from other systems too (glycolytic, oxidative). However, PCr depletion prevents the fast replace of ATP leading to low levels of ATP and therefore exhaustion where both PCr and ATP levels may be depleted (Karatzaferi, De Haan, Ferguson, Van Mechelen, & Sargeant, 2001; Karatzaferi, de Haan, van Mechelen, & Sargeant, 2001).

On the other hand, glycogen which is the main source of ATP in aerobic and anaerobic conditions lasting more than a few seconds may also be depleted fast while muscle glycogen reserves are limited. Glycogen depletion rate is higher in high intensity activities and during the first minutes of the activity. Thus, in long lasting activities exhaustion coincides with muscle glycogen levels' decrease (Wilmore & Costill, 1994). Glycogen depletion also depends on the fiber type which is recruited during specific activities while different fiber types have different metabolic profile. In light intensity exercise, slow fibers are recruited first and fast fibers are later recruited if tension requirements are increased and glycogen is depleted in a relative manner. In low intensity exercise slow fibers are mainly recruited and fast fibers are almost inactive whereas at high intensity exercise, fast fibers are more frequently recruited and depleted in a higher rate compared to slow fibers. This means that fast fibers have a higher glycogen demand (Egan & Zierath, 2013; Westerblad et al., 2010; Wilmore & Costill, 1994).

Metabolites accumulation theory (H^+, Pi, ADP)

Accumulation of lactic acid, a by-product of glycolysis, has been considered as a major cause of fatigue for many years. However, lactic acid accumulates in the fibers only in brief and high intensity activities whereas at the end of long lasting and low intensity activities lactic acid levels are equal to resting levels. Therefore, fatigue in this kind of activities (long lasting and low intensity activities) is the result of energy depletion. On the other hand, in brief and high intensity exercise, where lactic acid accumulates in the fibers, excess of lactic acid per se is not the reason for muscle fatigue. Instead, its breakdown to lactate causes accumulation of hydrogen ions H^+ leading to lowering of intracellular pH (acidosis) (Wilmore & Costill, 1994).

During fatigue, intracellular pH falls from about pH7 to pH6.2. Early studies examining the contractile properties of permeabilized single muscle fibers, have shown that acidosis (pH6.2) leads to major reductions in isometric force and shortening velocity indicating that low pH inhibits muscle contraction at the motor proteins level (Chase & Kushmerick, 1988; Cooke, Franks, Luciani, & Pate, 1988; Metzger & Moss, 1987; Pate & Cooke, 1989). In more recent studies where the effect of acidosis was examined at near physiological temperatures (≥30°C) (see below for the role of temperature) it was found a smaller effect of acidosis in muscle contraction. For example, in the study of Pate et al (Pate, Bhimani, Franks-Skiba, & Cooke, 1995), it was found that although at 10°C acidosis caused reductions in both isometric force and maximal shortening velocity by about 55% and 30% respectively, at 30°C isometric force was reduced by 20% without any change in shortening velocity. However, even though recent studies have shown a smaller effect of acidosis in muscle fatigue, low pH remains a key factor since in acidic conditions muscle contraction of single muscle fibers is undoubtedly inhibited.

The mechanisms underlying inhibition of muscle contraction due to acidosis remain complicated. However it has been shown that low pH inhibits the action of a glycolytic enzyme (phosphofructokinase-PFK) causing a delay in the rate of glycolysis and therefore ATP production (Nelson & Fitts, 2014; Wilmore & Costill, 1994). Besides the lower rate of ATP hydrolysis, low pH may also inhibit muscle function due to its effect in the myofilament lattice spacing (Umazume, Onodera, & Higuchi, 1986). In addition, during acidosis, force generation at high force states of cross bridge cycle is significantly reduced, indicating a direct effect of H^{+} in the interaction between myosin-actin (Fabiato & Fabiato, 1978). Moreover the force per cross-bridge and the number of cross-bridges that are in the high-force states have

been found to be reduced in the presence of low pH (Metzger & Moss, 1990a, 1990b). It has been also reported that low pH in combination with accumulation of inorganic phosphate (Pi) plays a critical role in muscle fatigue (Karatzaferi, Franks-Skiba, & Cooke, 2008).

In high energy activities, ATP concentration is initially constant and PCr breaks down to creatine (Cr) and Pi. In resting conditions, Pi concentration is almost 5mM and during fatigue may reach 30mM. This increase is related to the intensity of exercise and fiber type with the fast fibers showing the greatest values of Pi and therefore the greatest levels of fatigue (Fitts, 2008). The accumulation of Pi at high concentrations may inhibit muscle function by affecting the cross bridge cycle. Specifically, under normal conditions, release of Pi turns the cross bridge into the strongly bound high-force state leading to force increase. In contrast, during fatigue the increase of Pi concentration accelerates a backward step in cross bridge cycle leading to force reduction (Allen & Trajanovska, 2012). Furthermore, Fryer et al (Fryer, Owen, Lamb, & Stephenson, 1995) first suggested that Pi may enter the sarcoplasmic reticulum and precipitate calcium (CaPi) resulting in reduced calcium availability for release. In later studies using permeabilized single muscle fibers it was also suggested that accumulation of Pi leads to the reduction of calcium sensitivity (Allen & Trajanovska, 2012) indicating again the critical role of Pi in muscle fatigue.

An additional factor which has been implicated in muscle fatigue is ADP. In resting conditions, intracellular concentration of ADP is maintained almost at 20μM due to the creatine kinase which contributes to the formation of ATP. In high intensity activities where PCr is decreased and creatine is increased, concentration of ADP may reach 200μM (Dawson, Gadian, & Wilkie, 1978; Karatzaferi, Myburgh, Chinn, Franks-Skiba, & Cooke, 2003). Many studies using permeabilized muscle fibers have

shown that ADP rise can cause a decrease in unloaded shortening velocity, increase in isometric force and increase in calcium sensitivity. These opposite reactions can be explained from the well known “competition” between ADP and ATP for the nucleotide binding site of myosin. Therefore, when concentration of ADP is increased it can bind to myosin pushing the cross bridge back to the strongly bound state which slows down velocity and increases force and calcium sensitivity (Cooke, 2007; Debold, Longyear, & Turner, 2012; Karatzaferi et al., 2003).

Other contributors (ROS, phosphorylation, temperature)

Others factors implicated in muscle fatigue include the reactive oxygen species (ROS), phosphorylation of myosin light chains (MLC) and Ca^{2+} availability. ROS are molecules containing oxygen such as superoxide (O_2^-) having unpaired electrons and this makes them reactive enough to damage membranes and impair contractile proteins (Debold, 2012). It is generally believed that mitochondria are the main source of superoxide. High levels of superoxide may be broken down to H_2O_2 or enter in the myoplasm through the mitochondrial membrane even at resting conditions (Allen et al., 2008). It has been well established that exercise leads to an increase in ROS. In many studies using permeabilized single muscle fibers it has been observed that ROS significantly contribute to the onset of muscle fatigue and their effect is more intense in experiments conducted at high temperatures (Fitts, 2008). However, at low concentrations (in resting conditions or early fatigue), ROS appear to enhance muscle performance whereas high concentrations of ROS such as H_2O_2 (in fatigue conditions) appear to inhibit muscle function (Debold, 2012). It has been suggested that the mechanism underlying muscle fatigue in the presence of ROS is the

depression of Ca^{2+} sensitivity and that myofibril proteins which may be affected from ROS are troponin-C, troponin-I, tropomyosin, actin and myosin (Fitts, 2008).

Phosphorylatable regulatory myosin light chains (RLCs) are also involved in muscle fatigue. Although at resting conditions RLCs remain unphosphorylated, during repetitive muscle activation where Ca^{2+} release increases, they are progressively phosphorylated by myosin light chain kinase (MLCK). In contrast, when Ca^{2+} decreases, MLCK is deactivated and therefore RLCs are dephosphorylated after a few minutes (MacIntosh, Holash, & Renaud, 2012). It has been observed that after a tetanus of 1-second long, there is a twitch potentiation (post-tetanic twitch potentiation) and this is the result of RLC phosphorylation. In addition, RLC phosphorylation has been associated with a phenomenon known as staircase where in repetitive low frequency activations, force is progressively higher in the first seconds. Experiments in skinned fibers have also shown that RLC phosphorylation causes an increase in calcium sensitivity and therefore can affect cross bridge kinetics. In the study of Karatzaferi et al (Karatzaferi et al., 2008) it was found that although under resting conditions phosphorylation of RLCs led to a faster rate of cross bridge cycle, under fatigue conditions (low pH and high Pi concentration) RLC phosphorylation led to an even lower rate of cross bridge cycle. All of the above, give evidence that RLC phosphorylation plays a key role in muscle contraction and any abnormalities in phosphorylation or dephosphorylation may lead to muscle fatigue (MacIntosh et al., 2012).

Factors inhibiting muscle function or fatigue, including pH, Pi or ROS are temperature sensitive and fatigue may occur faster at high temperatures. Therefore, it is important to know the physiological temperature of an active muscle which depends on many factors such as core temperature, environmental temperature, blood

flow and the type of the activity. For this reason in studies examining mechanisms of muscle function or muscle fatigue it is important to evaluate contractile properties at, or near physiological temperatures when possible (Allen et al., 2008). In the case of using isolated skinned muscle fibers (see below) this has become possible through the use of the temperature jump (t-jump) methodology (Coupland, Puchert, & Ranatunga, 2001; Karatzaferi, Chinn, & Cooke, 2004; Karatzaferi et al., 2008), in order to have an in vitro assay that mimics well the physiological environment.

Understanding muscle contraction and fatigue

Skinned fibers

In order to understand the mechanisms of muscle contraction and fatigue, researchers have used for many years a range of methods including the method of skinned fibers. In this method the surface membrane of fibers is chemically or mechanically skinned and muscle samples are stored at -20°C until functional assessment. The main advantage of this method is the control of intracellular solutions. Therefore, researchers can study the reactions of single muscle cells not only in normal conditions but also in conditions that mimic fatigue by examining the effects of many factors such as pH and Pi in contractile properties of single fibers (Allen et al., 2008). Contractile properties include maximal isometric tension, force-pCa relationship and stretch activation kinetics. Depending on the protocol it is important to make conclusions with respect to morphological parameters of the fibers such as diameter or cross sectional area (CSA) and sarcomere length. Temperature is an additional important parameter in these studies while results from studies performed at low temperatures have shown different results from studies conducted at near physiological temperatures (>30°C) (Karatzaferi et al., 2008). The results of this

kind of studies can lead to important conclusions about mechanisms of muscle contraction and/or fatigue.

Maximal isometric tension (Po)

After determining the necessary morphological aspects with a cell in an ATP containing relaxing solution (the degree of detail depending on microscopy resolution and other technical prowess), a fiber is immersed in a solution containing ATP and saturated calcium. Then, Po (mN) is recorded continuously through specific softwares until reach a stable force. This is considered as the absolute force while specific force is defined as Po/CSA (mN/mm²) (Malisoux, Francaux, & Theisen, 2007). The Po of a single cell is determined from the number of active cross bridges during contraction and the force exerted from each cross bridge (Huxley, 1957). An increase or decrease of Po can be the result of muscle hypertrophy or muscle atrophy respectively. However, changes in Po/CSA are independent from alterations in CSA and may indicate alterations of the sarcomeric proteins (Malisoux et al., 2007).

Force-pCa relationship

Using the single fiber approach it is also possible to evaluate the isometric force generated in various submaximal concentrations of Ca^{2+}. Consequently, the relationship between various Ca^{2+} concentrations and generated force is determined using the Hill equation. Specifically, the relationship which is usually determined is between pCa and force where pCa= $-\log10[Ca^{2+}]$ and the Hill equation is $F=F_0/[1+10^{n(pCa_{50}-pCa)}]$ (Hill, 1913). The pCa_{50} estimates the calcium concentration which is required for muscle activation known as calcium sensitivity whereas n_H estimates the cooperativity of calcium activation.

Increased Ca^{2+} sensitivity may be the result of increased pH, sarcomere length, MgADP as well as of decreased ionic strength, lattice spacing, Mg^{++}, MgATP, Pi and myosin RLC phosphorylation. Changes in pH and ions such as K^{+} can lead to alterations in Ca^{2+} binding to TnC whereas Mg^{++} is able to affect thin filaments' protein interactions. Alterations in factors affecting the distance between myosin heads and thin filament such as sarcomere length, lattice spacing and RLC phosphorylation may also affect calcium sensitivity through the alterations in the level of myosin binding to actin. In addition, decreases in ionic strength, Pi, MgATP and increases in MgADP also lead to increased myosin binding to actin and therefore force generation (A. M. Gordon et al., 2000).

The n_H (steepness of force-pCa relationship) gives information about the cooperativity between proteins of thin filament. High n_H values correspond to high levels of cooperativity. Thin filament cooperativity may be affected by many factors including Ca^{2+} binding on TnC or along the thin filament as well as level of Ca^{2+} binding to regulatory units, movement of tropomyosin (A. M. Gordon et al., 2000) and temperature.

Stretch activation kinetics

During maximal activation of a single fiber under isometric conditions, a quick rectangular stretch of the fiber lasting only a few seconds (1-3 s) may be performed. This length change results in a series of different functional reactions due to stretch. Specifically, the stretch results in an immediate force increase (phase 1) followed by a force decline (phase 2) and then by a delayed force increase (phase 3) which is known as stretch activation (Galler, Hilber, & Pette, 1996). Phase 3 and especially the time from the beginning of the stretch to the peak value of the delayed

force increase (known as t_3) has been studied a lot as it has been well established that this length time is strongly associated with the myosin heavy chain isoforms of the fibers (ie. smaller t_3 values-faster myosins, larger t_3 values-slower myosins) (Galler et al., 1994). The mechanism underlying stretch activation kinetics has not been fully understood but it is strongly believed that the delayed force increase is the result of new actin-myosin cross bridges. According to Linari et al (Linari, Reedy, Reedy, Lombardi, & Piazzesi, 2004), lengthening results in the displacement of tropomyosin segments which uncovers new binding sites for myosin.

Chronic Kidney Disease and Uremia

Definition

In general, the human body contains 2 kidneys with a critical role in many vital body functions including the maintenance of solute and water transport balance, excretion of metabolic waste products and regulation of acids and bases. When the kidneys' function is impaired, renal insufficiency develops which may gradually lead to a chronic state known as chronic kidney disease (CKD) (Chikotas, Gunderman, & Oman, 2006).

CKD is now recognized as a major global health problem, expected to affect almost the 50% of European and American population during lifetime (Grams et al., 2013; Zoccali et al., 2010). This "silent" epidemic leads 440.000 of patients to renal replacement therapy (RRT) each year and it has been identified as one of the leading causes of death worldwide (Ortiz et al., 2014).

The disease is also known as renal insufficiency, end stage renal disease (ESRD) and uremia which are in general synonyms. Nevertheless, the term renal insufficiency is usually referred to a moderate decline of renal function and is presented with mildly high levels of creatinine, urea and potassium. The term "chronic" is used when there is an additional loss of renal function (more than 25% of the normal one) while ESRD is used when renal function finally remains in a percentage of 10%. On the other hand, the term uremia is used to describe the situations presented in patients due to the progressive accumulation of uremic toxins in plasma which are the result of the progressive loss of renal function. These situations include the accumulation of toxic wastes and electrolyte disorders which

lead to symptoms such as fatigue, vomiting and loss of appetite (Chikotas et al., 2006).

The disease has been categorized into five stages according to the level of kidney function which is reflected in glomerular filtration rate (GFR) (Levey et al., 2005). In the first two stages of kidney disease (stage 1 and 2) where GFR is above 60 mL/min/1.73 m^2, renal function is adequate to maintain patients without symptoms. Due to the progression of the disease (stage 3 and 4) where GFR is between 15-59 mL/min/1.73 m^2, renal function is significantly reduced and at stage 5 where GFR is up to 15 mL/min/1.73 m^2 (ESRD), the kidneys cannot perform almost any function and RRT is required (Levey et al., 2005). There are three methods of RRT and particularly hemodialysis (HD), peritoneal dialysis (PD) and kidney transplantation. HD and PD are the most frequent treatment modalities and dialysates act as artificial kidneys for the regulation of body fluids' balance and excretion of uremic toxins while kidney transplantation offers the possibility of a nature organ which offers a full or partial correction of renal function (Gokal, Figueras, Ollé, Rovira, & Badia, 1999; Sakkas et al., 2004).

Impact on health and functional capacity

CKD is associated with many comorbidities including diabetes mellitus, anemia, chronic inflammation, sleep disorders, depression and cardiovascular disease (CVD) with CVD being the most common cause of death in this clinical population (Tonelli, 2006). Patients undergoing HD therapy present high percentages of coronary artery disease and abnormalities in left ventricular structure and function (Foley et al., 1995).

Left ventricular hypertrophy (LVH), dilatation, systolic and diastolic dysfunction, are components of the condition that is used to describe the effects of CKD on myocardium, termed as uremic cardiomyopathy. Uremic cardiomyopathy leads to changes in the systemic hemodynamics that in turn affect the structural and functional characteristics of the myocardium. The main result of uremic cardiomyopathy is LVH, which is manifested in 26% of patients in stage 3 of CKD and in 75% of patients on hemodialysis therapy (Foley et al., 1995) and is considered an independent predictor of survival in CKD patients (Silberberg, Rahal, Patton, & Sniderman, 1989). LVH is the result of hypertension and arteriosclerosis, which occur due to pressure and/or volume overload induced by anemia, arteriovenous fistula and hypervolemia (Alhaj et al., 2013), while hypertension and increased volume overload, probably induce cardiomyocyte hypertrophy and vascular remodeling (London, 2002).

A host of additional complications also influence the development of cardiac diseases in CKD patients such as increased levels of homocysteine, hyperparathyroidism, hypoalbuminemia, oxidative stress and inflammation (Rigatto & Parfrey, 2001). Anemia and impaired mineral metabolism, stimulate hyperphosphatemia and elevated parathyroid hormone levels, leading to vascular calcification by altering the phenotype of vascular smooth muscle cells (Pecoits-Filho, Bucharles, & Barberato, 2012). Hypovitaminosis D, which is a common disturbance of CKD patients, contributes to myocardial hypertrophy and it has been associated with cardiovascular mortality and sudden cardiac death (Drechsler et al., 2010).

Given the high prevalence of cardiac abnormalities and complications in blood circulation, CKD patients are characterized by very low levels of aerobic and functional capacity with many factors being implicated. For example, peak oxygen

uptake (VO_2 peak) in ESRD patients is decreased by ~50% compared to healthy age-matched individuals and therefore the activities of these patients are limited to those intensities that require low levels of aerobic capacity (Painter, 1994). Limitations in oxygen delivery during exercise training are considered the major causes of the reduced VO_2 peak that CKD patients present. It has been suggested that many parameters interact and are responsible for the low levels of aerobic capacity in CKD patients including the decreased cardiac output, which is attributed to the low heart rate, and the low arterial oxygen content induced from anemia and abnormal muscle function, which is attributed to uremic myopathy and neuropathy (Alhaj et al., 2013; Painter, 2009).

Impact on skeletal muscle

Uremia itself contributes to functional and structural muscle abnormalities in patients with CKD, which are more conspicuous in ESRD patients and are considered characteristics of uremic myopathy. Uremic myopathy is a common abnormality presented in CKD patients and is strongly associated with muscle weakness, muscle atrophy and cachexia, which in turn lead to morbidity and mortality and worsen in patients undergoing HD therapy (Floyd et al., 1974). Specifically, muscle wasting, limited endurance, exercise limitation and easy fatigability are common characteristics of uremic myopathy with muscle weakness and muscle atrophy running parallel to the decline of renal function (Campistol, 2002).

Morphological and physiological abnormalities met in CKD patients lead to muscle wasting and loss of skeletal muscle strength. Many studies have shown various factors involved in muscle dysfunction presented in CKD patients and most of them agree that there are several morphological and metabolic factors implicated as

well as other parameters including dialysis modality and stage of the disease. In the following section, a variety of factors affecting muscle function in CKD are presented.

Factors Affecting Muscle Function in CKD

Cross sectional area (CSA) and muscle atrophy

Muscle area is an important determinant of force generation and it has been widely evaluated in many studies. Reduced muscle area has been reported in all muscle fibers types of CKD patients but mainly in type II. Specifically, Crowe et al (Crowe et al., 2007) found that type I and type II fibers from quadriceps femoris muscles had significantly smaller mean diameters in HD patients by 15% and 20% respectively compared to healthy controls. Similarly, Wagner et al (Wagner et al., 2001) who examined rectus femoris muscle biopsies from young HD patients compared to healthy controls found that CSA of type I and type II fibers were smaller in patients by 5% and 18% respectively although the difference between groups was not statistically significant. The histological assessments of Fahal et al 1997 (Fahal, Bell, Bone, & Edwards, 1997) who examined muscle biopsies from the right quadriceps of dialysis patients and healthy controls also showed that type I, IIA and IIB fibers were more atrophic in dialysis patients by 13%, 26% and 31% respectively with a significant difference between groups only in type IIA fibers. In contrast, in the study of Molsted et al (Molsted et al., 2007) it was found that fiber areas from vastus lateralis muscles were smaller in HD patients compared to controls by 21% and 14% in fiber type I and II respectively with significant differences between groups only in type I fibers.

Muscle atrophy in HD patients was also reported in the study of Wang et al (H.-L. Wang et al., 2013) who examined muscle quality and quantity, using magnetic resonance imaging (MRI) and histochemical analysis. From MRI of right thigh muscle it was found that HD patients had less muscle mass and muscle/total ratio

compared to healthy controls. Similarly, histochemical analysis from biceps showed atrophy and irregular fiber shape in HD patients compared to healthy controls (values are not reported in this study).

It is obvious that most of the studies have shown that CKD patients present with muscle atrophy but it has been also reported that CKD patients have larger CSAs compared to controls. Specifically Lewis et al (Lewis et al., 2012) who studied muscle biopsies from vastus lateralis muscle, reported that fibers' CSAs were significantly larger in HD patients compared to controls by 33%, 26% and 28% in type I, IIA and IIX fibers respectively. The authors suggested that this was probably due to fiber edema presented in HD patients but it represents "real life".

Fat accumulation

Fat accumulation has also been observed in skeletal muscle of CKD patients and is an additional factor contributing to muscle dysfunction observed in this clinical population. Wang et al (H.-L. Wang et al., 2013) examined muscle samples of HD patients using MRI and histochemical analysis. From MRI of right thigh muscle it was found that HD patients had higher fat/muscle ratio and intermuscular adipose tissue (IMAT) compared to healthy controls. Furthermore, histochemical analysis in muscle samples from biceps showed an increased adipose accumulation in HD patients. In this study it was also observed an increase in plasma fasting insulin and homeostatic model assessment of insulin resistance (HOMA-IR) which had a positive correlation with fat/muscle and a negative correlation with muscle/total ratio evaluated from MRI.

Capillaries

Capillarization is also a critical factor affecting muscle function since impaired capillaries can impair energy production and oxygen delivery in muscle tissue. In the study of Lewis et al (Lewis et al., 2012) it was found that the number of capillaries contacting each fiber excised from vastus lateralis muscles was significantly higher in HD patients compared to controls by 9%, 10% and 23% for type I, IIA and IIX respectively. Additionally, capillary/fiber ratio (total number of capillaries per total number of fibers within a muscle section) was significantly greater in HD patients by 11% but capillary density (number of capillaries per square millimeter of fiber) was significantly lower by 34% in HD patients. According to the authors, although capillarity was found to be increased in HD patients, their larger fibers' CSAs [see "Cross sectional area (CSA) and muscle atrophy" section] was more intense finally leading to low capillary density and therefore insufficient oxygen supply (oxygen and nutrient exchange) especially under conditions with high oxygen demands (eg exercise).

In contrast, Wagner et al (Wagner et al., 2001) who examined rectus femoris muscle biopsies from young HD patients and healthy controls found that mitochondrial oxidative capacity was not different between groups but capillaries/fiber were reduced (not significantly) in patients by 10% and 14% in fiber type I and II respectively. Additionally, capillary density of HD patients was similar to healthy controls (4% higher in patients).

Myosin Heavy Chain (MHC) expression

One of the well studied parameters in skeletal muscle of CKD patients is the MHC composition and proportion but the results remain controversial. For example,

Molsted et al (Molsted et al., 2007) who examined the MHC composition of vastus lateralis muscle in HD patients found that the relative distribution of MHCI (type I) was significantly lower in HD patients (~35% *vs* ~59%) while MHCIIX (type IIX) was significantly higher in HD patients (~29% *vs* ~7%) compared to controls. Additionally, the relative distribution of MHCIIA (type IIA) was similar between groups (~35%). Furthermore in HD patients, the number of muscle fibers expressed MHCIIX was higher from the number of fibers expressed MHCI. As it is discussed by the authors, an increase in fibers expressing MHCIIX has been linked to physical inactivity in healthy individuals and the physical inactivity which has been observed in HD patients probably leads to the higher content of this MHC isoform. In addition, the low content of MHCI and high content of MHCIIX may affect muscle endurance, aerobic capacity and insulin sensitivity (while type I fibers express higher levels of GLUT4). The histochemical assessments of Fahal et al (Fahal et al., 1997) also revealed a lower prevalence of type I (by 10%) and higher prevalence of type IIA (by 4%) and IIB (by 6%) fibers in muscle biopsies from the right quadriceps of dialysis patients when compared to healthy controls but without any significant difference between groups.

In contrast, Lewis et al (Lewis et al., 2012) who studied individual muscle fibers from biopsies of vastus lateralis muscle found different results. According to their results, HD patients had a significantly higher proportion of type I fibers and lower proportion of type IIX compared to controls while there was not observed any difference in IIA fiber type between groups. As it is discussed, the higher proportion of type I fibers may act as an adaptive response in order to maintain the endurance oxidative capacity.

On the other hand, in the study of Crowe et al (Crowe et al., 2007) who examined muscle fibers' type using staining for ATPase activity there was not found any significant difference between HD and control subjects in the proportion of type I nor of type II fibers excised from quadriceps femoris muscles. Similarly, Wagner et al (Wagner et al., 2001) who examined rectus femoris muscle biopsies from young HD patients compared to healthy controls did not observe any difference in fiber type composition between groups.

Oxidative stress

While oxidative stress is an additional factor which may lead to muscle dysfunction, Crowe et al (Crowe et al., 2007) evaluated the generation of ROS in skeletal muscle (quadriceps femoris) of HD patients. Regarding muscle antioxidant enzymes, catalase activity was significantly reduced in HD patients but there was not observed any difference in superoxide dismutase (SOD) activity. In addition, markers of ROS activity and specifically total glutathione was significantly higher in HD patients but the absolute amount of oxidized glutathione was not different between groups. MDA content was lower in HD patients while protein thiol content was not different between groups. Muscle heat shock protein HSP27 content was significantly higher in HD patients but there was not any difference in HSP60 and HSP70. Laslty, although it has been suggested that oxidative stress is partially responsible for muscle loss presented in HD patients in this study there was not found any relationship between oxidative stress markers and muscle atrophy (see at CSA section) probably due to the muscle chronic adaptations to this stress. However, there is not enough evidence regarding ROS in skeletal muscle of CKD patients.

Metabolic byproducts - Substrates

In 1994, Thomson and coworkers (Thompson et al., 1994) based on the idea of hyperphosphataemia accompanying CKD, conducted a study to examine the metabolism of uremic skeletal muscle at resting conditions and during exercise and recovery using 31P magnetic resonance spectroscopy. The results from intracellular high energy phosphates of patients were compared to age and activity-matched healthy control subjects who underwent the same exercise session. At resting muscle they found that the ration of cell Pi levels relative to ATP levels (Pi/ATP) was significantly higher in CKD patients compared to controls by 25,6% while pH, PCr/ATP, [ADP] and phosphorylation potential were not different between groups. However, exercise duration was significantly lower in CKD patients by 24.8%. The exercise included plantarflexion of the right ankle lifting a progressively higher weight until complaints of fatigue. After the exercise and during recovery there was not any significant difference between groups. It has to be noted that after exercise, mitochondrial ATP synthesis was not different between CKD patients and healthy subjects and this is in contrast with findings in hemodialysis patients.

Neuromuscular function

Another factor which has been studied in order to explain muscle dysfunction in CKD is neuromuscular function. Z'Graggen et al (Z'Graggen et al., 2010) examined the hypothesis that muscle function of HD patients is negatively affected from membrane depolarization of muscle fibers. For the purpose of their study they recorded the velocity recovery cycles (VRC) of brachioradialis muscle immediately before and after HD as well as 1 hour after HD and they compared the results with healthy control subjects. Evaluation of VRC consists of 4 parameters [relative refractory

period (RRP), early supernormality (ESN), late supernormality (SN100) and extra late supernormality (XSN100)] and all of them can provide information about axonal membrane potential and ion channel function. According to the results, before HD treatment RRP was significantly prolonged in HD patients compared to controls. In contrast, ESN and XSN100 were significantly reduced in HD patients while SN100 tended to be significantly reduced in patients. Immediately after HD treatment all parameters were corrected reaching control values except SN100 while 1 hour after HD, RCV values tended to return at the pre HD values. Interestingly, ESN found to be negatively correlated with serum potassium while XSN100 was negatively correlated with sodium and chloride concentrations. Overall, HD patients presented with depolarization of muscle membranes which was corrected immediately after HD treatment. This leads to a small number of available sodium channels and therefore supernormality and superexcitability reductions. The negative correlation between serum potassium and early supernormality as well as the normalization of both of them after HD suggest that depolarization is mainly caused by hyperkalemia. The authors also suggested that the reduction of SN100 in HD patients revealed abnormalities in t-tubule function and therefore in calcium release which is not corrected sufficiently after HD treatment.

Dialysis modalities

It has been also reported that the stage of the disease as well the dialysis modalities play a crucial role in muscle function through alterations in muscle morphology. For example McIntyre et al (McIntyre et al., 2006) evaluated muscle and fat CSAs in slices of thigh muscle from CKD (stage 4), PD and HD patients. Dialysis patients (PD and HD) presented with significantly lower muscle CSAs compared to

CKD patients by 9% without any difference between PD and HD patients. This lower muscle mass of dialysis patients was also reflected at their lower (significantly only for males) functional capacity assessed by sit to stand tests. It was also observed that fat CSA tended to be significantly increased in PD patients compared to CKD and HD patients by 9% and 15% respectively and as it is discussed by the authors this could happen due to glucose-based dialysis solutions.

Sakkas et al (Sakkas et al., 2004) examined the effect of dialysis modality in muscle morphology and capillarization of gastrocnemius muscle. It was not found any difference in fiber type distribution and MHC expression nor in central nuclei between PD and HD patients. Nevertheless, it was found a trend for larger CSAs in HD patients which could be explained by the fact that most of the HD patients were males. Furthermore HD patients presented with significantly higher capillary contact/fiber by 33%, capillary to fiber ratio by 19% and cytochrome c activity in fast fibers by 33% compared to PD patients. According to the authors this may be due to heparin treatment in routine HD treatment and together with anemia and acidosis which were more prominent in HD group may lead to angiogenesis. However, the differences in capillarization were not reflected in physical capacity assessed by sit to stand and walking tests while the results were similar between groups.

In addition it has been found that not only the uremic environment but also the HD treatment per se leads to dynamic changes in genes responsible for inflammation and apoptosis. Specifically, Shah et al (Shah et al., 2006) who examined vastus lateralis muscle of HD patients before and after HD treatment found that before HD treatment 91 genes (83 of them upregulated) were altered in HD patients' skeletal muscle compared to healthy controls. Furthermore, HD treatment caused changes in 23 genes (21 of them upregulated), 21 of them are biologically interactive and

partially responsible for various processes such as cell signaling, cell proliferation, protein metabolism, inflammation and apoptosis. Furthermore Raj et al (Raj et al., 2005) also examined oxidative stress markers and cytokines in muscle biopsies from vastus lateralis of HD patients. Hemodialysis led to a significant increase in skeletal muscle interleukin-6 (IL-6) (from 0.028±0.02 to 6.69±0.21), heme oxygenase-1 (HO-1) (0.96 ± 0.01 to 5.08 ± 1.11) and suppressors of cytokine signaling (SOCS-2) (0.63 ± 0.12 to 0.82 ± 0.14) mRNA levels indicating that HD treatment itself is a source of inflammation and oxidative stress and therefore is responsible for muscle wasting presented in this group of patients. Gene expression of additional markers of inflammation in skeletal muscle was also increased but not significantly [IL-1β and tumor necrosis factor-a (TNF-a)] while IL-10 was not detectable in skeletal muscle. Lastly, expression of IL-6 in skeletal muscle of HD patients, examined by immunohistochemical staining, was found to be increased compared to expression before HD treatment and control subjects.

CKD patients also appear with low aminoacids' (AA) concentrations leading to decreased protein synthesis and propably to increased protein degradation and therefore muscle weakness (Johansen, 2009). Asola et al (Asola et al., 2008) compared the effects of two peritoneal dialysis modalities (1.1% AA solution and glucose based solution, lasted six-weeks each one) in thigh skeletal muscle AA uptake. AA uptake was evaluated at both fasting and insulin stimulation conditions using positron emission tomography (PET). It was found that AA solution caused a higher AA uptake by 32% and 26% in the fasting and insulin stimulation respectively compared to glucose based solution. AA solution also caused a higher uptake of the six "system A AAs" by 24% and 16% in fasting and during insulin stimulation respectively compared to the conventional glucose based solution.

The dialysis bath has also been examined as a possible factor affecting neuromuscular function in HD patients. Coppolino et al (Coppolino et al., 2007) examined the hypothesis that plasma potassium reduction due to standard acetate-free biofiltration negatively affects neuromuscular function. Therefore they studied the effect of 2 techniques of acetate-free biofiltration (AFB) on the motor action potential (cMAP) and force production of HD patients before, during and at the end of HD treatment (6 time points). Specifically the first one was the standard technique with constant AFB and the second one with variable potassium concentration (AFB_k) in the hemodialysis bath. Neuromuscular excitability was assessed at the right abductor digiti minimi muscle (ADM) which was stimulated from the wrist ulnear nerve of the opposite arm and force generation was evaluated in the right index finger and thumb using a dynamometer. It was found that in contrast to AFBk, AFB caused a significant reduction in cMAP after 15 and 45 minutes from the beginning of dialysis treatment where force was also significantly reduced (values are not reported). Additionally, both techniques caused significant reductions in electrical membrane potential at rest (REMP) in all time points (compared to baseline) but AFB caused a significantly greater reduction at 15 and 120 minutes compared to AFBk. Potassium reduction leads to membranes' potential changes which depend on the potassium concentration. According to the authors, AFB caused reductions in cMAP and force because of the sudden drop of serum potassium concentrations which leads to the blocking of cellular depolarization and sodium channel, finally leading to low force generation.

Other factors

In the study of Lewis et al (Lewis et al., 2012) muscle fibers of HD patients from vastus lateralis muscle also presented with significantly lower succinate

dehydrogenase (SDH) activity compared to control subjects by 29%, 40%, and 47% in type I, IIA and IIX fibers respectively. In this study a few biopsies were also underwent an ultrastructural analysis in order to observe differences in mitochondria, myocyte and Z-band disruptions as well as glycogen depletion. In two-thirds of biopsies which were analyzed, the authors observed changes in mitochondria and this is in agreement with the low oxidative capacity observed from SDH activity in HD patients. Furthermore, in the biopsies with mitochondrial abnormalities there was also observed myocyte and Z band disruption by 9% and glycogen depletion by 18%.

Lopez et al (López et al., 2005) evaluated the calcium concentration in skeletal muscle of HD patients using Ca^{2+} selective microelectrodes. It was found that resting intracellular calcium concentration was significantly higher in all uremic muscle fibers from intercostal muscle biopsies (48 hours after hemodialysis) compared to controls by ~235% but resting membrane potential was not different between groups. Additionally, removal of extracellular Ca^{2+} or incubation with a dihydropyridine receptors' specific antagonist (antagonist named nifedipine) did not affect intracellular calcium neither in uremic nor in control fibers indicating that the source of elevated intracellular calcium was mainly of intracellular origin. Indeed, the intracellular calcium elevation was reversible after applying an antagonist of cyclic adenosine diphosphate-ribose (cADPR) (antagonist named 8-bromo-cADPR) indicating that impaired regulation of intracellular Ca^{2+} was at least partially affected by cADPR. cADPR is a regulator of intracellular Ca^{2+} concentration synthesized by ADP-ribosul cyclases and degraded by c-ADPR-hydrolase to ADP- ribose. Therefore, these results indicate that uremic toxins may affect the cADPR signaling pathway in skeletal muscle causing elevated intracellular calcium concentration and therefore impairing muscle function in CKD patients.

Counteracting Measures - Exercise interventions

Many studies have studied the effect of exercise training in correcting muscle function and morphology of CKD patients with encouraging results. For example Molsted et al (Molsted, Andersen, Harrison, Eidemak, & Mackey, 2015) examined the effect of a 16-week high intensity resistance exercise training (3 times per week) in satellite cells' (SC) content of muscle biopsies from vastus lateralis of HD patients. Myonuclei content and domain were also assessed. At baseline, SC content was significantly higher in type I compared to type II fibers but after correcting for fiber area the difference was not significant between fiber types. During the training period, SC content was significantly increased by 15% in type I fibers without any change in type II fibers. On the other hand, myonuclei content of type I fibers did not alter after the training period but in type II fibers, myonuclei content was significantly increased by 13%. Regarding myonuclei domain there was not found any difference before nor after training period. Fiber size and distribution between fiber types also remained unchanged but knee extension torque was significantly improved by ~23%.

Castaneda et al examined the effect of a low protein diet in combination with resistance training or low protein diet alone for 3 months in vastus lateralis morphology of pre-dialysis CKD patients. After the exercise period, CSAs of type I and II fibers were significantly increased by 24% and 22% respectively compared to non exercised patients. These improvements were accompanied by reductions in C-reactive protein (CRP) and IL-6 (Castaneda et al., 2004). The same intervention also led to a significant increase of total body potassium by 4% through the anabolic effect of resistance training (Castaneda et al., 2001).

Sakkas et al (Sakkas, Sargeant, et al., 2003b) studied the effect of a six-month aerobic exercise training in skeletal muscle morphology of dialysis patients (HD and PD). Gastrocnemius muscle biopsies were obtained and analyzed before and after the training period. Although it was not found any change in MHC distribution and cytochrome c oxidase concentration between pre and post training periods, fibers' CSAs were significantly increased by 32% in type I, 54% in type IIA and 36% in type IIX fibers. In addition, the proportion of atrophic fibers was also decreased from 51 to 15% in type I, from 58 to 21% in type IIa and from 62 to 32% for type IIX muscle fibers. Capillary contact was also improved by 24%. Thus, 6 months of aerobic exercise training corrected muscle atrophy and capilarization in skeletal muscle fibers of dialysis patients.

Wagner et al (Wagner et al., 2001) also examined the angiogenic growth factor gene responses due to a session of exercise in young hemodialysis patients. Patients underwent a one-hour training session at a leg-kick ergometer (50% of maximal capacity). Rectus femoris muscle biopsies were collected during rest and exercise. Although vascular endothelial growth factor (VEGF) mRNA was significantly increased after exercise in both groups, basic fibroblast growth factor (bFGF) and transforming growth factor (TGF-β1) were not altered after exercise in either group.

Balakrishnan et al (Balakrishnan et al., 2010) investigated the effect of a twelve-week resistance exercise training program in mitochondrial biogenesis of vastus lateralis muscle biopsies from CKD patients (stage 3 and 4). Patients, were randomly assigned to the resistance training group or the attention control group (only stretching and flexibility exercises) and both groups followed a low protein diet 2 to 8 week before the beginning and until the end of the exercise training period. In the

current study CSAs of type I and type II fibers, whole-body muscle strength and metabolic factors (i.e. GFR, CRP, IL-6, HOMA-IR, IGF-1) were also assessed. At baseline, mitochondrial DNA copy number (mtDNA copy number) was higher in the attention control group (P=0.08) while IL-6 was higher in the training group (P=0.07). After the 12-week period, exercise group presented with a significant increase in median mtDNA copy number by 7.5% compared to baseline, while a significant reduction was observed for the control group (18%). Additionally, the changes in mtDNA copy number were significantly associated (positively) with alterations in CSAs of both fiber types (values are not reported). Thus, despite the low protein diet, resistance training improved mitochondrial content and muscle mass of patients. However, in some patients the mtDNA copy number was reduced after exercise period as it was observed in the control group (probably due to low protein diet without exercise training).

Molsted et al (Molsted, Andersen, Eidemak, Harrison, & Jørgensen, 2014) examined the association of hormone levels [testosterone, luteinizing hormone (LH), insulin-like growth factor 1 (IGF-1) and insulin-like growth factor-binding protein 3 (IGF-BP3)] with muscle fiber size and muscle strength before and after a 16-week supervised heavy load resistance training program (3 times per week) of male dialysis (HD and PD) patients. At baseline, total testosterone found to be in a normal range compared to age-matched reference population (not from this study) and this finding was in contrast to other studies reporting low levels of testosterone in dialysis patients. In contrast, free testosterone, LH, IGF-1 and IGF-BP3 were higher in patients compared to reference population. Regarding the association of hormone levels with muscle fibers' size from vastus lateralis muscle it was found that free testosterone (but not age-adjusted free testosterone) had a significant positive

association with type II muscle fibers' size whereas IGF-1 (and age-adjusted IGF-1 too) had a significant positive association with both type I and II fiber size. After the training period all hormones remained unchanged but when comparing delta values of the control and training period total testosterone/LH was changed. Muscle strength measured by knee extension improved after training period by 19-25% without any significant change in muscle fibers' size neither of type I nor of type II fibers. According to the authors this was an unexpected result while testosterone levels were normal and it is known that resistance training causes an increase in muscle mass in subjects with normal testosterone levels compared to subjects with low levels. The authors also explain that since the increase in muscle strength was not due to an increase in muscle mass it was probably due to neuromuscular improvements.

Molsted et al (Molsted, Eiken, Andersen, Eidemak, & Harrison, 2014) studied the proinflammatory cytokine IL-6 and vitamin D (reflected in 25-OH D) and their associations with muscle size and function of dialysis patients (HD and PD) after a 16-week high intensity resistance training. Muscle power (leg extensor power) was significantly increased after training period by 20-23% and physical function measured through the chair stand test was also significantly improved by ~22%. However the training period had not significant effects on muscle fiber size of vastus lateralis muscle. In addition, levels of IL-6 were not changed after training period but according to the authors this could be explained by the type of exercise or the origin of low grade inflammation. High levels of IL-6 were associated with older patients, low muscle power, low protein intake but not with low muscle fiber size. As it is suggested, the fact that IL-6 was not found to act as an inhibitor in muscle hypertrophy it was surprising but it may act synergistically with other factors such as uremia, insulin resistance, anabolic hormone deficiency and acidosis. Regarding

levels of 25-OH D, at baseline, 51% of patients had vitamin D deficiency. 25-OH D was significantly decreased after the 16-week training program but this may happened because the end of the training program was during winter where 25-OH D was found to be significantly lower. Normal values of 25-OH D were correlated with lower percentage of type IIX fibers and increased size of type I fibers. After adjustment for age and season, normal values of 25-OH-D were still associated with the reduced percentage of type IIX fibers but reduced size of type I fibers. 25-OH-D had not any correlation with muscle power and morphology throughout the study but lower levels during training period had a correlation with an increase in type II muscle fiber size. The improvements in muscle power and physical function after the training period were not correlated with IL-6 nor with 25-OH D values.

The Practicality of Animal Models

Several animal models of CKD have been introduced in order to overcome the confounding factors of human disease (such as years from diagnosis, comorbidities, pharmaceuticals and nutritional status) and to study particular mechanisms in a limited time frame (Becker & Hewitson, 2013). Using such experimental models, researchers are able to understand the physiology and pathophysiology of the disease and therefore to examine potential novel therapies, or to improve the existing ones (Becker & Hewitson, 2013).

Depending on the mechanism of interest, CKD can be induced by *in vitro* or *in vivo* models and the choice of the model is of crucial role for the outcomes of each study. *In vitro* models are of small utility while results may vary depending on the cell type examined and therefore studies examined this kind of models are limited (Yang, Zuo, & Fogo, 2010). On the other hand, *in vivo* models are of larger interest while outcomes have a better reflection in *in vivo* physiology. As a result, a lot of *in vivo* animal models of CKD have been introduced such as genetically engineered models, spontaneous models and acquired models (immune or non-immune induced models). Non-immune models can be induced in many ways including the reduction of renal mass by surgical intervention, known as nephrectomy, which leads to a CKD animal model of known etiology (Yang et al., 2010).

Another critical factor for animal models is the choice of the right animal. There has been introduced a variety of species (mice, rats, rabbits, dogs) as animal models of CKD, but the right choice depends on the particular aspect of the disease which is going to be reproduced each time. Murine models of kidney disease are increasingly preferred because of their small size, their low cost housing and the genetically defined strains (Anders & Schlöndorff, 2000). However, rodent models,

remain the closest to human disease species (Becker & Hewitson, 2013) and especially rabbits are viable and adequate models for surgical interventions (Calasans-Maia, Monteiro, Áscoli, & Granjeiro, 2009).

One of the surgical interventions of renal mass reduction was developed in 1982 by a two-step partial nephrectomy (Gotloib et al., 1982). First, partial nephrectomy of left kidney was induced by electrocauterization keeping 1/3 of the kidney functional and after an interval of two weeks, a total removal of the right kidney was performed through a flank incision and by extraperitoneal approach. In this way the authors established a reliable animal model for investigating the metabolic complications of continuous ambulatory peritoneal dialysis. This surgical approach that aims reproducing CKD by reduction of renal mass has been achieved by using different protocols and in various animal species (Dobbie, 1993; Gotloib et al., 1982; Ma et al., 2005; Ma & Fogo, 2003; Oreopoulos, Balaskas, Rodela, Anderson, & Oreopoulos, 1993; T. Wang et al., 1997).

Our research interest focuses on uremic myopathy. Therefore, we chose the animal model of White New Zealand rabbit (WNZ) that could provide us the necessary experimental material for a variety of experiments, while remaining were shared for further experiments respecting the “Three R’s” (Institute for Laboratory Animal Research, 2011).

Unanswered Questions

From the current literature review, it can be concluded that while conditions affecting muscle function in uremic patients have been widely described examining a variety of possible factors implicated, results have been usually dealing with macroscopic parameters, clouded by comorbidities, and there is not yet a clear explanation about the exact mechanism underlying muscle dysfunction in CKD. Specifically it is not clear whether the muscle cell *per se* is affected in early disease and whether its mechanical operation, as for example reflected by its ability to generate isometric force, may be discernibly impaired.

Clearly, there is a gap in the literature regarding the contractile properties of uremic single muscle fibers not only in resting conditions but also in conditions mimicking fatigue such as acidosis and oxidative stress.

To answer such questions it is important to use methodology that avoids confounding factors (such as years from diagnosis, comorbidities, pharmaceuticals, gender, and nutritional status) which are unavoidable when studying patients. This issue can be satisfactorily addressed by employing an animal model mimicking chronic renal insufficiency. Moreover, to exert precise control over the factors affecting muscle function at rest and fatigue, independently of any degree of neuropathy, we herein propose to address such questions using the skinned fiber method where the surface membrane of fibers is chemically or mechanically skinned and intracellular solutions can be altered and controlled according to the conditions researchers need to mimic. The results of skinned fiber studies may lead to important conclusions about mechanisms of muscle contraction and/or fatigue and give information regarding the sarcomeric proteins' function, an area which has not been yet addressed in the literature.

CHAPTER 3: RESEARCH PAPER 1

Evidence of muscle fiber functional deficits in experimentally-induced renal insufficiency

Aims addressed in this paper: using tissue from an animal model of uremia to examine the effect of chronic renal insufficiency on

1. force generation of isolated muscle fiber preparations in conditions mimicking the resting state
2. force generation of isolated muscle fiber preparations in conditions mimicking acidosis
3. morphology of isolated muscle fiber preparations

ABSTRACT

Introduction: Chronic kidney disease (CKD) patients present with muscle abnormalities (uremic myopathy) whose mechanisms have not yet been fully elucidated. The aim of this study was to investigate whether renal insufficiency affects skeletal muscle contractile properties at the cellular level. **Materials and Methods:** Renal insufficiency was induced surgically in New Zealand rabbits (UREM), with sham-operation for controls (CON), using ethically approved procedures. At 3 months post-surgery, following euthanasia, psoas muscle samples were excised. Sample treatments, dissection and all evaluations were conducted in a blind fashion. Maximal isometric forces were recorded from maximally calcium activated single fibers (n=142 CON, n=240 UREM) at 'standard' baseline conditions (pH 7, 10°C), and at a near physiological temperature (pH 7, 30°C) in a subset of fibers; the effect of acidosis (pH 6.2) was also evaluated. **Results:** Renal insufficiency resulted in significantly smaller average CSA (~11%) for UREM muscle fibers compared to CONs ($P<0.01$). At standard conditions, UREM fibers produced lower absolute and specific forces (vs CON, $P<0.01$); force increased by exposure to 30°C ($P<0.01$), percentage-wise more so in UREM fibers vs CONs, however the initially observed force disparity remained significant. For both groups, acidosis significantly reduced force production (vs pH 7, 10°C $P<0.01$), with a similar percent force decline in both groups (UREM -48% vs CON -43%, $P>0.05$). **Conclusion:** Chronic renal insufficiency induced significant impairments in single psoas muscle fibers' tension that were only partially explained by atrophy. Further investigation is warranted to pinpoint the contributions of possible changes in sarcomeric protein properties to the evident functional deficit.

Key words: Chronic kidney disease, isometric tension, skinned fibers, specific force, animal model

INTRODUCTION

Chronic kidney disease (CKD) is increasingly recognized as a major global health problem affecting 40-50% of EU and USA populations (Grams et al., 2013; Zoccali et al., 2010), as well millions in Asia (Abraham et al., 2016), Latin America (Cusumano & González Bedat, 2008) and elsewhere. Whole body function and skeletal muscle phenotype are heavily impacted by CKD (Kaltsatou et al., 2015; Sakkas, Ball, et al., 2003; Sakkas, Sargeant, et al., 2003a) with patients presenting with muscle weakness, limited endurance and fatigue intolerance (Campistol, 2002), neuropathy (Adams & Vaziri, 2006) and a host of other striated muscle problems. Collectively described as *uremic myopathy* (Campistol, 2002), this muscular dysfunction promotes loss of independence and contributes to patients' high cardiovascular and metabolic morbidity, resulting to poor quality of life and early mortality (Johansen, Chertow, Jin, & Kutner, 2007; Pereira et al., 2015). Thus, there is an urgent need to generate new knowledge to enhance our understanding of the characteristics and progression of striated muscle pathophysiology in CKD in order to support the design of appropriate interventions to prevent or alleviate its devastating impact.

The safeguarding of skeletal muscle mass and function has been recognized as a critical challenge in the end-stage CKD, as indicated by the existing literature. In patients, muscle pathology is exacerbated but only partly explained by inactivity (Sakkas, Sargeant, et al., 2003a).

While both intramuscular energetics disturbances and central activation failure are implicated (Johansen et al., 2005), the mechanisms underlying peripheral fatigue in CKD have not yet been fully understood. A number of interventions implemented so far, while greatly beneficial (P. L. Gordon, Sakkas, Doyle, Shubert, & Johansen, 2007; Johansen et al., 2006; Sakkas, Sargeant, et al., 2003a), have failed to fully

correct for muscular deficits. Moreover, patients present with neurological and quality of life problems (Giannaki et al., 2011), sleep disturbances (Sakkas, Karatzaferi, et al., 2008), anxiety or symptoms of depression (Grigoriou, Karatzaferi, & Sakkas, 2015; Mitrou et al., 2013). Additionally, along with the individual patient's characteristics, the interaction of disease specific and/or toxicity factors and disuse, as in other chronic conditions, is not easily untangled (Malavaki et al., 2015). All of the above are adding on the complexity of the challenge to *explain* and ultimately *prevent and/or ameliorate* uremic myopathy. Given most research so far has been done at the end-stage, key issues related to disease progression and contractile mechanisms are still unanswered, such as; *do sarcomeric muscle deficits develop early during the disease progression*? and, if yes, *could they have a measurable effect on muscle contractile properties per se*?

To answer such questions it is important to use methodologies that a) avoid confounding factors (such as years from diagnosis, comorbidities, pharmaceuticals, gender, and nutritional status) which are unavoidable when studying patients, and b) allow for the functional assessment of the contractile machinery *per se* and has relevance to physiology *in vivo*.

The first issue can be adequately addressed by employing an animal model mimicking chronic renal insufficiency [e.g. surgically induced (Gotloib et al., 1982)] to provide muscle tissue. The second issue requires an evaluation that focuses on the sarcomeric level of function (and is thus acutely independent of metabolic and neural factors). This can be addressed by using the isolated single, muscle cell preparation with a permeabilized sarcolemma (*aka* skinned fiber) to assess *in vitro* the effects of selected conditions on muscle contraction at the single cell level (Cooke & Bialek, 1979). This type of cellular preparation allows for the reliable study of sarcomeric

function (e.g. by assessing maximal isometric tension and the evaluation of individual or combinations of factors, such as pH (Fitts, McDonald, & Schluter, 1991), temperature, etc (Karatzaferi et al., 2004; Ranatunga, 2010) with high physiological relevance (Karatzaferi et al., 2004, 2008).

Therefore, the aim of this study was to evaluate, for the first time, the effects of chronic renal insufficiency on the contractile properties of isolated muscle fiber preparations from an animal model, in a blind design. We examined whether chronic renal insufficiency affected the ability of the cell to produce maximal isometric force. We also evaluated whether the contractile 'response' to acute acidosis was altered. Moreover, considering the importance of temperature in translating our *in vitro* findings to *in vivo* function we appropriately employed the temperature-jump method to examine isometric tension at a temperature closer to physiological.

MATERIALS AND METHODS

Animal model

All animal procedures, including surgery and euthanasia for this project 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, in accordance to the national directives for the care and use of laboratory animals. Nine New Zealand young adult white female rabbits (N=9) with a body weight (BW) of ~ 3200g were housed in a controlled environment with stable conditions (temperature 22–24°C, 12:12 h light-dark cycle) and were acclimatized for 48 hours. Rabbits were fed ad libitum a special rabbit chow containing low levels of protein, potassium, calcium, phosphorus and sodium (prepared by Research Diets, Inc. USA) and water ad libitum.

After acclimatization, surgical procedures were performed (sham operation for control animals - CON and partial nephrectomy for experimental animals –UREM). Animals were anaesthetized by intravenous administration of a solution mixture of ketamine hydrochloride 100 mg/ml (Imalgene® 1000; Merial, Duluth, Georgia, USA) and xylazine 20 mg/ml (Rompun®; Bayer, Leverkusen, Germany), 87% and 13% respectively (proportion 6.69:1 approximately). The initial dosage for the induction of anesthesia was 0.3 ml/Kg BW of the above solution mixture, i.e. Imalgene® (87%) and Rompun® (13%), intravenously (i.v.). The maintenance of anesthesia was achieved by a dose of 0.2 to 0.3 ml i.v. of the solution mixture. Animal temperature was maintained via a heating pad. Three hours before the intervention, each animal had only access to water and its weight was measured on a precision scale. The induction of renal insufficiency was performed surgically (using a surgical protocol modified from Gotloib et al., 1982)(Gotloib et al., 1982). For the UREM group six

animals (N=6) underwent removal of the left kidney after careful ligation of the left renal artery and vein; and partial nephrectomy (¾) of right kidney. For the CON group, three age –matched animals (N=3) underwent sham operation and were considered as the control group (CON animals). In a previous pilot study (data not shown) sham-operated muscle contractility was comparable to non-procedure animals. Thus, in agreement with the principle of Reduction in Animal Research sham-operated animals were used as controls in the present study.

Twelve weeks after surgery, animals were weighted and then sacrificed by injection of sodium pentobarbital solution (50 mg/ml) which was applied in a dosage of 100 mg/Kg BW followed by bilateral thoracotomy. Immediately after cardiac arrest, blood samples were collected from heart and aorta using a heparinized syringe for subsequent serum urea and creatinine determination using standard photometric protocols. Urea concentration in serum was determined with the colorimetric method using a commercially available kit (ab83362, Abcam), a 96-well microtiter plate and a programmable microplate reader (Biochrom, Asys Expert 96). Urea concentration in unknown samples was determined by comparison with the standard curve. Creatinine concentration in serum was determined with the colorimetric method using a commercially available kit (ab65340, Abcam), a 96-well microtiter plate and a programmable microplate reader (Biochrom, Asys Expert 96). Creatinine concentration in unknown samples was determined by comparison with the standard curve.

Psoas muscle was fast excised and processed for contractility studies (see below) while the remaining of the animal's tissues was shared for other approved protocols. Sample collections were done in a blind fashion.

Muscle Samples

Psoas muscle samples from UREM and CON animals were permeabilized as previously described (Karatzaferi et al., 2008). Briefly, thin bundles of rabbit PSOAS muscle (~2 mm diameter) were dissected and tied to wooden sticks using surgical thread. The samples were placed in falcon tubes containing skinning buffer solution ($0^{o}C$) [120 mM KAc, 50 mM 3-(N-Morpholino) propanesulfonic acid (MOPS), 5 mM $MgAc_2$, 4 mM ethylene glycol tetraacetic acid (EGTA) and 50% glycerol (v/v); pH 7] with the addition of 100 μl, per 50 ml end volume, of a protease inhibitor cocktail (104 mM AEBSF, 80 microns Aprotinin, 4 mM Bestatin, 1.4 mM E-64, 2 mM Leupeptin and 1.5 mM Pepstatin). Samples were placed on a vibrating platform shaker (Heidolph-Titramax 100) in a parallel to the ground position and shaken at 350 rpm for 24 hours at 0°C. Thereafter the skinning solution was replaced with fresh solution and muscle samples were stored at $-20^{o}C$ until mechanical assessments. Chemicals were purchased from Sigma-Aldrich (via national retailers Life Science Chemilab SA, Athens, Greece and Anadrasis, Thessaloniki, Greece).

Experimental setup for single fiber mechanics

For their mechanical assessment, each single fiber was dissected from the muscle bundle on a cold stage under a stereomicroscope and the fiber ends were attached between two tissue mounts of a muscle micro dynamometer. The tissue mounts were connected to a force transducer and a motor arm (used as a fixed end). The fiber was then immersed in baths each holding ~200 μl of experimental solution (refer to the experimental solutions section). Data were continuously recorded and later exported for further analysis.

The customized micro dynamometer with a resolution of 0.4 μN to 4 N (SI Heidelberg/WPI) consists of a horizontally translocating platform with five independently temperature controlled baths (via peltier) supported by a cooling water circulator (Thermo Electron Haake WKL 26 re-circulator chiller 3L capacity bath). A He-Ne laser and a system of mirrors with the use of a camera allow for sarcomere length measurements to be taken. Fibers can be switched between baths in less than 250 ms allowing for rapid temperature jumps (T-Jump).

For this study the temperature of the baths was maintained at 10°C and 30°C. As detailed elsewhere (Karatzaferi et al., 2004), the advantage of the T-Jump method is that the fiber may be first activated at a low temperature where the sarcomere arrangement is stable and it can then be rapidly transferred to a higher, closer to physiological, temperature in order to generate the maximal force without ruining the sarcomere arrangement (for an indicative experiment see Figure 1).

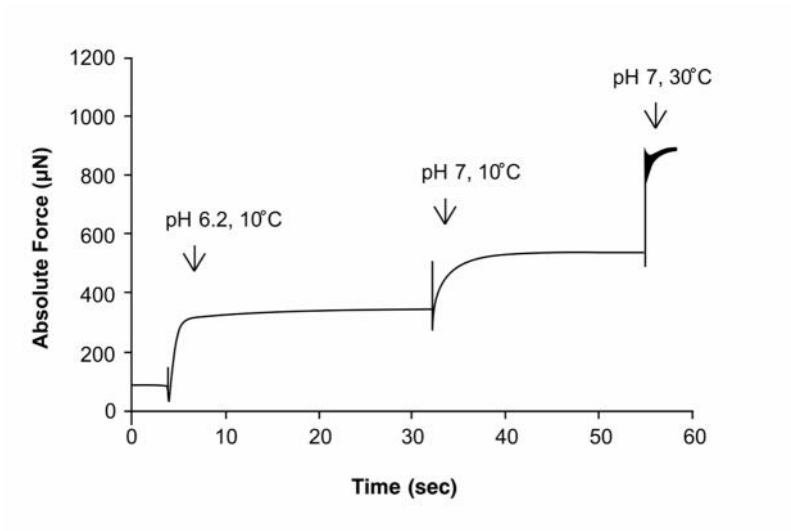

Figure 1. Indicative example of single fiber force recordings: A fiber was first immersed in a relaxing solution with pH 6.2 and then in an activating solution with pH 6.2, 10°C. Once a steady state force was reached, the fiber was transferred in the activating solution with pH 7, 10°C and was allowed again to reach a steady state

force. After the low temperature activation, the fiber was activated in an activating solution with pH 7, 30°C (T-Jump). Fibers were returned to a relaxing solution of the pH of interest in between and reactivated to verify stability (not shown). Up to five maximal activations were possible.

Experimental solutions

For contractile assessments the basic rigor buffer contained 120 mM KAc, 50 mM MOPS, 5 mM $MgAc_2$ and 1 mM EGTA for pH 7 or 120 mM KAc, 50 mM MES, 5 mM $MgAc_2$ and 1 mM EGTA for pH 6.2. Relaxing solution was achieved with the addition of 5 mM ATP in the corresponding rigor buffer (pH 7 or pH 6.2). Lastly, maximal calcium activation was achieved with the addition of 1.1 mM $CaCl_2$ in the relaxing solution. The ionic strength of the solutions was ~0.2 M.

To exclude the possibility that the calcium concentration used to maximally activate control fibers might not be sufficient for uremic fibers, a small pilot study was first undertaken where approx. 40 control and uremic fibers (data not shown) were blindly assessed in three to four pCa concentrations at pH 7, 10°C. From that pilot study it was concluded that the standard addition of 1.1 mM $CaCl_2$ (pCa 4.4) provided maximal isometric tension for both groups and was thereafter used.

Maximal Isometric Tension measurements

All assessments and initial data reductions were done in a blind fashion. Maximal isometric tension was first evaluated at resting conditions (pH 7) and 10°C at resting sarcomere lengths (2.2-2.4 μm). Each fiber was first immersed for 1 minute in a bath containing rigor solution (to wash out excess glycerol) and then it was transferred and equilibrated for 2 minutes in a bath containing relaxing solution. Average diameter was determined for subsequent cross-sectional area (CSA)

calculations assuming a cylindrical shape. Consequently, a fiber was maximally activated to contract isometrically at pH 7, 10°C and 30°C.

A subset of fibers underwent an assessment at 10°C, in both resting (pH 7) and acidic (pH 6.2) conditions while few fibers were also assessed at 30°C. In this set of measurements each fiber was maximally activated to contract isometrically first in one pH condition, at 10°C and it was then activated in the other pH condition, at 10°C. Thereafter, the fiber was maximally activated at 30°C for 3 to 5 sec at the corresponding pH of the latter activating solution. Finally, the fiber was re-assessed in the initial pH and temperature solution (to fulfill criteria of stability, e.g. ≤10% initial force decline). To avoid an order effect, fibers were randomly assigned to be first activated in pH 7 and then in pH 6.2 and vice versa. Depending on the pH of the first activating solution (pH 7 or pH 6.2) the fibers were first immersed in the corresponding rigor and relaxing solutions.

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. To examine possible differences in the response to either temperature or pH, force change was calculated in percentages and the differences between groups were tested using unpaired analysis (t-test for independent samples). All statistical analyses were performed using a

commercially available statistical package (SPSS 15.0 for Windows) and the significance level was set at $P<0.05$.

RESULTS

Both surgery procedures (¾ partial nephrectomy-UREM animals and sham operation-CON animals) were well-tolerated and animals presented with a normal after-surgery recovery. At the end of the twelve-week period post-surgery, body weight ranged between 3,500-4,965 for CON (with mean ± SD of 4,105 ± 765) g and 1,970-4,585 (2,988 ± 1,045) g for UREM animals ($P>0.05$). Renal insufficiency in experimental animals, compared to controls, was reflected in raised serum creatinine (CON, 1.38 ± 0.09 mg/dl, vs UREM, 2.67 ± 1.15 mg/dl, $P<0.05$) and urea levels (CON 40.67 ± 4.62 mg/dl vs UREM 67.33 ± 32.02 mg/dl, $P>0.05$).

A total of 142 CON and 240 UREM single fibers were assessed at 10°C and pH 7 (pH 7 representing a 'resting' state) under maximal calcium activation ('pool of fibers' in 'standard in vitro conditions'). Of those, a subset of 41 CON and 73 UREM fibers were also assessed at 30°C using the t-jump method ('temperature effect').

In order to examine any differences in functional response to acidosis (pH 6.2), another subset of the pool fibers (25 CON and 48 UREM fibers) were exposed to both resting (pH 7) and acidic (pH 6.2) conditions at 10°C. It was also possible for 13 CON and 21 UREM fibers to be assessed at 30°C, pH 6.2.

Morphological characteristics of single fibers

Average fiber diameters differed significantly between groups as revealed by unpaired t-test analysis (Mean ± SD CON 84.5 ± 9.3 vs 79.6 ± 9.4 μm, $P<0.001$). Likewise, calculated cross sectional areas (CSAs) of UREM fibers were significantly lower compared to CONs (CON 5,671 ± 1,259 *vs* 5,040 ± 1,189 μm^2, $P<0.001$), indicating a level of ~11% atrophy. Thus, thereafter force analysis data will be presented for both absolute and specific values (i.e. corrected for calculated CSA).

Contractile properties of single fibers at resting conditions (pH 7)

Descriptive and inferential statistical analysis results of main data are presented in Table 1 and Figure 2. Whether on absolute or specific force values, GLM analysis indicated a statistically significant main effect of group [CON vs UREM, $F(1,492)=83.6$, $P<0.001$ and $F(1,492)=33.1$, $P<0.001$ respectively] and temperature [$10^{o}C$ vs $30^{o}C$, $F(1,492)=114.6$, $P<0.001$ and $F(1,492)=108.2$, $P<0.001$ respectively] as well a significant interaction (group x temperature) [$F(1,492)=19.9$, $P<0.001$ and $F(1,492)=10.5$, $P=0.001$ respectively].

Table 1. Contractile properties of UREM and CON single psoas muscle fibers at 10°C and 30°C, pH 7.

Dependent Variable	**Group**	**Temperature (ºC)**	**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

Descriptive (Mean ± SD) and Inferential statistics (SEM and exact 95% CIs) are reported for the collected isometric force data. The value for n represents fibers assessed. GLM analysis indicated a significant main effect of either group, temperature and their interaction (group * temperature) ($P \leq 0.001$).

The pairwise comparisons showed consistent statistically significant functional deficits in UREM fibers compared to CONs. At the standard

experimentation conditions of 10°C, pH 7, absolute isometric tension (Figure 2A) of UREM fibers was significantly lower compared to CONs (P<0.001) roughly by approx. -25%. After accounting for fiber CSA, UREM fibers were still found to produce lower isometric forces compared to CON fibers (P<0.01), roughly by approx. -14% (Figure 2B).

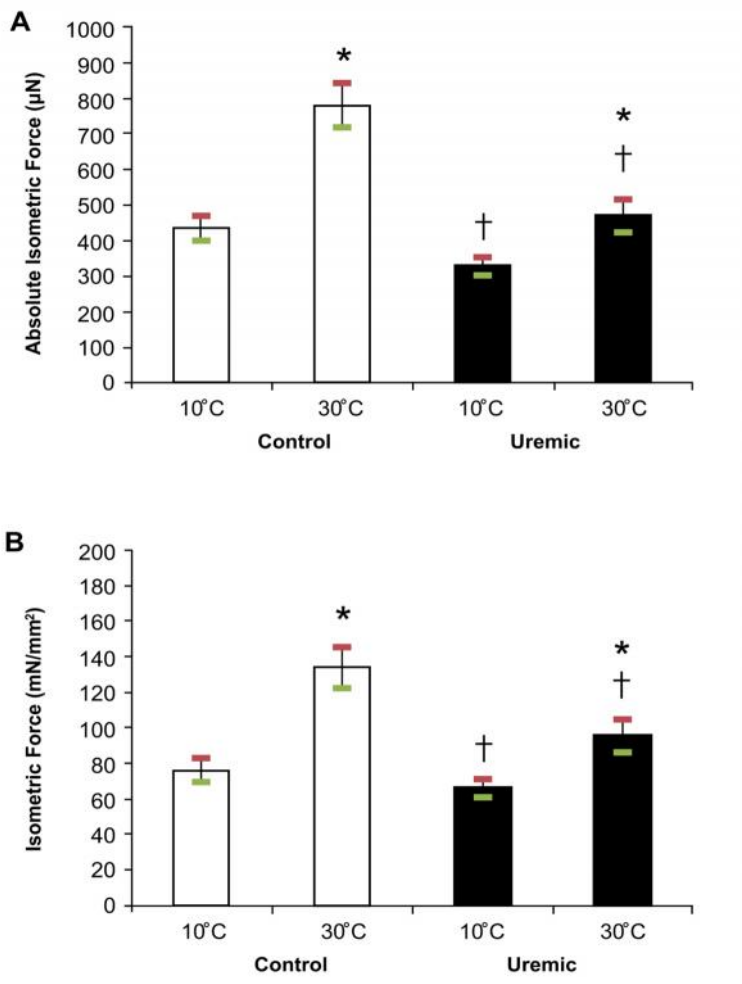

Figure 2. Isometric force for CON (open bars) and UREM (filled bars) psoas muscle fibers. Data collected at 10°C (10°C) and 30°C (30°C) pH 7 are presented as Mean values with exact Upper and Lower 95% CIs for A. absolute and for B. specific isometric forces. * Denotes significant difference from corresponding 10°C value

(P<0.001); † Denotes significant difference from corresponding control values (P<0.01).

Exposing fibers to a temperature jump from 10°C to 30°C caused an expected significant force rise in both fiber groups (P<0.001). Still, at 30°C and pH 7, UREM fibers (n=73) produced significantly lower forces (P<0.001) than CON fibers (n=41), in both absolute force (lower by approx.-40%) and specific force levels (by approx.-28%), (Figure 2). Examining the temperature effect as a percentage of a fiber's own baseline force value at pH 7 and 10°C, UREM fibers appeared to gain more, as their average temperature-induced force increase (+167 ± 170%) was 2.2 fold that of CON fibers (+76 ± 42%) (P<0.005, unpaired t-test). However, the significant force disparity between groups remained at 30°C, as mentioned above.

Contractile properties of single fibers in acidic conditions (pH 6.2)

In a different subset of fibers (n=25 CON and n=48 UREM) we assessed the effect of lowering the pH (from 7 to 6.2) on isometric force, which caused an expected significant force reduction in both groups (P<0.005). More specifically, for CON fibers absolute and specific forces at pH 6.2, 10°C, were (Mean ± SD) 316 ± 116 μN and 55 ± 20 mN/mm^2 respectively. For UREM fibers, absolute and specific forces at pH 6.2, 10°C, were (Mean ± SD) 211 ± 125 μN and 46 ± 26 mN/mm^2 respectively.

Whether on absolute or specific force data, GLM analysis indicated a statistically significant main effect of pH [pH7 vs pH 6.2, 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]. A non-

significant result was found for the interaction effect (group x pH) [F(1,451)=0.003, P>0.05, and F(1,451)=0.028, P>0.05 respectively].

The pairwise comparisons showed consistent functional deficits in UREM fibers compared to CONs in absolute values. At 10°C and pH 6.2, absolute isometric tension (Figure 3A) of UREM fibers was significantly lower compared to CONs (P<0.05) roughly by approx. -33%. After accounting for fiber CSA, at 10°C pH 6.2 UREM fibers were still found to produce lower specific isometric forces compared to CON fibers roughly by approx. -17% (Figure 3B) but non significantly (P>0.05).

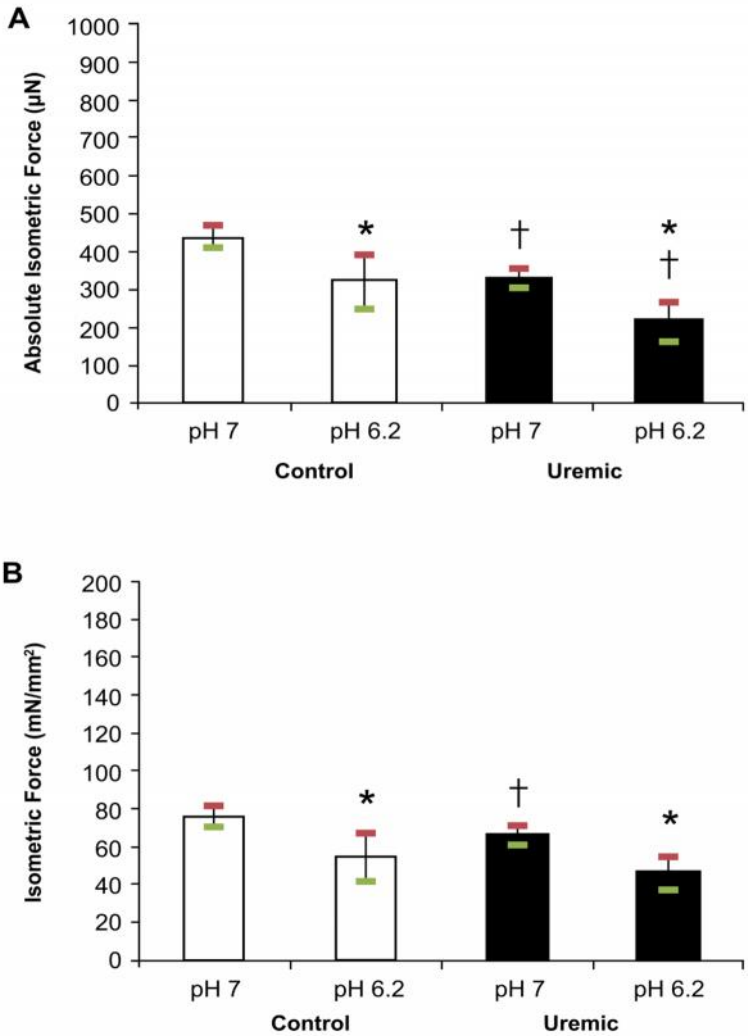

Figure 3. Effect of pH on isometric force for CON (open bars) and UREM (filled bars) psoas muscle fibers. Data collected at standard (pH 7) and acidic (pH 6.2)

conditions, at 10°C, are presented as Mean values with exact Upper and Lower 95% CIs for A. absolute and for B. specific isometric forces. * Denotes significant difference from corresponding pH 7 value (P<0.005); † Denotes significant difference from corresponding control value (P<0.05).

When examining the pH effect as a percentage of a fiber's own baseline force value at pH 7 10°C the average % force decline due to the lowering of pH showed a non-significant tendency to be larger for UREM (-43 ± 9% for CON vs -48 ± 14% for UREM, P=0.06, unpaired t-test).

It was possible in a small number of fibers (n=13 CON, n=21 UREM) to examine the temperature effect at pH 6.2. Given that it was not possible to perform an experiment of lowering the pH from 7 to 6.2 while at 30°C, we did not include those values in the global statistical assessment mentioned above. The absolute and specific force values at pH 6.2, 30°C, were 700 ± 238 μN and 117 ± 33 mN/mm^2 for CON and 550 ± 248 μN and 114 ± 52 mN/mm^2 for UREM fibers respectively. We also expressed the effect of a temperature jump from 10°C to 30°C at pH 6.2 as a percentage of a fiber's own force value at pH 6.2, 10°C. In response to the temperature jump, UREM fibers on average increased their force by +219 ± 160% while CON by +143 ± 62% however no significant differences in this response were found between groups (P>0.05, unpaired t-test).

DISCUSSION

To the best of our knowledge this is the first study to examine the effect of chronic renal insufficiency on the contractile properties of single muscle fibers in an animal model mimicking CKD. We found that renal insufficiency not only induced muscle remodeling as evidenced by atrophy, but that it also resulted in a significantly impaired force generation capacity at the single fiber level. We provide, for the first time, compelling evidence of uremia-induced direct effects on sarcomeric protein interaction that could help explain aspects of the disturbed functional capacity and muscle weakness often seen in CKD patients.

In our study UREM psoas fibers presented with significantly smaller CSAs compared to CONs by ~11%. In CKD patients, muscle weakness may be partially explained by atrophy which has been reported to be exacerbated but only partly explained by inactivity (Sakkas, Sargeant, et al., 2003a), pointing to a disease specific effect. It has been reported that atrophy in CKD is mainly presented in type II fibers (Sakkas, Ball, et al., 2003; Sawant, Garland, House, & Overend, 2011) and that was an important reason for assessing psoas muscle in the current study [which in NZ rabbits expresses > 95% the IIX(IId) myosin heavy chain isoform (Aigner et al., 1993; Hämäläinen & Pette, 1993)]. In an examination of the rectus abdominis muscle (Sakkas, Ball, et al., 2003), it was reported that in end-stage pre-dialysis patients, fast muscle fibers exhibited substantial atrophy (26% for those expressing type IIA myosin and 28% atrophy for the type IIX), with slower fibers also affected, as compared to matched-controls. A recent uremic rat study by Acevedo et al (Acevedo et al., 2015), reported no significant differences in fiber CSA of tibialis anterior (a mixed fast hindlimb muscle) of surgically-induced uremic vs control animals. In contrast, Organ et al 2016 (Organ et al., 2016), using the Cy/+ CKD rat model

reported that the extensor digitorum longus (another mixed fast muscle) of CKD rats showed atrophy in all fiber types. As we studied a larger species and a muscle with different metabolic properties and myosin composition our results are not directly comparable, but together these findings could indicate a muscle type specificity of early atrophy in CKD. Our findings are consistent with human studies reporting loss of muscle mass and muscle atrophy in CKD patients (Johansen et al., 2003; Sakkas, Ball, et al., 2003; Sawant et al., 2011). Our results support the notion that atrophy could start earlier during the disease progress and could appreciably affect fast muscles which are normally tasked to provide high levels of muscle power.

Regarding maximal isometric force at pH 7, 10°C and 30°C, our analysis revealed statistically significant differences in both absolute and specific forces between uremic and control fibers at both temperatures. Specifically, at 10°C, absolute and specific forces were lower in uremic fibers compared to controls by 25% and 14%, respectively; at 30°C the corresponding values for uremic fibers were lower than controls by 40% and 28%, respectively. The significantly smaller CSA of uremic fibers vs control could not thus account for the depressed specific forces of uremic fibers we observed. Organ et al (Organ et al., 2016), attributed the reduced ankle dorsiflexion torque they observed in Cy/+ uremic rats mostly to atrophy. These authors did not assess single cell contractile properties and their *in vivo* model and muscle group studied differed from ours. The specific force deficit of single uremic muscle fibers observed in our study could indicate a reduced capacity of cross bridges to generate force or perhaps possible disturbances on how generated force is transmitted across the sarcomeric arrangements. With regards to force transmission across the sarcomere, possible changes in viscoelastic properties e.g. as indicated in chronic heart failure (Miller et al., 2010), could play a role in reducing the measurable

isometric force. We cannot exclude such changes in our study (see comments on qualitative properties further on) and this remains a direction to pursue in the future.

The maximal isometric tension of a single muscle cell is ultimately determined from the number of active cross bridges during contraction and the force exerted from each cross bridge (Fitts et al., 1991) essentially dependent on the strength of the actomyosin bond (Karatzaferi et al., 2004). Moreover, the ability of the cell to produce force can be affected by modifications influencing actomyosin interaction kinetics [such as the duty ratio e.g. in (Miller et al., 2010)]. With a turnover of approx. 30 days (Kay, 1978), myosin can be a target for posttranslational modifications that affect its functional properties. One of the biochemical repercussions of renal insufficiency is oxidative stress (Kaltsatou et al., 2015) which can act on its own or in combination with other molecules to cause muscle dysfunction at multiple levels. Studies with skinned fibers have indicated that acute oxidative stress can impair isometric force (Gilliver, Jones, Rittweger, & Degens, 2010; Murphy, Dutka, & Lamb, 2008) and actin activated myosin ATPase with measurable alterations in transition from weak to strong binding states (Prochniewicz, Spakowicz, & Thomas, 2008) affecting perhaps the concerted strength production by active cross-bridges. Whether chronic oxidative stress in renal insufficiency (Poulianiti et al., 2015) may cause similar irreversible structural modifications affecting the actomyosin interaction cannot be excluded. Oxidative stress can also affect function by promoting the formation of advanced glycation end products, AGEs (Miyata et al., 1997). In an examination of the effects of reversible glycation on myosin structural and functional properties, Ramamurthi et al (Ramamurthy, Höök, Jones, & Larsson, 2001), observed ‘glycation-related structural alterations’ which were paralleled by a significant reduction in *in vitro* motility speed. The same group also observed that glutathione

rescued myosin functional properties (Ramamurthy, Jones, & Larsson, 2003). As glycosylation is often observed in renal disease (Thornalley, 2006) along with oxidative stress (Kaltsatou et al., 2015) such combination of factors may lead to AGEs formation causing structural alterations that could contribute to our observations of lower isometric forces produced by uremic fibers.

Muscle contraction is temperature sensitive as it is driven by an enzymatic reaction, the myosin ATPase (Ranatunga, 2010). Both uremic and control fibers substantially increased force generation in response to temperature increase from 10°C to 30°C in agreement to previous observations by us (Karatzaferi et al., 2004, 2008) and others (Coupland et al., 2001; Pate et al., 1995). At pH 7, tension rise in control fibers was ~76% which is consistent with other studies evaluating the temperature effect on the contractile properties of rabbit psoas skinned fibers (Coupland et al., 2001; Pate et al., 1995). Although the temperature-induced tension rise was percent-wise higher in uremic fibers, force at 30°C values remained significantly lower in uremic fibers than in control fibers. Thus the functional deficit of the uremic fibers observed at standard experimental conditions held true in a temperature closer to physiological.

Fatigue intolerance in CKD has also been associated with the rapid development of acidosis (Johansen et al., 2005; Moore, Bertocci, & Painter, 1993). This would be consistent to observations that low pH alone or in combination with other metabolites (such as phosphate), plays a crucial role in the inhibition of muscle contraction and force generation at the cross-bridge level (Allen et al., 2008; Karatzaferi et al., 2008; Nelson & Fitts, 2014). We examined if uremic fibers demonstrated a differential response to the low pH as compared to controls. Both uremic and control fibers responded in a similar manner to the lowering of pH from 7

to 6.2 (which reduced tension approx. 45%). Such magnitude of force decline is in agreement to previous studies investigating the effect of pH in control psoas skinned fibers (reporting declines in the range of 45-65%) (Cooke et al., 1988; Karatzaferi et al., 2003; Pate et al., 1995). The effect of pH is less pronounced at more physiological temperatures [in skinned fibers (Karatzaferi et al., 2008; Pate et al., 1995), in intact fibers (Westerblad, Bruton, & Lännergren, 1997)] with temperature increases partially negating its reductive effect on single fiber force, and this was also supported in the present study. The functional consequence of acidosis-induced force reduction however may well differ between control and uremic muscle *in vivo*. As uremic fibers produce lower absolute and specific force even at baseline conditions, one can fathom that in acidosis, which is rapidly developing in renal patients even during low to moderate exercise intensities (Johansen et al., 2005), uremic muscle may be at a severe disadvantage during any fatiguing activity.

Other factors, related to toxicity effects may be also at play, either promoting atrophy or affecting muscle properties. As CKD progresses, uremia develops in parallel with the accumulation of related toxic waste products (Richet, 1988). These are implicated in the etiology of abnormal muscle function and fatigue intolerance experienced by renal patients (Campistol, 2002; Davis et al., 1983) with research so far indicating a multiple of candidates with a possible role (Vanholder et al., 2003, 2009). Not much is known about their possible direct role on skeletal muscle function, however they have been shown to affect smooth muscle function via endothelium mediated effects (Jourde-Chiche, Dou, Cerini, Dignat-George, & Brunet, 2011). The so- called ‘uremic toxins’ (Vanholder et al., 2009) i.e. solutes that would be cleared by the kidney but instead gradually accumulate, often protein-bound themselves, like indoxyl sulfate which binds to albumin or p-cresyl sulfate, could worsen oxidative

stress and inflammation and hasten muscle loss and mitochondrial dysfunction, thus contributing to the moderate atrophy observed in this study as well as to further, hitherto undefined, changes in sarcomeric protein properties.

Our study had many strengths but also some limitations that we have to address. Despite implementing the same surgical approach, the biochemical responses were somewhat variable which perhaps explains why the uremic fibers presented with greater variability in force values than controls. Also, it was not possible to evaluate all psoas fibers at 30°C since fibers often broke (or did not fulfill pre-set criteria upon return to 10°C and were thus discarded). This is not surprising as exactly due to this known sensitivity of skinned fibers we and others have developed such t-jump protocols (Karatzaferi et al., 2004, 2008; Pate et al., 1995), while the majority of skinned single fiber studies are performed at lower temperatures (e.g.10°C-20°C) (Fitzsimons, Patel, Campbell, & Moss, 2001; Gilliver et al., 2010; Mollica et al., 2012; Murphy, Stephenson, & Lamb, 2004; Reggiani et al., 1997; Stienen, Versteeg, Papp, & Elzinga, 1992; Sweeney, Kushmerick, Mabuchi, Sréter, & Gergely, 1988). Additionally, fibers' cross sectional areas were calculated assuming a cylindrical and not an elliptical shape and this may be why our baseline control force values were lower compared to other studies (Coupland, Pinniger, & Ranatunga, 2005). Nevertheless, this happened in a systematic way in all single fibers and could not affect the interpretation of our results, as for example our control fibers response to the drop of pH was similar to what observed previously (Cooke et al., 1988; Karatzaferi et al., 2003; Pate et al., 1995). Another weakness of our study was that a 'positive' bias has been inadvertently introduced, due to the blind design, as uremic fibers were in retrospect proven difficult to dissect and handle. I.e. the fibers that withstood our handling were assessed but many more that could not be handled were

not assessed due to the bad quality of muscle tissue. Also, given some standard criteria that we and others use [e.g. (Karatzaferi et al., 2003; Liang et al., 2008)] for force data quality, it later transpired that more uremic fibers were discarded than controls. Thus, the 'better' fibers were measured by each round of assessments. If we could ascribe 'qualitative' descriptors, we could describe the discarded psoas fibers as 'mussy', 'messy', 'sticky'. In addition, although myosin heavy chain identification to verify fiber typing was in our initial design, SDS-page analysis proved to be unattainable since proteins from uremic fibers could not been resolved, instead a smear was produced. When grouping fibers similar problems transpired. Upon un-blinding the study this problem became apparent to us but we had not had available tissue to further address it.

The latter two observations reveal important information regarding the quality of uremic muscle, and point to new directions for further research. On the one hand possible changes in passive elastic properties warrants further study [for example changes in titin or nebulin may be implicated (Horowits, Kempner, Bisher, & Podolsky, 1986)]. It is noteworthy that in studies evaluating properties of skeletal muscle fibers from heart failure patients, sinusoidal analysis indicated changes in viscoelastic properties at the sarcomere level (Miller et al., 2010; Toth et al., 2012), thus such lattice properties changes could be possible in our model. On the other hand, glycosylation which has been observed in uremia (Thornalley, 2006), might have been at play affecting the resolving power of SDS page through the attachment of sugar residues in proteins leading to changes in the proteins' nature (Spiro, 2002). Advanced glycosylation has also been associated with glycation of type IV collagen of endothelial cells in ESRD (Thornalley & Rabbani, 2009) and further changes in overall muscle elastic properties cannot be excluded.

Our study had also some important strengths, foremost, the employment of an animal model of chronic renal disease and not of acute kidney injury. Our animal model was allowed to develop renal insufficiency for 3 months after nephrectomy which corresponds to a sufficient period considering rabbit lifespan whereas an acute model of CKD may not induce realistically relevant modifications on muscle properties. Moreover, as the model was representative of a pre-dialysis stage, our results may hold larger clinical relevance given the extent of 'silent' kidney disease among the general population (Stenvinkel, 2010). Furthermore, we used the single fiber technique, which allows forming conclusions about sarcomeric function by acutely isolating other factors such as muscle atrophy and excitation-contraction coupling issues. Another fact that strengthens our observations is that we examined contractile properties of samples in a blind fashion and that we used sham operated instead of purely control animals in order to avoid differences in results due to the different animal handling.

The understanding of the possible underlying mechanisms to explain muscle dysfunction in CKD is of major clinical relevance as skeletal muscle is associated with many aspects of life and health. Not only skeletal muscle produces force, power, to maintain body control and allows for safe mobility, it also plays a crucial role in the regulation of protein and energy metabolism and many other vital body functions (Frontera & Ochala, 2014). It is obvious from the above that any abnormality of skeletal muscle status can lead to a series of physical disturbances, as well as low quality of life, and dangerous falls (Wolfe, 2006). CKD is associated with serious muscle abnormalities such as skeletal muscle atrophy, muscle weakness, limited endurance and fatigue intolerance (Adams & Vaziri, 2006; Campistol, 2002). These situations lead patients to reduced functional capacity, jeopardizing their

independence in their daily life, leading to poor quality of life and in the long term resulting in premature death (Johansen, 2007). It is known that neuropathy can contribute to muscle dysfunction in renal disease, especially at the end stage (Adams & Vaziri, 2006; Brouns & De Deyn, 2004) and its effects have made difficult to distinguish excitation-contraction coupling from sarcomeric function problems.

Our study gives for the first time evidence that muscle contractile dysfunction in renal insufficiency can manifest early at the single muscle fiber level. Given the extent of 'silent' kidney disease among the general population (Stenvinkel, 2010) and that our model was on a 'pre-dialysis' disease state, this and other observations from our group [e.g. increased protein carbonylation (Poulianiti et al., 2015)] point to the need to consider interventions early during kidney disease progression as to safeguard skeletal muscle properties. Such measures would allow patients to reach the end-stage at the best possible metabolic profile. Thus, on the one hand, future work should focus on detailed examinations of the structure and function of uremic sarcomeric proteins in order to understand the exact mechanisms leading to the observed muscle dysfunction. In parallel, work is needed as well on developing interventions to counteract functional deteriorations, and thus by ameliorating muscle weakness, to prevent further atrophy and metabolic morbidity and mortality in CKD.

To conclude, in a model of chronic renal insufficiency, uremia can induce some remodeling of muscle fiber size and significantly impair force generation at the single muscle fiber level. The impairment in force generation of uremic muscle fibers could not be explained by atrophy and was present in conditions mimicking the *in vivo* cellular environment at rest and acidosis. Our observations indicate possible uremia-induced changes in sarcomeric protein properties and warrant further investigation.

CHAPTER 4: RESEARCH PAPER 2

Effect of chronic renal insufficiency on force-pCa relationship of permeabilized single fibers

Aims addressed in this paper: using tissue from an animal model of uremia to examine the effect of chronic renal insufficiency on

1. force-pCa relationship of isolated muscle fiber preparations in conditions mimicking the resting state
2. force-pCa relationship of isolated muscle fiber preparations in conditions mimicking acidosis

ABSTRACT

Introduction: Chronic kidney disease (CKD) is highly associated with muscle dysfunction, such as reduced tension generation and premature fatigue. The causes of muscle dysfunction in CKD have not been yet established. We aimed to evaluate the difference in force-pCa relationship of permeabilized single psoas fibers from healthy and uremic rabbits. **Materials and Methods:** We induced renal insufficiency (via partial nephrectomy) in 5 NZ rabbits. Psoas muscle samples harvested from control (sham-operated, CON) and uremic (UREM) animals, at 3 months, were chemically skinned and stored in 50% glycerol solution at -20°C until mechanical assessment. Isometric tension was assessed in single psoas fibers (n=128 CON; n=195 UREM) at 10°C, 30°C and at pH 7 and pH 6.2, using an SI Heidelberg micro dynamometer, in various concentrations of calcium chloride. Force data expressed as percentage of Po at 10°C and pH 7 and free calcium expressed in pCa values were fitted in the Hill equation. **Results:** At standard conditions 10°C pH7, UREM fibers presented with quite similar calcium sensitivity (pCa_{50} UREM 6.12±0.02 vs CON 6.20±0.03) and cooperativity (n_H UREM 2.11± 0.14 vs CON 2.36±0.3). Acidosis (pH 6.2) at 10°C caused a loss of calcium sensitivity for both groups, more so for UREM (pCa_{50} UREM 5.32±0.06 vs CON 5.58±0.02). At 30°C pH7, UREM fibers presented with lower sensitivity than CON (pCa_{50} UREM 6.00±0.25 vs CON 6.42±0.19). At 30°C acidosis reduced calcium sensitivity similarly for both groups (pCa_{50} UREM 5.71± 0.13 vs CON 5.80± 0.05). Changes in cooperativity followed a similar pattern. **Conclusion:** It appears that chronic renal insufficiency may depress calcium sensitivity, the magnitude of this depression being dependent on prevailing experimental conditions. It is important to consider temperature and acidosis parameters when assessing calcium sensitivity in chronic disease.

Key words: uremia, calcium sensitivity, thin filament, acidosis, isometric tension

INTRODUCTION

Skeletal muscle form and function is heavily impacted from chronic kidney disease (CKD). This “silent” epidemic leads 440.000 of patients to renal replacement therapy each year and it has been identified as one of the leading causes of death worldwide (Ortiz et al., 2014). The disease is characterized by many muscle abnormalities such as skeletal muscle atrophy, muscle weakness, limited endurance and fatigue intolerance (Adams & Vaziri, 2006; Campistol, 2002). These situations lead patients to reduced functional capacity ameliorating their independency in their daily life, leading to poor quality of life and in the long term to premature death (Johansen, 2007).

Many factors may inhibit muscle function in CKD including mitochondrial dysfunction, substrate availability and neuropathy (Adams & Vaziri, 2006). In addition, accumulation of toxic waste products (Richet, 1988) may be implicated in the muscle abnormalities experienced by CKD patients which are collectively termed as “*uremic myopathy*” (Campistol, 2002; Davis et al., 1983). Fatigue intolerance in CKD has been also associated with acidosis (low pH) (Johansen et al., 2005; Moore et al., 1993) which is well known to play a crucial role in the inhibition of muscle contraction (Allen et al., 2008; Karatzaferi et al., 2008; Nelson & Fitts, 2014).

The mechanism underlying fatigue intolerance in CKD has not yet been fully understood. Our previous data (Research paper 1) showed reduced maximally activated isometric tension for uremic psoas skinned fibers. However, it is not clear how uremic muscle responds in conditions of suboptimal calcium activation. Calcium triggers contraction by binding to troponin and shifting the troponin-tropomyosin complex uncovering actin binding sites to myosin heads. By determining the relationship between the level of calcium activation and tension generation one can

evaluate sarcomeric function at submaximal calcium levels (expressed as the negative logarithm of free calcium). Thus, force-pCa data can be fit to the Hill equation to obtain pCa_{50} and Hill coefficient (n_H) values as indicators of contractile proteins' calcium sensitivity and thin filament cooperativity and can reveal functional and structural alterations in sarcomeric proteins (Schiaffino & Reggiani, 1996).

Interestingly, intracellular measurements of calcium levels have shown that low-frequency fatigue (a type of fatigue related to everyday activities and breathing) is mostly due to a reduction in Ca^{2+} release (Jones, 1996). Moreover, in heart failure the sensitivity of contractile proteins to Ca^{2+} appears to be reduced, as reflected by a requirement for more calcium to achieve 50% relative force (pCa_{50}), resulting in a overall reduced force generation at a sub-maximal Ca^{2+} level (e.g. in rat diaphragm fibers (van Hees, Andrade Acuña, Linkels, Dekhuijzen, & Heunks, 2011). Additionally, it has been proposed that myosin loss can be reflected by a disturbed force-calcium (force-pCa) relationship (Ochala & Larsson, 2008), i.e. a rightward shift in the force-pCa relationship.

If one considers that CKD patients present with excessive fatigue even during moderate activity (Johansen et al., 2005), it becomes of interest to examine whether force generation capacity at submaximal calcium activation may be affected, pointing perhaps to functional and structural alterations in sarcomeric proteins (Schiaffino & Reggiani, 1996). We employed the skinned fiber preparation using an animal model mimicking CKD (Gotloib et al., 1982; Bagcivan et al., 2003) to examine the effects of chronic insufficiency on the force-calcium relationship. To improve physiological relevance of our results we examined calcium sensitivity at resting and acidotic conditions at low (10^oC) and high (30^oC) temperatures.

MATERIALS AND METHODS

Animal model

All animal procedures, including surgery and euthanasia 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, in accordance to the national directives for the care and use of laboratory animals. Eight New Zealand young adult white female rabbits (N=8) with a body weight (BW) of ~3200g were housed in a controlled environment with stable conditions (temperature 22–24°C, 12:12 h light-dark cycle) and acclimatized for 48 hours. Rabbits were fed ad libitum a special rabbit chow containing low levels of protein, potassium, calcium, phosphorus and sodium (prepared by Research Diets, Inc. USA) and water ad libitum.

After acclimatization, surgical procedures were performed (sham operation for control animals - CON and partial nephrectomy for experimental animals –UREM). Animals were anaesthetized by intravenous administration of a solution mixture of ketamine hydrochloride 100 mg/ml (Imalgene® 1000; Merial, Duluth, Georgia, USA) and xylazine 20 mg/ml (Rompun®; Bayer, Leverkusen, Germany), 87% and 13% respectively (proportion 6.69:1 approximately). The initial dosage for the induction of anesthesia was 0.3 ml/Kg BW of the above solution mixture, i.e. Imalgene® (87%) and Rompun® (13%), intravenously (i.v.). The maintenance of anesthesia was achieved by a dose of 0.2 to 0.3 ml i.v. of the solution mixture. Animal temperature was maintained via a heating pad. Three hours before the intervention, each animal had only access to water and its weight was measured on a precision scale. The induction of renal insufficiency was performed surgically (using a surgical protocol modified from Gotloib et al., 1982) (Gotloib et al., 1982). For the UREM group five

animals (N=5) underwent removal of the left kidney after careful ligation of the left renal artery and vein; and partial nephrectomy (¾) of right kidney. For the CON group, three age –matched animals (N=3) underwent sham operation and were considered as the control group (CON animals). In a previous pilot study (data not shown) sham-operated muscle contractility was comparable to non-procedure animals. Thus, in agreement with the principle of Reduction in Animal Research sham-operated animals were used as controls in the present study.

Twelve weeks after surgery, animals were sacrificed (after determining their BW) by injection of sodium pentobarbital solution (50 mg/ml) which was applied in a dosage of 100 mg/Kg BW followed by bilateral thoracotomy. Immediately after cardiac arrest, blood samples were collected from heart and aorta using a heparinized syringe and were placed into ethylene diamine tetra-acetic acid (K2EDTA)-containing tubes (Vacutainer Plus Plastic K2EDTA; Becton Dickinson) for subsequent serum urea and creatinine determination using standard photometric protocols. Thereafter, psoas muscle fast excised and processed for contractility studies (see below) while the remaining of the tissue was shared for other approved protocols.

Muscle Samples

Psoas muscle samples from UREM and CON animals were permeabilized as previously described (Karatzaferi et al., 2008). Briefly, thin bundles of rabbit PSOAS muscle (~2 mm diameter) were dissected and tied to wooden sticks using surgical thread. The samples were placed in falcon tubes containing skinning buffer solution (0°C) [120 mM KAc, 50 mM 3-(N-Morpholino) propanesulfonic acid (MOPS), 5 mM $MgAc_2$, 4 mM ethylene glycol tetraacetic acid (EGTA) and 50% glycerol (v/v); pH 7] with the addition of 0.2% of a protease inhibitor cocktail (104 mM AEBSF, 80

microns Aprotinin, 4 mM Bestatin, 1.4 mM E-64, 2 mM Leupeptin and 1.5 mM Pepstatin). After a 24-hour permeabilization treatment [muscle samples (in falcon tubes) were tied along a vibrating platform shaker (Heidolph-Titramax 100) in a parallel to the ground position and shacked at 350 rpm for 24 hours at 0°C], skinning solution was replaced with fresh solution and protease inhibitor cocktail and then the muscle samples were stored at -20°C until mechanical assessments. All chemicals were purchased from Sigma-Aldrich national retailer.

Experimental setup for single fiber mechanics

For the mechanical assessment, each single fiber was dissected from the muscle bundle on a cold stage under a stereomicroscope and the fiber ends were attached between 2 tissue mounts of a muscle micro dynamometer with resolution of 0.4 μN to 4 N (SI Heidelberg/WPI). The tissue mounts were connected to a force transducer and a motor arm (used as a fixed end). The fiber was then immersed in baths containing ~200 μl of various solutions (see in the solution section). This procedure was achieved using an adapted stereomicroscope on the top of the micro dynamometer. Data were continuously recorded in a computer using the particular software of SI Heidelberg/WPI and later exported for further analysis.

The particular micro dynamometer consists of a platform with 5 baths and fibers can be automatically switched between them in less than 250 ms allowing for rapid temperature jumps (t-jumps). The temperature of the baths was adjusted at 10°C and 30°C using a cooling/heating water circulator (Thermo Electron Haake WKL 26 Recirculator Chiller 3L Capacity Bath). The advantage of the T-Jump method is that the fiber may be first activated at a low temperature where the sarcomere arrangement is stable and it can then be rapidly transferred to a near-physiological temperature in

order to generate the maximal force without damaging the sarcomere arrangement (Karatzaferi et al., 2004).

Experimental solutions

The basic rigor buffer contained 120 mM KAc, 50 mM MOPS, 5 mM $MgAc_2$ and 1 mM EGTA for pH 7 or 120 mM KAc, 50 mM MES, 5 mM $MgAc_2$ and 1 mM EGTA for pH 6.2. Relaxing solution was achieved with the addition of 5 mM ATP in the corresponding rigor buffer (pH 7 or pH 6.2). Activating solutions: by mixing relaxing solution with a 2 mM $CaCl_2$ activating solution to obtain various concentrations. Total and free calcium concentrations were calculated according to "Maxchelator" computer program (C. Patton, Stanford University, Pacific Grove, CA, USA). The ionic strength of the solutions was ~0.2 M.

Force-pCa measurements

Maximal isometric tension (P_o) was first evaluated at pH 7 and 10°C (control conditions) at resting sarcomere lengths (no measurable tension vs slack, 2.2-2.4 μm). Specifically, each fiber was first immersed for 1 minute in a bath containing rigor solution (to wash out excess glycerol) and then it was transferred and equilibrated for 2 minutes in a bath containing relaxing solution of pH 7. Average diameter was determined assuming a cylindrical shape. Consequently, the fiber was maximally activated in control conditions (pCa 4.4) and after equilibration in a fresh relaxing solution of pH 7 it was submaximally activated in solutions with variable calcium concentrations (pCa 7.2-pCa 4.7). Some of the fibers were also assessed at 30°C. In this case, after each submaximal activation at 10°C the fiber was immediately

activated in the same total calcium concentration at 30°C (which corresponded to a range of pCa 5.8-pCa 4.02) for 3 to 5 sec using the t-jump protocol.

Measurements were also performed at pH 6.2. In this set of measurements a fiber was first maximally activated in control conditions (10°C and pH 7- as described above) and after equilibration in relaxing solution of pH 6.2 it was (submaximally and/or maximally) activated in various calcium concentrations at 10°C and pH 6.2 (which corresponded to a range of pCa 5.6-pCa 3.96). Some of the fibers were also assessed at 30°C. In this case after each submaximal activation at 10°C the fiber was immediately activated in the same total calcium concentration at 30°C (pCa 5.8-pCa 4.02) using the t-jump protocol.

Except from the initial maximal activation, all measurements were performed from low to high calcium concentrations. After each maximal or submaximal activation, fibers were equilibrated in relaxing solution of the pH of interest. However this could not happen in the case of a t-jump as the evaluation at 30°C had to occur immediately after activation at 10°C. Each fiber was activated up to six times and the final activation was achieved in control conditions to verify the condition of the fiber (to satisfy criteria of stability).

Data and statistical analysis

Force data distribution was tested using the Kolmogorov-Smirnov test of normality. Due to the normal data distribution, statistical analysis was performed using parametric tests. Differences at baseline forces (pH 7, 10°C) between fiber groups were estimated using unpaired analysis on absolute force and specific force, i.e. force values corrected for cross-sectional area (t-test for independent samples). The statistical analysis software used is commercially available (SPSS 15.0 for

Windows). For the force-pCa analysis, and to facilitate comparisons, relative force values were used. Thus, individual force data were expressed for each fiber as a percentage of P_o achieved by each fiber in control conditions (pH 7, 10°C, pCa 4.4) and free calcium was expressed in pCa values. Force data were then averaged for each pCa achieved for the two separate groups, per condition of pH and of temperature. In plots force per pCa were plotted as Mean ± SD. Average force-pCa data were fitted with the commercially available software (KaleidaGraph version 3.0.5) where the Hill equation was expressed in "y=m1*(10^(-m0))^m3/(10^(-m2))^m3+(10^(-m0))^m3" [m2=pCa_{50} and m3=Hill coefficient].

RESULTS

Both surgery procedures (¾ partial nephrectomy-UREM animals and sham operation-CON animals) were well tolerated and animals presented with a normal after-surgery recovery. At the end of the twelve-week period post-surgery, body weight ranged between 1,970-4,585 (with mean ± SD of 3,200 ± 1,076) g for UREM and 3,500-4,965 (4,105±765) g for CON animals (P>0.05). Renal insufficiency in experimental animals, compared to CONs, was reflected in raised blood creatinine (UREM, 2.7±1.28 mg/dl *vs* CON, 1.43±0.04 mg/dl, P>0.05) and urea levels (UREM 70.0±37.7 mg/dl *vs* CON 42.0±5.66 mg/dl, P>0.05).

In order to express force data from various calcium concentrations as a percentage of baseline forces, a total of 164 UREM and 109 CON single fibers were first assessed at 10°C and pH 7 (baseline values) under maximal calcium activation. Of those, 140 UREM and 86 CON fibers were also assessed submaximally at pH 7 and 10°C while 19 UREM and 18 CON fibers were also assessed at 30°C (submaximally and maximally) using the t-jump method. In order to examine any difference in force-pCa curves due to acidosis (pH 6.2), from the pool of fibers reported above (164 UREM and 109 CON), 36 UREM and 30 CON fibers were also assessed submaximally and/or maximally at pH 6.2 and 10°C while 24 UREM and 22 CONs were also assessed at 30°C (submaximally and/or maximally).

Morphological characteristics of single fibers

Average fiber diameters differed significantly between groups as revealed by unpaired t-test analysis (Mean ± SD UREM 78.6 ± 9.7 vs CON 82.1 ± 9.0 μm, P<0.005). Likewise, calculated cross sectional areas (CSAs) of UREM fibers were significantly lower compared to CONs (UREM 4,916 ± 1,217 *vs* CON 5,354 ± 1,175

μm^2, P<0.005), indicating a level of ~8% atrophy. Thus, force analysis data will be presented for both absolute and specific values (i.e. corrected for calculated CSA).

Contractile properties of single fibers at resting conditions (pH 7)

Baseline force values (10°C, pH 7) of all fibers assessed at both pH 7 and pH 6.2 are presented in Table 1. UREM fibers presented with significantly lower absolute isometric forces compared to controls by approximately 14% (P<0.05).

Table 1. Contractile properties of UREM and CON single psoas muscle fibers at standard conditions (10°C, pH 7).

	Group	**n**	**Mean**	**SD**
Absolute Force (μN)	CONTROL	109	385	168
	UREMIC	164	330	182
Specific Force (mN/mm²)	CONTROL	109	71	24
	UREMIC	164	67	33

Descriptive (Mean ± SD) statistics are reported for the collected isometric force data. The value for n represents fibers assessed. Unpaired analysis indicated a significant difference in forces (μN) between fiber groups (P<0.05)

Force-pCa relationship

Force values at variable pCa values were expressed as percentage of Po achieved at maximal calcium activation. Best fits using the Hill equation ranged from 95 to 99%. At pH 7 and 10°C, UREM fibers presented with similar pCa_{50} (UREM 6.12±0.02 *vs* CON 6.20±0.03) compared to CONs and slightly reduced cooperativity n_H (UREM 2.11±0.14 *vs* CON 2.36±0.30). Acidosis (pH 6.2 and 10°C) resulted in a loss of calcium sensitivity, more so for UREM fibers (pCa_{50} UREM 5.32±0.06 *vs* CON 5.58±0.02). Cooperativity was also reduced for both groups, more so for UREM fibers (n_H UREM 1.08±0.16 *vs* CON 1.93±0.22) (Figure 1).

When fibers were assessed at 30°C, UREM fibers presented with lower calcium sensitivity (pCa_{50} UREM 6.00±0.25 *vs* CON 6.42±0.19). For both groups n_H values indicated a loss of cooperativity, with UREM values being lower compared to CONs (n_H UREM 0.64±0.15 *vs* CON 0.75±0.21) respectively. Acidosis at 30°C resulted again in a loss of calcium sensitivity, compared to 30°C pH7, for both groups (pCa_{50}UREM 5.71±0.13 *vs* CON 5.80±0.05) but n_H values were 'restored' to values close to those achieved in standard conditions (UREM 2.21±1.57 *vs* CON 2.14±0.88), (Figure 2).

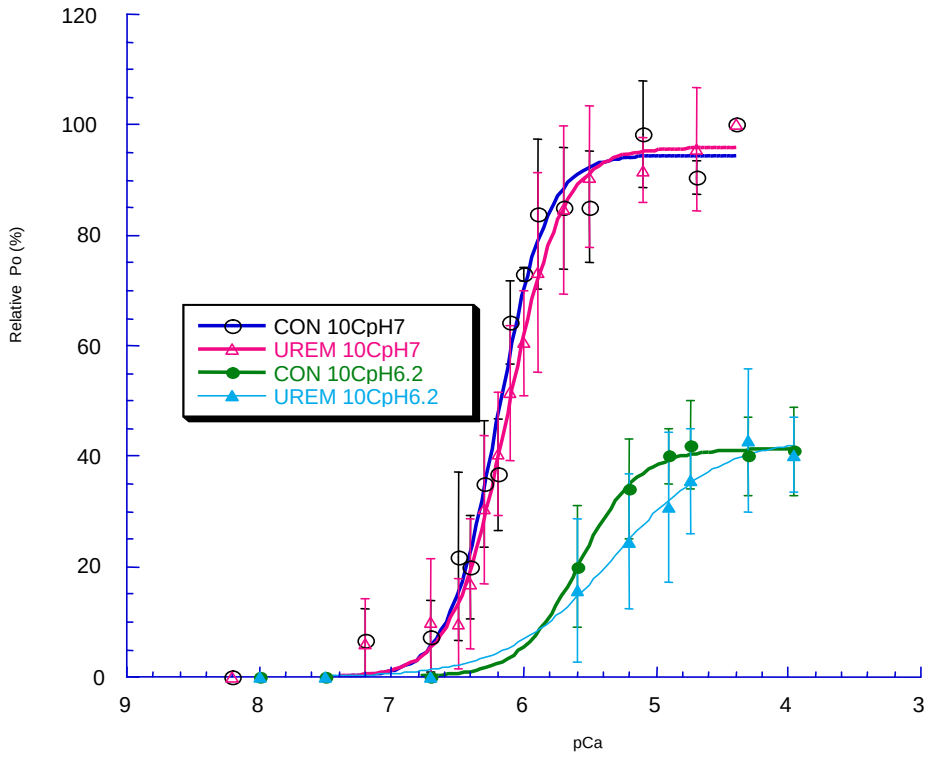

Figure 1. Average relative forces (%) versus free Ca^{2+} expressed in pCa at 10°C. Data points are presented as Mean ± SD for **pH 7** (CON-blue; UREM-pink) and **pH 6.2** (CON-green; UREM-light blue).

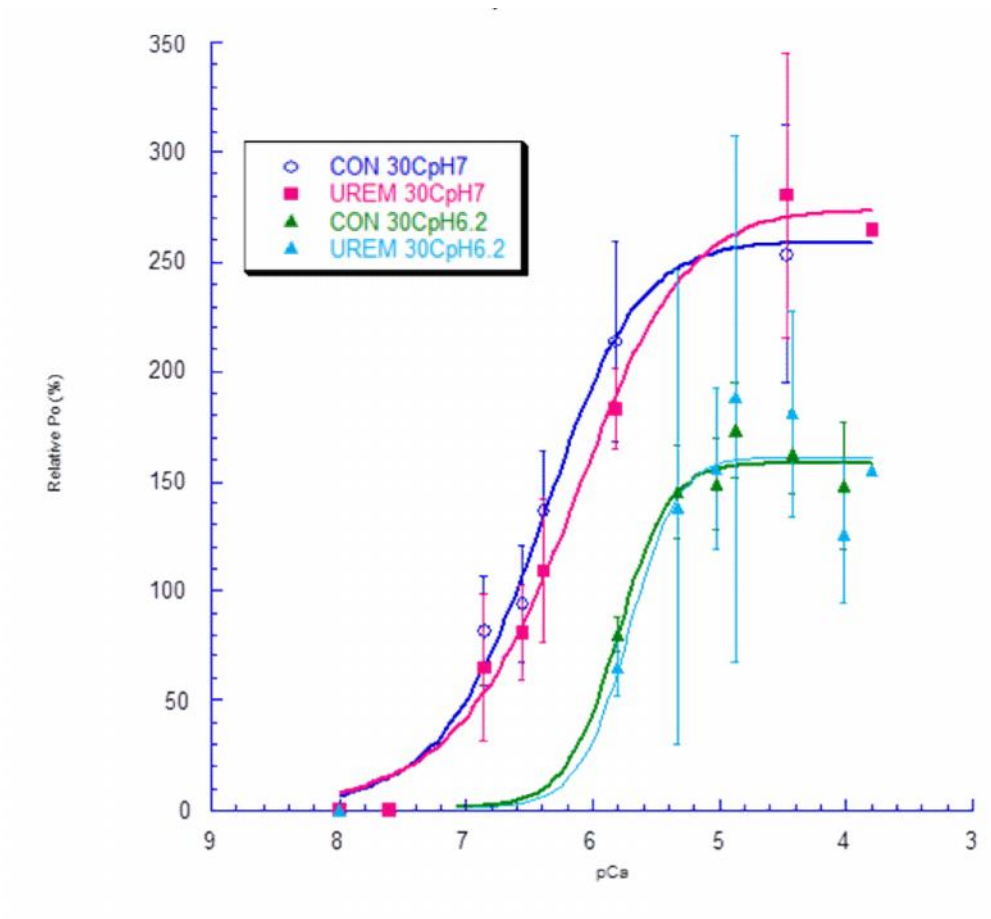

Figure 2. Average relative forces (%) versus free Ca^{2+} expressed in pCa at 30°C. Data points are presented as Mean ± SD for **pH 7** (CON-blue; UREM-pink) and **pH 6.2** (CON-green; UREM-light blue).

DISCUSSION

We studied for the first time the effect of uremia on the force-pCa relationship of skeletal muscle from an animal model of renal insufficiency at the single fiber level. We assessed force generation in various calcium concentrations in two sets of experiments. At the first set, force generation was assessed at resting conditions (pH 7) and at both 10°C and 30°C while at the second experimental set we assessed force generation in acidic conditions (pH 6.2), at 10°C and 30°C. We found that overall UREM fibers exhibited less calcium sensitivity than CONs as reflected by a consistent rightward shift of the force-pCa relationship, with the magnitude of pCa_{50} difference from CON being influenced by prevailing temperature and pH conditions.

According to the pCa_{50} values in resting conditions (pH 7), UREM fibers presented with lower calcium sensitivity compared to CONs at both 10°C and 30°C but the difference between fiber groups were more evident at 30°C. In acidosis UREM fibers presented again with lower calcium sensitivity than CONs by -4.6% at 10°C and by -1.5% at 30°C. The decrease in calcium sensitivity of a single fiber indicates that more calcium is required in order to achieve the 50% of maximal isometric force (Walker, Li, & Buttrick, 2011).

Our pCa_{50} values of CON fibers were in agreement with previous studies evaluating force-pCa relationship in skinned rabbit psoas fibers at both low (i.e. 5.35-5.98 vs 6.2 at 10°C-15°C) (Lu, Swartz, Metzger, Moss, & Walker, 2001; Vinogradova et al., 2005) and higher temperatures (i.e 5.76-5.86 vs 6.4 at 20°C-25°C) (Blanchard, Pan, & Solaro, 1984; Morimoto & Ohtsuki, 1994; Palmer & Kentish, 1994). Our CON n_H values were also consistent with previous studies at 10°C (2.65 vs 2.36) (Vinogradova et al., 2005) but at higher temperatures were lower compared to previous studies (1.9-2.7 vs 0.75) (Blanchard et al., 1984; Morimoto & Ohtsuki, 1994;

Palmer & Kentish, 1994). Furthermore, our values at pH 6.2 were consistent with previous studies at 10°C and higher temperatures (1.92 vs 2.14) (Blanchard et al., 1984; Palmer & Kentish, 1994). We cannot explain the discrepancy in n_H values at the high temperature. It should be noted though that as the Hill equation has a descriptive role (Walker et al., 2011) it is possible that it cannot adequately fit data obtained at high temperatures. Moreover, the high variability of force values and a difficulty in obtaining measurements at pCa values in the range of 8 to 6.5 (data not shown) may have contributed. Still, differences in cooperativity between UREM and CON were mostly consistent.

Calcium sensitivity derived from the Hill equation is considered a robust index to characterize functional properties (Walker et al., 2011). It depends mainly on the function of troponin-C (TNC) protein which holds the regulatory calcium binding sites. However, thin filament proteins may also affect calcium sensitivity such as tropomyosin (TM) since, for example, overlap of adjacent TM ends may act as a signal along the thin filament (cooperativity) (Schiaffino & Reggiani, 1996). Nebulin also plays an important role while it has been reported that nebulin deficiency leads to extremely low calcium sensitivity in mouse muscle (Lee et al., 2013). Thick filament proteins are also implicated since strong cross bridge attachment causes the stabilization of the thin filament in a state with high Ca^{2+} affinity (Schiaffino & Reggiani, 1996). Moreover, the sarcomere length can affect calcium sensitivity (Stephenson & Williams, 1982). Given that we have observed slightly longer sarcomere lengths in UREM fibers (Research paper 3), despite these being at the optimal range for isometric force production, we cannot exclude that part of our observations may reflect differences in elastic forces maintaining the sarcomere length during contraction.

In addition to calcium sensitivity, in resting conditions (pH 7), n_H values revealed a lower thin filament cooperativity for UREM fibers compared to CONs which was more intense at 30°C. More precisely, n_H values were lower in UREM fibers compared to CONs by -10.3% and -15.1% at 10°C and 30°C respectively. Interestingly, in acidic conditions and 10°C UREM fibers presented with lower n_H values by -44.2% compared to CONs. However, this important functional limitation of UREM fibers disappeared when fibers were assessed at near physiological temperature where UREM fibers had almost similar n_H values with CONs.

It is obvious from the above that although in resting conditions, the lowest pCa_{50} and n_H values were observed at 30°C, in acidic conditions the lowest values were observed at 10°C. This could be explained by the fact that in muscle mechanics low pH causes significant functional deficits but at near physiological temperatures the effect of pH is reduced (Karatzaferi et al., 2008; Pate et al., 1995; Westerblad et al., 1997).

The cooperativity which is reflected in n_H values depends on the thin filament proteins tropomyosin, troponin complex (TNC, TNT, TNI), actin and myosin heads. All of the above need to act cooperatively for the achievement of muscle contraction (Boussouf & Geeves, 2007) and even a small degree of change in the n_H value of the force-pCa relationship can have profound implications. Decreases in n_H have been mainly correlated with alterations in troponin complex. For example, replacement of TNC slow to TNC fast has led to n_H reduction (Brandt, Diamond, Rutchik, & Schachat, 1987; Moss, Lauer, Giulian, & Greaser, 1986). Similar results have been reported for TNT, while replacement of slow to fast TNT isoform has caused a significant increase in n_H values in transgenic mouse cardiac muscle without changes in calcium sensitivity (Huang, Brozovich, & Jin, 1999) and it is of interest that CKD

has been associated with increased levels of cardiac serum TNT which has been attributed to the uremic myopathy (Freda, Tang, Van Lente, Peacock, & Francis, 2002). Therefore, a possible explanation for the lower $_{nH}$ values of UREM fibers may be due to alterations in the troponin complex.

Regarding maximal baseline (control solutions) isometric forces at the pool of fibers, unpaired analysis showed that UREM fibers had lower absolute forces by 14% compared to CONs. This functional limitation of uremic fibers was consistent with our previous results (Research paper 1) and it is probably due to the atrophy of uremic fibers which was reflected in the significantly smaller (by 8%) CSAs of UREM fibers compared to CONs. Specific forces produced by UREM fibers were not statistically significantly lower than the values of the CON fibers but this could be due to the smaller size of fibers used in this study. Moreover, if one source of tissue was excluded (fibers originating from an animal that had a better biochemical, mechanical and morphological profile), the extent and magnitude of force deficit was similar to that of research paper 1. It appears that this one better maintained animal (ie that in which for some technical or physiological reason the nephrectomy did not cause an equal degree of overall renal impairment as to the other UREM animals) fiber morphology and maximal force generation capacity was similar to that of fibers from CON animals.

From a functional point of view, loss of sensitivity to calcium may render a muscle effectively weaker in conditions where calcium availability may be low or impaired. For example under normal conditions, diaphragm is submaximally activated during normal breathing. Therefore reduced calcium sensitivity could lead to impaired breath as reported by studies in patients with chronic obstructive pulmonary disease (van Hees et al., 2011). Likewise, in CKD patients who experience functional

incapacity and their daily activities include submaximal calcium activation, in fatigue these 4.6% and 44.2% shifts on pCa_{50} and n_H values would make an appreciable contribution to an overall disturbed functional profile.

From a therapeutic point of view, various medications exist to potentiate force production at submaximal calcium. For example to better support respiratory function in COPD patients, the calcium sensitizer levosimendan exerts its effects through restoring of reduced calcium sensitivity of contractile proteins (van Hees, Dekhuijzen, & Heunks, 2009). Levosindeman has also been found to improve calcium sensitivity of diaphragm muscle fibers in heart failure (van Hees et al., 2011).

While at a first glance the observed differences in pCa_{50} and n_H may not appear of a great magnitude one should consider the overall weakened and atrophic status of skeletal muscle in chronic renal insufficiency.

Our study had many strengths but also some limitations that we have to address and foremost, the employment of an animal model of chronic kidney disease and not of acute kidney injury. Our animal model was allowed to develop renal insufficiency for 3 months after nephrectomy which corresponds to a sufficient period considering rabbit lifespan whereas an acute model of CKD may not induce realistically relevant modifications on muscle properties. Moreover, as the model was representative of a pre-dialysis stage our results may hold larger clinical relevance given the extent of ‘silent’ kidney disease among the general population (Stenvinkel, 2010). Furthermore, we used the single fiber technique, which allows forming conclusions about sarcomeric function by acutely isolating other factors such as muscle atrophy and excitation-contraction coupling issues. We also considered the importance of high temperature and we thus employed the Temperature-Jump (t-jump) method. The advantage of t-jump is that the fiber may be first activated at low

temperature where the sarcomere arrangement is stable and then it can be rapidly transferred to a near-physiological temperature in order to generate the maximal force without ruining the sarcomere arrangement (Karatzaferi et al., 2004). Another fact that strengthens our observations is that we examined contractile properties of samples in a blind fashion and that we used sham operated instead of purely control animals in order to avoid differences in results due to the different animal handling.

In conclusion, force-pCa relationship was determined in CON and UREM skinned psoas fibers at pH 7 and pH 6.2 and at both 10°C and 30°C. Our data imply a reduction of calcium sensitivity and mainly of thin filament cooperativity in UREM muscle without excluding the contribution of myosin loss or elastic proteins' problems. Our results indicate that the acute effect of low pH in UREM muscle which is a known condition in CKD patients (Moore et al., 1993) is of crucial role to negatively affect the calcium regulation. However, the lower pCa_{50} and n_H values also in normal pH indicate a chronic effect of uremia in contractile proteins which seem to mainly affect thin filament cooperativity. Future studies could investigate the possibility of alterations in sarcomeric proteins' expression affecting thin filament cooperativity such as troponin and tropomyosin (A. M. Gordon et al., 2000; Morimoto & Ohtsuki, 1994).

CHAPTER 5: RESEARCH PAPER 3

Effects of chronic renal insufficiency on stretch induced force potentiation

Aims addressed in this paper: using tissue from an animal model of uremia to examine the effect of chronic renal insufficiency on

1. stretch activation kinetics relationship of isolated muscle fiber preparations in conditions mimicking the resting state
2. morphology of isolated muscle fiber preparations from an animal model of uremia

ABSTRACT

Introduction: Chronic Kidney Disease is characterized by functional abnormalities of skeletal muscle, such as muscle weakness and easy fatigability. These abnormalities are strongly associated with an increase of waste products in the blood (uremia). Chronic uremia can lead to reduced functional capacity and premature death. The mechanisms underlying muscle dysfunction due to chronic uremia are unclear. We investigated the effects of uremia on myosin head cross-bridge kinetics, sarcomere length and diameter of fibers from rabbit psoas muscle. **Materials and Methods:** Renal insufficiency was induced surgically (removal of right kidney and partial nephrectomy of left one) in New Zealand female rabbits. Surgery and euthanasia protocols were approved by the University of Thessaly ethics committee. Psoas muscle samples were excised from control (sham-operated, CON) and uremic (UREM) animals at 3 months post-surgery. After 24-hour permeabilization treatment fibers were stored in 50% glycerol solution at -20°C until mechanical assessment. Single skinned fibers (n=21 CON; n=42 UREM) were investigated in solutions containing 5 mM ATP, 10 mM phosphocreatine and 20 U/ml creatine kinase at pH 7 and 22°C. After attachment, the maximal and minimal diameter of the fiber and the resting sarcomere length were measured in relaxation solution at a near slack position. Subsequently, fibers were maximally activated under isometric conditions (pCa 4.4). When force reached a plateau, step-like stretches of 0.3% fiber length were performed to induce isometric force transients. The time to peak of stretch-induced delayed force increase (t_3) was evaluated as a measure of cross-bridge kinetics. **Results:** Our results (mean±SD) derive from 42 UREM fibers and 21 CON fibers. Fibers of the UREM animals exhibited significantly larger t_3 values (UREM: 67±18 ms, CON: 57±16 ms; $P<0.05$). Furthermore, fibers of the UREM animals exhibited larger resting sarcomere

lengths (UREM: 2.25±0.33 μm, CON: 2.05±0.17 μm; P<0.01) and smaller mean diameters (UREM: 70±19 μm, CON: 79±13 μm; P<0.05). **Conclusion:** In conclusion, our results suggest that uremia can induce a slowing of myosin head cross-bridge kinetics and remodelling changes concerning fiber diameters (atrophy) and sarcomere structure. The larger sarcomere lengths in fibers of UREM animals could be due to a decrease of forces restoring the sarcomere length at resting conditions.

Key words: Chronic kidney disease, stretch activation kinetics, permeabilized fibers, sarcomere length, animal model

INTRODUCTION

Chronic kidney disease (CKD) is a new “epidemic’ across both sides of the Atlantic, expected to affect millions during their life time (Grams et al., 2013; Zoccali et al., 2010). CKD is characterized by skeletal muscle functional and metabolic abnormalities, such as muscle weakness and easy fatigability collectively termed *uremic myopathy* (Campistol, 2002; Fahal, 2014). Such functional abnormalities lead patients to low mobility, low levels of independence and quality of life and, in the long term, to premature death (Johansen et al., 2013).

Various factors have been reported to inhibit muscle function in CKD including uremia (Richet, 1988). Uremia-associated toxicity has been suggested to be implicated in the abnormal muscle function and fatigue intolerance experienced by CKD patients (Campistol, 2002; Davis et al., 1983). Other factors implicated in the etiology of uremic myopathy include inflammation, muscle atrophy, mitochondrial and neural dysfunction as well as oxidative stress (Campistol, 2002; Davis et al., 1983; Kaltsatou et al., 2015; Sakkas et al., 2004). However, it is not known if the contractility of skeletal muscle *per se* is altered.

Force is produced via the cyclical interaction of myosin to actin and depends on the number of formed cross-bridges, the force generated per cross-bridge and the duty ratio (i.e. time spend with myosin bound on actin) (Fitts et al., 1991; Karatzaferi et al., 2004; Miller et al., 2010). One approach to mechanically study cross-bridge kinetics is to induce a rapid stretch in fully activated fibers. The time from the beginning of the stretch to the peak value of the delayed force increase is known as t_3 and can give important information regarding the cross bridge kinetics, with larger values, thus slower kinetics, observed for the slower myosin isoforms (Galler et al., 1994; Linari, Bottinelli, et al., 2004).

From a functional point of view a muscle's response to a sudden stretch is crucial for an efficacious and safe locomotion. One should consider the important role of fast-twitch muscle in locomotion and the loss or qualitative alterations of this muscle type with ageing and/or disease (Lexell, 1995). A possible impairment in the ability of a muscle to maintain its length and respond to sudden stretches in CKD [where a preferentially atrophy of type II fibers has been reported (Sakkas, Ball, et al., 2003)] could lead to injury and falls.

To investigate effects of uremia on muscle mechanics, while avoiding a variety of confounding factors common in patient studies we employ a surgically induced CKD animal model (Gotloib et al., 1982) and we use the skinned fiber preparation (Cooke & Bialek, 1979; Degens & Larsson, 2007). This approach allows us to study the contractile machinery directly under controlled conditions (Karatzaferi et al., 2008). Using this approach we have observed that uremic muscle produces lower maximally activated isometric force (Research paper 1), at rest and in acidosis, which was only partially accounted by the observed moderate atrophy. We have also observed a slowing of the velocity of contraction, whether in the resting state or acidosis (Karatzaferi, Geeves, & Mitrou, 2014). We do not however know what the force generation capacity of uremic fibers would be in response to a sudden stretch.

To our knowledge there have not been yet any studies examining the effect of chronic renal insufficiency on stretch-potentiation and the underlying cross-bridge kinetics of skeletal muscle. Thus, the aim of the study was to examine the effect of chronic renal insufficiency on stretch-activation measurements of permeabilized single muscle fibers.

MATERIALS AND METHODS

All animal procedures, including surgery and euthanasia 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, in accordance to the national directives for the care and use of laboratory animals. Six New Zealand young adult white female rabbits (N=6) with a body weight (BW) of ~3200g were housed in a controlled environment with stable conditions (temperature 22–24°C, 12:12 h light-dark cycle) and acclimatized for 48 hours. Rabbits were fed ad libitum a special rabbit chow containing low levels of protein, potassium, calcium, phosphorus and sodium (prepared by Research Diets, Inc. USA) and water ad libitum.

After acclimatization, surgical procedures were performed (sham operation for control animals - CON and partial nephrectomy for experimental animals –UREM). Animals were anaesthetized by intravenous administration of a solution mixture of ketamine hydrochloride 100 mg/ml (Imalgene® 1000; Merial, Duluth, Georgia, USA) and xylazine 20 mg/ml (Rompun®; Bayer, Leverkusen, Germany), 87% and 13% respectively (proportion 6.69:1 approximately). The initial dosage for the induction of anesthesia was 0.3 ml/Kg BW of the above solution mixture, i.e. Imalgene® (87%) and Rompun® (13%), intravenously (i.v.). The maintenance of anaesthesia was achieved by a dose of 0.2 to 0.3 ml i.v. of the solution mixture. Animal temperature was maintained via a heating pad. Three hours before the intervention, each animal had only access to water and its weight was measured on a precision scale. The induction of renal insufficiency was performed surgically (using a surgical protocol modified from Gotloib et al., 1982) (Gotloib et al., 1982). For the UREM group four animals (N=4) underwent removal of the left kidney after careful ligation of the left

renal artery and vein; and partial nephrectomy (¾) of right kidney. For the CON group, two age –matched animals (N=2) underwent sham operation and were considered as the control group (CON animals). In a previous pilot study (data not shown) sham-operated muscle contractility was comparable to non-procedure animals. Thus, in agreement with the principle of Reduction in Animal Research sham-operated animals were used as controls in the present study.

Twelve weeks after surgery, animals were sacrificed (after determining their BW) by injection of sodium pentobarbital solution (50 mg/ml) which was applied in a dosage of 100 mg/Kg BW followed by bilateral thoracotomy. Immediately after cardiac arrest, blood samples were collected from heart and aorta using a heparinized syringe and were placed into ethylene diamine tetra-acetic acid (K2EDTA)-containing tubes (Vacutainer Plus Plastic K2EDTA; Becton Dickinson) for subsequent serum urea and creatinine determination using standard photometric protocols. Thereafter, psoas muscle was fast excised and processed for contractility studies (see below) while the remaining of the tissue was shared for other approved protocols.

Muscle Samples

Psoas muscle samples from UREM and CON animals were permeabilized as previously described (Karatzaferi et al., 2008). Briefly, thin bundles of rabbit PSOAS muscle (~2 mm diameter) were dissected and tied to wooden sticks using surgical thread. The samples were placed in falcon tubes containing skinning buffer solution (0°C) [120 mM KAc, 50 mM 3-(N-Morpholino) propanesulfonic acid (MOPS), 5 mM $MgAc_2$, 4 mM ethylene glycol tetraacetic acid (EGTA) and 50% glycerol (v/v); pH 7] with the addition of 100μl, per 50 ml end volume, of a protease inhibitor cocktail (104 mM AEBSF, 80 microns Aprotinin, 4 mM Bestatin, 1.4 mM E-64, 2 mM Leupeptin

and 1.5 mM Pepstatin). Muscle samples underwent a 24-hour permeabilization treatment [muscle samples (in falcon tubes) were placed on a vibrating platform shaker (Heidolph-Titramax 100) in a parallel to the ground position and shacked at 350 rpm for 24 hours at 0°C]. Thereafter the skinning solution was replaced with fresh solution and muscle samples were stored at -20°C until mechanical assessments.

Experimental setup for single fiber mechanics

Mechanical assessment was achieved using a home-made micro dynamometer which included an 8-bath platform (Galler lab). Each single fiber was dissected from the muscle bundle on a cold stage under a stereomicroscope. Consequently, the fiber ends were glued between the arms of a Piezo stepping motor (Physik Instrumente, Karlsruhe) and a force transducer (Scientific Instruments, Heidelberg) and manually switched between solutions.

Each fiber was first immersed for 1 minute in a bath containing rigor solution and then was incubated for 2 minutes in relaxing solution where fiber length and diameter were measured using the eyepiece of the stereoscope. Resting sarcomere length was also determined at a near slack position by laser diffraction (633 nm wavelength).

The fiber was maximally activated in an activating solution and once steady force was reached, a quick stretch of 0,3% fiber length was performed. Absolute maximal isometric force was recorded in mN. The force was related to the cross-sectional area of the muscle fiber to obtain the maximal isometric tension in mN/mm². Also the time from the beginning of the stretch to the peak value of the delayed force increase (t_3) (Figure 1) and the time from the beginning of the stretch to the lowest

force before the onset of the delayed force increase were recorded (t_2), using LabChart software and home written routines. Experiments were performed at 22°C, pH 7.

Experimental solutions

The basic rigor buffer contained 120 mM KAc, 50 mM MOPS, 5 mM $MgAc_2$ and 1 mM EGTA, pH 7. Relaxing solution was achieved with the addition of 5mM ATP, 10mM PCr and 20 U/ml ck. Lastly, activating solution was achieved with the addition of 1.1 mM $CaCl_2$ in the relaxing solution. The ionic strength of the solutions was ~0.2 M.

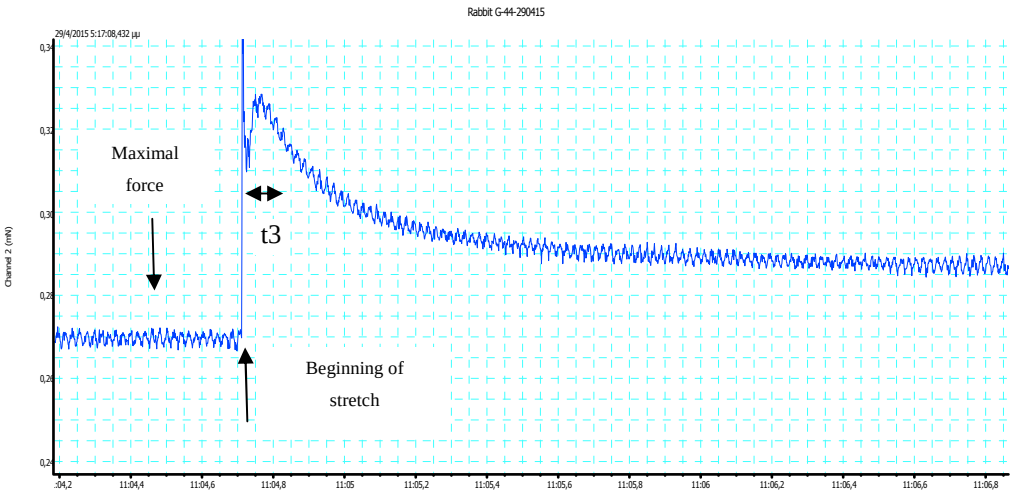

Figure 1. A representative experiment of stretch activation in a single fiber from rabbit psoas muscle. Fibers were maximally activated under isometric conditions. Once steady force was reached (maximal force), a quick stretch of 0.3% fiber length was performed. t_3 is the time from the beginning of stretch imposed on an isometrically contracting fiber to the peak value of the delayed force increase; X axis represents the time (ms) and Y the force (mN).

Data and Statistical analysis

Force transients were analyzed using the commercially available software "LabChart". Using this software we recorded force values (mN) in particular activation states of a fiber as well as time (ms) from a particular state (e.g. beginning of stretch) to another one (e.g. the peak value of the delayed force increase), as described in Galler et al (Galler et al., 1994). Therefore, after determining the maximal isometric force of each fiber, the time from the beginning of the stretch to the peak value of the delayed force increase was manually determined. In cases where force transients were unclear, t_3 values could not be determined and these data were not included in the 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 statistics (Mean ± SD) were first obtained for the diameters, CSAs, sarcomere lengths, tension and stretch activation collected data of CON and UREM single skinned psoas fibers. Differences between groups were tested using unpaired analysis (t-test for independent samples). All statistical analyses were performed using a commercially available statistical package (SPSS 15.0 for Windows) and significance level was set at $P<0.05$. Values are presented as mean ± standard deviation (SD).

RESULTS

Both surgery procedures (¾ partial nephrectomy-UREM animals and sham operation-CON animals) were well-tolerated and animals presented with a normal after-surgery recovery. At the end of the twelve-week period post-surgery, body weight ranged between 3,500-4,965 g for CON (with mean ±SD of 4,233 ± 1,036) g and 1,970-4,585 (3,200 ± 1,076) g for UREM animals. Renal insufficiency in experimental animals, compared to controls, was reflected in raised blood creatinine (CON, 1.40 ± 0.0 mg/dl, vs UREM, 2.97 ± 1.33 mg/dl) and urea levels (CON 46.0 ± 0.0 mg/dl vs UREM 71.3 ± 43.4 mg/dl).

Morphological characteristics of single fibers

Fibers of the UREM animals (n=42) exhibited smaller mean diameters compared to CONs (n=21) (Mean ± SD UREM 70.0 ± 19.0 vs 79.0 ± 13.0 μm, $P<0.05$). Calculated cross sectional areas of UREM fibers were also lower compared to CONs but the difference between groups was not statistically significant (UREM 4,117 ± 2,157 vs 5,020 ± 1,727 μm^2 , $P>0.05$). Furthermore, UREM fibers presented with significantly larger resting sarcomere lengths compared to CONs (UREM 2.25 ± 0.33 vs 2.05 ± 0.17 μm, $P<0.01$).

Contractile properties of single fibers

Both absolute and specific forces were weaker in the UREM fibers studied but not in a statistically significant way (Table 1).

Table 1. Isometric tension of UREM and CON single psoas muscle fibers at baseline (22°C, pH 7).

	Force (μN)	**Specific Force (mN/mm²)**
Control	326 ± 78	70 ± 25
Uremic	287 ± 167	72 ± 32
Descriptive (Mean ± SD) statistics are reported for the collected isometric force data.		

However, CON fibers showed a better response to stretch activation compared to UREMs as it is shown in representative experiments of Figure 2. On average CON fibers achieved a higher delayed force increase (by ~8% of baseline force) while UREM fibers often did not achieve a measurable delayed force increase (of those measured, ~3% of baseline). Due to noise and the low resolution of our analysis tools we could not reliably measure those small force differences. Thus force potentiation values will not be discussed thereafter.

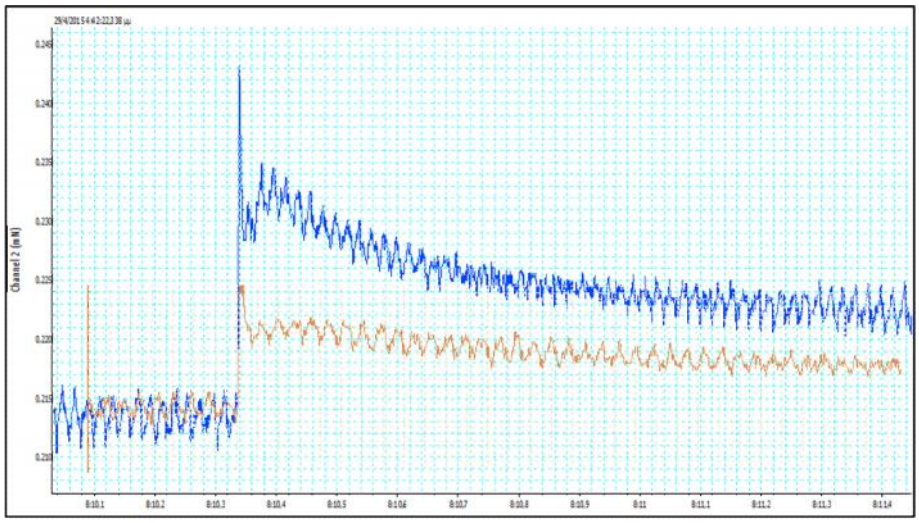

Figure 2. Force response following a step like stretch in a CON (blue) and a UREM (orange) fiber; X axis represents the time (ms) and Y the force (mN).

Our analysis showed that average t_3 values were significantly larger in UREM compared to CON fibers, P<0.05 (Figure 3). Values of t_2 did not differ between groups (UREM 19.05± 5.5 ms vs CON 20.55±6.3 ms, P>0.05).

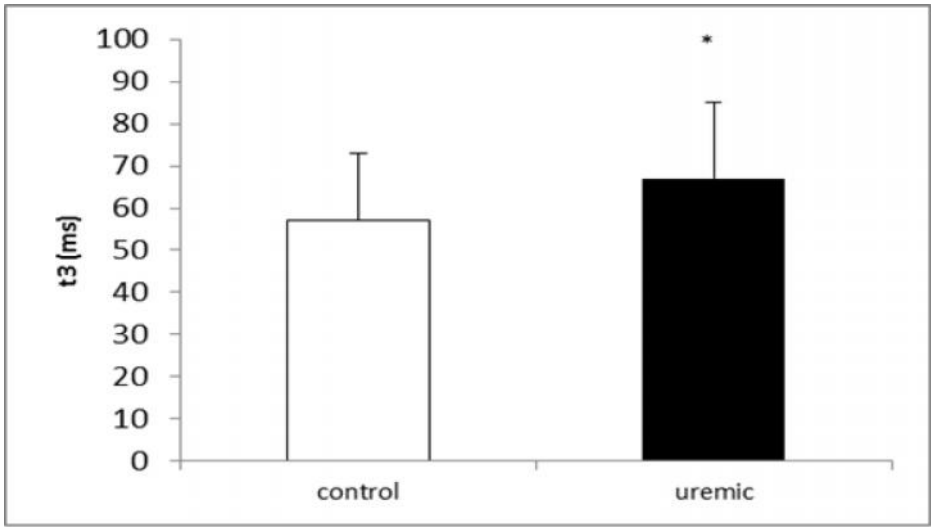

Figure 3. Stretch activation (t_3) in CON (open bars) and UREM (filled bars) fibers. From measurements at 22°C, pH 7; Data are presented as MEAN±SD; * Indicates statistically significant difference compared to CON (P<0.05).

The two stretch activation parameters t_2 and t_3 were strongly correlated (Pearson correlation coefficient, r =0.60, P<0.01) for the CON fibers. However, this correlation while remaining significant, became weaker for UREM fibers (r=0.37, P<0.05).

DISCUSSION

To the best of our knowledge, this is the first time that the effects of chronic renal insufficiency on myosin head cross-bridge kinetics of skeletal muscle single psoas fibers were examined. Uremic muscle fibers presented with slower cross bridge kinetics compared to controls, indicating an impaired ability to effectively respond to a sudden stretch while activated. Moreover, the observation of larger sarcomere lengths and smaller cross sectional areas in uremic fibers provided evidence for uremia-induced structural alterations in sarcomeric proteins.

Our stretch activation measurements revealed approx. 15% higher t_3 values in UREM fibers compared to CONs. A key parameter of the stretch activation protocol used in this study, t_3, has been proposed to primarily reflect cross bridge kinetics of myosin re-attachment and force development (Galler et al., 1994). Rapid stretches lead to a series of events, i.e. initially a simultaneous force rise (due to stretch forces on attached cross-bridges), followed by a sudden force reduction (as myosin heads are forcibly dissociated from actin binding sites) and then a delayed force increase above the initial force, i.e. force potentiation [due to cross-bridges re-attaching, (Kawai & Zhao, 1993)], followed by stabilization on a new force level dependent on final length. Assuming thus that stretch induced force transients are associated with the elementary steps of cross bridge cycle then, the larger t_3 values may indicate slower cross bridge kinetics and *vice versa*. Such kinetics are dependent on the myosin heavy chain (MHC) isoforms of the fibers (ie. smaller t_3 values-faster myosins, larger t_3 values-slower myosins) (Galler et al., 1994) in accordance to the known differences in myosin types with regards to mechano-chemical coupling steps of actomyosin (Geeves, 2016). Thus, based on t_3 results alone, UREM fibers' slower kinetics could indicate a shift to slower myosin properties.

Moreover, t_2 and t_3 values correlated strongly and significantly in our CON samples, as reported in other studies (Andruchova, Stephenson, Andruchov, Stephenson, & Galler, 2006). However, while still significant that association was weakened in UREM fibers. Past research using sinusoidal analysis and stretch activation kinetics has provided strong evidence that the force decay after stepwise stretch (t_2) is associated with detachment of myosin heads following ATP binding and the delayed force increase (t_3) is associated with their re-attachment and force generation prior to the release of phosphate [e.g. (Kawai & Zhao, 1993)] and are thus expected to correlate well (Andruchova et al., 2006). The latter and others have reported that, as these two time parameters reflect two distinct steps of the cross-bridge cycle (detachment and attachment) and do not incorporate the whole cross-bridge cycle sequence of steps (as e.g. when measuring velocity or the time development of the twitch force response), there is a tight correlation between MHC isoform and the values of both t_2 and t_3. Our observation of a discrepancy in the relationship between t_2 and t_3 in UREM fibers may be suggestive of either changes in MHC properties or other elements, such as elastic proteins, affecting sarcomeric protein interactions, perhaps weaken.

Therefore our results could be initially explained by changes in cross bridge kinetics due to some changes in myosin isoform properties of UREM fibers, with a shift to slower myosin properties. Such change of properties could relate to either MHC alterations (from a pure IIX to other type II isoforms) or myosin light chain changes (in the ratio of slow and fast isoforms). For the former, while we were unsuccessful due to technical difficulties in determining MHC expression, these could not be excluded in uremia [e.g. given the disturbed muscle type ‘mosaic’ observed in human studies of end-stage patients, (Sakkas, Ball, et al., 2003)]. Moreover, slower

cross bridge kinetics have also been observed in aged skeletal muscle fibers compared to young (Ochala, Dorer, Frontera, & Krivickas, 2006). It is also noteworthy that changes in the myosin light chain could affect the overall velocity of contraction of a single fiber, which in turn relates to properties of actomyosin dissociation step and the ability for rapid myosin binding in shortening conditions (Karatzaferi et al., 2008), steps which were more specifically probed with the performed stretches. While not shown here, we have observed a slowing of velocity of contraction of UREM fibers vs CON in an earlier pilot study (Mitrou et al., 2014) in line with the present observations.

Other changes in sarcomeric proteins of UREM fibers could occur. One site of interest would be at the troponin-tropomyosin regulatory level considering that the delayed force increase could be the result of problems in the displacement of tropomyosin segments, which uncover new binding sites for myosin, and could allow previously weakly bound cross-bridges to now attach strongly (Linari, Reedy, et al., 2004).

At the whole fiber level but also at the single sarcomere level some contribution of passive elastic elements should be considered, especially in larger sarcomeric lengths (Edman & Tsuchiya, 1996) and an emerging role for titin has been highlighted (Labeit et al., 2003), which could affect overall tension under stretch. Titin tends to bind to actin (Li, Jin, & Granzier, 1995) and can increase its stiffness when its PEVK and Ig domain binds calcium, as it can occur with their unfolding during stretching (Labeit et al., 2003). We observed consistently larger resting sarcomere lengths (despite those still being in the theoretical range of optimal force production for rabbit psoas) which could reflect changes in sarcomeric elastic

proteins. Thus a titin-mediated mechanism could in part be responsible for the overall repressed tension under stretch observed in UREM fibers.

In some cases t_3 values could not be determined due to the unclear signal. More precisely, in these fibers the points of force transients which would indicate the transition from the beginning of the stretch to the delay force increase (t_3) were not discernible. Upon unblinding it was revealed that this happened only in UREM fibers indicating that in some cases cross bridges of UREM fibers were unable for reattachment after a sudden lengthening. Thus our results regarding t_3 values could be even worse for UREM fibers if it was possible to determine these values in the discarded data sets.

Our results gave also evidence for uremia-induced remodeling of UREM fibers presented with 10% larger resting sarcomere lengths and 10% smaller diameters (atrophy) compared to CONs. Resting sarcomere length is mainly regulated from the giant elastic protein titin (Kontrogianni-Konstantopoulos, Ackermann, Bowman, Yap, & Bloch, 2009). Therefore the larger resting sarcomere lengths of UREM fibers could be due to modifications in titin due to uremic toxicity.

Our findings regarding atrophy in uremic muscle are in accordance to previous studies reporting significant atrophy in fast twitch fibers of uremic muscle (Diesel et al., 1993; Fahal et al., 1997; Sakkas, Ball, et al., 2003). Psoas muscle is a fast-twitch muscle expressing mainly IIX myosin heavy chain (MHC) isoforms [95% IIX(IId)] (Aigner et al., 1993) and IIX fibers appear with greater atrophy among all fast MHC isoforms in uremic muscle (Fahal et al., 1997; Sakkas, Ball, et al., 2003). The present results are in accordance to previous observations (Research paper 1) of a significant but moderate atrophy in UREM muscle fibers.

Overall, uremic fibers exhibited lower absolute and specific forces (see Research paper 1 and present results). This observation and the above, described reduced ability to respond to stretch, leads to the conclusion that uremic muscle suffers from severe functional problems. Our results are of major clinical relevance as it is known that skeletal muscle is of crucial role in the maintenance of an independent daily life and high levels of quality of life. In CKD weakness, early fatigue and injury heavily impact on patients' quality of life and overall health status (Go, Chertow, Fan, McCulloch, & Hsu, 2004; Tonelli, 2006). As mentioned in the introduction, the optimal functionality of fast skeletal muscle is crucial for a safe and efficacious locomotion. Our findings could guide future interventions to improve skeletal muscle's mechanical function in CKD.

In conclusion, our kinetic measurements suggest a slowing of myosin head cross-bridge kinetics, indicating underlying post-translational modifications of myosin, not excluding also some changes in the muscle's elastic properties. Moreover, our results show evidence of uremia-induced remodeling (atrophy). The larger resting sarcomere lengths in fibers of uremic animals could be due to a decrease of forces restoring the sarcomere length at resting conditions, i.e. also being linked to some elastic structural change.

Future research should examine protein expression and isolated proteins interactions (perhaps through fast kinetics assays). Our findings open new avenues in the direction for designing rational interventions in promoting actomyosin interaction and potentiating force in chronic disease.

CHAPTER 6: RESEARCH PAPER 4

Functional responses of uremic single skeletal muscle fibers in redox imbalances

Aims addressed in this paper: using tissue from an animal model of uremia to examine the effect of chronic renal insufficiency on

1. force generation of isolated muscle fiber preparations in conditions of acute redox imbalance

ABSTRACT

Introduction: Chronic kidney disease is associated with accumulation of uremic toxins, negatively affecting skeletal muscle. Uremic (UREM) muscle presents with redox imbalances and functional incapacity. To understand whether UREM muscle has acquired any sensitivity in redox imbalances we examined the functional responses of UREM myocytes to an acute load of oxidized and/or reduced agents.

Materials and Methods: Single psoas fibers from 2 UREM animals (n=19 fibers) and 2 sham-operated (CON, n=18 fibers), were assessed on 10mM Hydrogen Peroxide (H_2O_2) and/or Dithiothreitol (DTT) in 2 experimental sets: A) Exposure to H_2O_2 during activation followed by DTT during relaxation and repeated activation (n=9 CON; n=9 UREM fibers; B) Exposure to H_2O_2 during relaxation preceded and followed by submaximal (pCa_{50}) and maximal activation (n=9 CON; n=10 UREM fibers). **Results:** A) Acute exposure to H_2O_2 during activation did not affect force generation ($P>0.05$). DTT pre-incubation caused 12% force reduction ($P<0.05$) only in UREM fibers. B) H_2O_2 during relaxation reduced subsequent maximal isometric forces in the Pool of fibers (both CON and UREM) by 3.5% ($P<0.05$) but not in fiber groups separately (UREM $P>0.05$; CON $P>0.05$). **Conclusion:** Force generation capacity of CON and UREM fibers is affected by oxidation similarly. However the observation that UREM muscle may have been in a more reduced state at baseline warrants further investigation as it could be linked to disease induced effects.

Key words: Chronic kidney disease, isometric tension, permeabilized fibers, redox balance, animal model

INTRODUCTION

Chronic kidney disease (CKD) is now recognized as a major global health problem, expected to affect almost the 50% of European and American population during lifetime (Grams et al., 2013; Zoccali et al., 2010). The disease is accompanied by a variety of clinical implications, ameliorating independency and quality of life including muscle abnormalities such as skeletal muscle atrophy, muscle weakness and limited endurance collectively described as *uremic myopathy* (Adams & Vaziri, 2006; Campistol, 2002). The term "uremic" is due to the association of CKD with uremia, a condition which is caused by the accumulation of toxic waste products due to impaired kidney function (Richet, 1988) and it is believed that uremic toxins may affect muscle tissue (Campistol, 2002). Skeletal muscle structure and function is of major role not only for body movement and control but also for many other vital body functions such as protein and energy metabolism (Frontera & Ochala, 2014). Therefore, many researchers have studied the effects of various interventions (pharmaceutical, non pharmaceutical or combination) in preserving muscle quality and quantity of CKD patients (Balakrishnan et al., 2010; Johansen et al., 2006; Sakkas, Sargeant, et al., 2003b). Despite the improvements, CKD patients cannot restore their muscle structure and function at the levels of age-matched healthy individuals with a sedentary lifestyle (Sakkas, Hadjigeorgiou, et al., 2008; Sakkas, Sargeant, et al., 2003b).

The mechanisms underlying uremic myopathy and especially muscle dysfunction remain unknown with many factors being implicated including a reduced antioxidant capacity (Hensley, Robinson, Gabbita, Salsman, & Floyd, 2000) and the generation of excess reactive oxygen species (ROS) (Adams & Vaziri, 2006). The antioxidant capacity (especially the levels of glutathione), has been shown to be

reduced by fasting, low-protein diets, or diets limiting in sulfur amino acids (Paterson & Juurlink, 1999). It is not unexpected thus that dietary restrictions and inter-individual differences in antioxidant nutritional intakes affect antioxidant capacity in pre-dialysis patients (Sahni, Gupta, Rana, Prasad, & Bhalla, 2012). On the other hand, ROS, molecules containing oxygen such as superoxide ($O2^-$) with unpaired electrons, can damage membranes and impair contractile proteins (Debold, 2012). In healthy individuals, ROS production is a physiological outcome of activities such as exercise, and play a role in adaptive mechanisms (Møller, Wallin, & Knudsen, 1996) but in disease states and especially in CKD, ROS over-production may be the result of various additional endogenous and exogenous factors including uremic toxins, inflammation and dialysis treatment (Massy, Stenvinkel, & Drueke, 2009). Among the most important ROS are superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2), which originates mainly from the mitochondria and from enzymatic activity (Allen et al., 2008). H_2O_2 can diffuse into cell membranes and act as a signal to the oxidation of thiol groups of proteins (Allen et al., 2008). Oxidation is also associated with the formation of advanced glycation products (AGEs) (Miyata et al., 1997), a condition known as glycosylation which is common in CKD patients (Thornalley, 2006). It has been reported that AGEs can induce modifications in myosin structure (Ramamurthy et al., 2001) which could lead in a sensitivity to oxidative stress presented in uremic muscle and therefore leading to muscle dysfunction.

It has been proposed that the negative effect of H_2O_2 in muscle cells are through the generation of hydroxide (OH^-) (Lamb & Posterino, 2003). In vitro studies using single fiber preparations have shown that excess H_2O_2 can cause significant functional deficits (Lamb & Posterino, 2003; Murphy et al., 2008) which are fully or partially reversible using antioxidant molecules such as dithiothreitol (DTT) (Lamb &

Posterino, 2003; Murphy et al., 2008). It has been shown that fast twitch muscle fibers are more prone to functional deficits in the presence of ROS (Spencer & Posterino, 2009). In a CKD animal model (Research paper 1) we reported that fast muscle fibers (psoas) present with lower maximally activated force, compared to control. We also showed (Research paper 2) that acidosis causes a greater loss of calcium sensitivity in uremic fibers, but the degree of force decline was similar between uremic and control fibers. Our group recently showed that uremic muscle presents with a disturbed redox profile as reflected by increased protein carbonylation and a tendency for increase glutathione content (Poulianiti et al, unpublished data). It is not however known whether chronic renal insufficiency causes the sarcomeric functional unit to be more sensitive to acute oxidative stress.

Therefore, the aim of the study was to examine for the first time whether uremic skeletal muscle fibers from psoas fast-twitch muscle responded to an acute load of oxidative stress and/or to reducing agent similarly compared to CON fibers. To avoid a variety of confounding factors in human patients such as years in dialysis, comorbidities and pharmaceuticals, we employ a surgically induced CKD animal model (Gotloib et al., 1982) to investigate effects of uremia on muscle mechanics using the skinned fiber preparation (Cooke & Bialek, 1979; Degens & Larsson, 2007). The main advantage of this approach is that one can study the performance of contractile machinery *per se* isolating factors such as excitation-contraction coupling and atrophy. This method also allows for the control of intracellular content including calcium concentration alone or in combination with other factors such as oxidized and reducing agents which were the main issue of the present study (Karatzaferi et al., 2008; Lamb & Posterino, 2003).

MATERIALS AND METHODS

Animal model

All animal procedures, including surgery and euthanasia for this project 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, in accordance to the national directives for the care and use of laboratory animals. Four New Zealand young adult white female rabbits (N=4) with a body weight (BW) of ~3200g were housed in a controlled environment with stable conditions (temperature 22–24°C, 12:12 h light-dark cycle) and were acclimatized for 48 hours. Rabbits were fed ad libitum a special rabbit chow containing low levels of protein, potassium, calcium, phosphorus and sodium (prepared by Research Diets, Inc. USA) and water ad libitum.

After acclimatization, surgical procedures were performed (sham operation for control animals - CON and partial nephrectomy for experimental animals –UREM). Animals were anaesthetized by intravenous administration of a solution mixture of ketamine hydrochloride 100 mg/ml (Imalgene® 1000; Merial, Duluth, Georgia, USA) and xylazine 20 mg/ml (Rompun®; Bayer, Leverkusen, Germany), 87% and 13% respectively (proportion 6.69:1 approximately). The initial dosage for the induction of anesthesia was 0.3 ml/Kg BW of the above solution mixture, i.e. Imalgene® (87%) and Rompun® (13%), intravenously (i.v.). The maintenance of anaesthesia was achieved by a dose of 0.2 to 0.3 ml i.v. of the solution mixture. Animal temperature was maintained via a heating pad. Three hours before the intervention, each animal had only access to water and its weight was measured on a precision scale. The induction of renal insufficiency was performed surgically (using a surgical protocol modified from Gotloib et al., 1982) (Gotloib et al., 1982). For the UREM group two

animals (N=2) underwent removal of the left kidney after careful ligation of the left renal artery and vein; and partial nephrectomy (¾) of right kidney. For the CON group, two age-matched animals (N=2) underwent sham operation and were considered as the control group (CON animals).

Twelve weeks after surgery, animals were weighted and then sacrificed by injection of sodium pentobarbital solution (50 mg/ml) which was applied in a dosage of 100 mg/Kg BW followed by bilateral thoracotomy. Immediately after cardiac arrest, blood samples were collected from heart and aorta using a heparinized syringe for subsequent serum urea and creatinine determination using standard photometric protocols. Urea and creatinine concentrations were determined using commercially available kits (Abcam-ab83362 and ab65340for urea and creatinine respectively), a 96-well microtiter plate and a programmable microplate reader (Biochrom, Asys Expert 96). Psoas muscle was fast excised and processed for contractility studies (see below). Sample collections were done in a blind fashion.

Muscle Samples

Psoas muscle samples from UREM and CON animals were permeabilized as previously described (Karatzaferi et al., 2008). Briefly, thin bundles of rabbit PSOAS muscle (~2 mm diameter) were dissected and tied to wooden sticks using surgical thread. The samples were placed in falcon tubes containing skinning buffer solution ($0^{o}C$) [120 mM KAc, 50 mM 3-(N-Morpholino) propanesulfonic acid (MOPS), 5 mM $MgAc_2$, 4 mM ethylene glycol tetraacetic acid (EGTA) and 50% glycerol (v/v); pH 7] with the addition of 100μl, per 50 ml end volume, of a protease inhibitor cocktail (104 mM AEBSF, 80 microns Aprotinin, 4 mM Bestatin, 1.4 mM E-64, 2 mM Leupeptin and 1.5 mM Pepstatin). Samples were placed on a vibrating platform shaker

(Heidolph-Titramax 100) in a parallel to the ground position and shaken at 350 rpm for 24 hours at 0°C. Thereafter the skinning solution was replaced with fresh solution and muscle samples were stored at -20°C until mechanical assessments. Chemicals were purchased from Sigma-Aldrich.

Experimental setup for single fiber mechanics

For the mechanical assessment, each single fiber was dissected from the muscle bundle on a cold stage under a stereomicroscope and the fiber ends were attached between 2 tissue mounts of a muscle micro dynamometer with resolution of 0.4 μN to 4 N (SI Heidelberg/WPI). The tissue mounts were connected to a force transducer and a motor arm (used as a fixed end). The fiber was then immersed in baths containing ~200 μl of various solutions (refer to the experimental solutions section). This procedure was achieved using an adapted stereomicroscope on the top of the micro dynamometer. Data were continuously recorded in a computer using the particular software of SI Heidelberg/WPI and later exported for further analysis

The particular micro dynamometer consists of a platform with 5 baths and fibers can be automatically switched between them in less than 250 ms allowing for rapid changes such as calcium concentration (for an indicative experiment see Figure 1). The temperature of the baths was adjusted at 10°C using a cooling/heating water circulator (Thermo Electron Haake WKL 26 Recirculator Chiller 3L Capacity Bath).

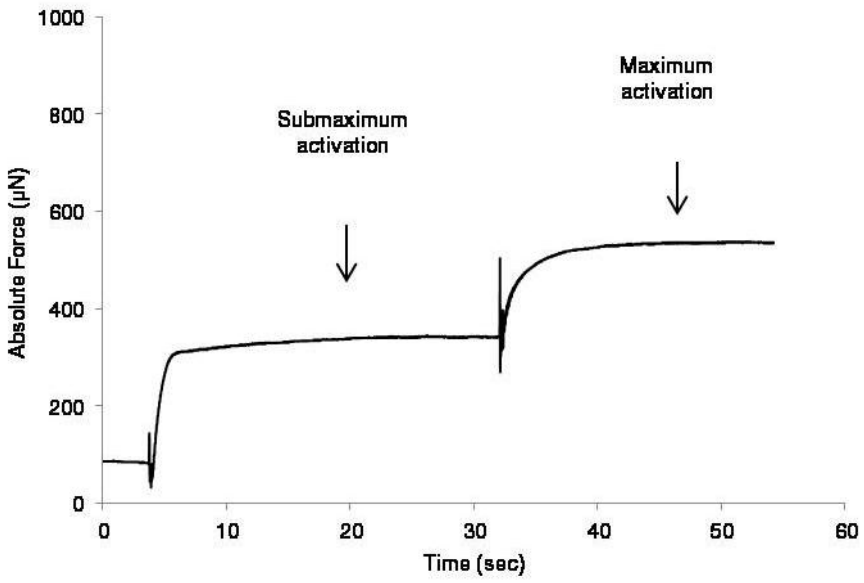

Figure 1. Indicative example of single fiber force recordings at 10°C, pH 7: A fiber was first immersed in a control relaxing solution and then in a submaximal activating solution. Once a steady state force was reached, the fiber was transferred in the maximal activating solution and was allowed again to reach a steady state force.

Experimental solutions

The basic rigor buffer contained 120 mM KAc, 50 mM MOPS, 5 mM $MgAc_2$ and 1 mM EGTA, pH 7; *Relaxing solutions.* Standard: with the inclusion of 5 mM ATP. Relaxing solution with reducing agent: addition of 10 mM dithiothreitol (DTT) in standard relaxing solution; Relaxing solution with oxidizing agent: addition of 10 mM H_2O_2 in standard relaxing solution; *Activating solutions.* Submaximal: addition of 0.53 mM $CaCl_2$ in standard relaxing solution (pCa 6.2). Maximal: addition of 1.1 mM $CaCl_2$ in standard relaxing solution (pCa 4.4). Maximal activating solution with oxidizing agent: addition of 10 mM H_2O_2 in Maximal activating solution. The ionic strength of the solutions was ~0.2M (Karatzaferi et al., 2004; Lamb & Posterino, 2003).

To exclude the possibility that the calcium concentration used to maximally activate CON fibers might not be sufficient for UREM fibers, a small pilot study was first undertaken where approx. 40 CON and UREM fibers (data not shown) were blindly assessed in three to four pCa concentrations in pH 7, 10°C. From that pilot study it was concluded that the standard addition of 1.1 mM $CaCl_2$ (pCa 4.4) provided maximal isometric tension for both groups and was thereafter used.

Isometric Tension measurements

A total of 37 fibers were first assessed under maximal isometric conditions at 10°C, pH 7 and baseline solutions. For this purpose, each fiber was first immersed for 1 minute in a bath containing rigor solution (to wash out excess glycerol) and then it was transferred and equilibrated for 2 minutes in a bath containing standard relaxing solution. Average diameter was determined assuming a cylindrical shape. Consequently, the fibers underwent a full assessment in one of the two protocols described below. Assessments were done in a blind fashion.

Experimental set A: “Exposure to H_2O_2 during activation and DTT during relaxation”. While fibers were maximally activated in standard solutions (n=18, 9 UREM and 9 CON) upon reaching a force plateau, 10mM H_2O_2 were added and forces were recorded for a further 5 minutes. After a wash-out fibers were returned to a relaxing condition. They were then exposed to 10mM DTT for 10 min. Following washing out, a final maximal activation in standard solutions was performed (Lamb & Posterino, 2003).

Experimental set B. “Exposure to H_2O_2 during relaxation-effect on submaximal and maximal force”: Another subset of fibers (n=19, 10 UREM and 9 CON fibers) was submaximally (pCa 6.2) and maximally activated (pCa 4.4) under

isometric conditions in standard activating solutions at resting sarcomere lengths. Then, the fibers were returned to a relaxing condition and exposed for 5 min to 10mM H_2O_2. After a wash-out, submaximal and maximal isometric forces were reassessed in fresh standard solutions (Lamb & Posterino, 2003).

In the results' section, force analysis data will be presented for both absolute and specific values (i.e. corrected for calculated CSA). In addition, effects of H_2O_2 or DTT on force will be presented separately for CON and UREM fibers but also as the pool of fibers.

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. One-way repeated measures ANOVAs were performed to examine the effects of experimental conditions in absolute and specific force of the Pool of fibers and of fiber groups separately. To examine possible differences between groups in their response to either H_2O_2 or DTT, force changes were calculated in percentage-change from respective standard force values and differences between groups were tested using the t-test for independent samples. Data are reported as MEAN±SEM. Statistical analyses were performed using SPSS 15.0 for Windows and significance level was set at $P<0.05$.

RESULTS

Both surgery procedures (¾ partial nephrectomy-UREM animals and sham operation-CON animals) were well-tolerated and animals presented with a normal after-surgery recovery. At the end of the twelve-week period post-surgery, body weight ranged between 3,000-3,245 (3,123±123gr) for UREM and 3,500-3,850gr for CON (3,675±175gr) animals. Renal insufficiency in experimental animals, compared to control, was reflected in raised serum creatinine (UREM 2.22±0.33 vs CON 1.46±0.00mg/dl) and urea levels (UREM 44.0±2.0 vs CON 38.0±0.0mg/dl).

Morphological characteristics of single fibers

Average fiber diameters differed significantly between groups (UREM 72.3±1.9 vs CON 77.9±2.0μm, $P<0.05$). Likewise, calculated cross sectional areas (CSAs) of UREM fibers were significantly lower compared to CONs (4,150±220 vs 4,817±237μm^2, $P<0.05$), indicating a level of ~14% atrophy in UREM fibers.

Functional characteristics of single fibers

Standard Conditions (10°C, pH 7): Baseline absolute maximal isometric force was significantly lower in UREM (n=19) fibers compared to CONs (n=18) by ~23% (UREM 316±17μN vs CON 410±22μN, $P<0.05$). Moreover, specific forces of UREM fibers tended to be lower (by ~9%) compared to CONs (UREM 78.2±4.4 vs CON 86.0±3.4 mN/mm^2) but not significantly ($P>0.05$). Similarly, in a subset of fibers (n=19, 9 CON/ 10 UREM) also assessed at standard submaximal calcium activation (pCa 6.2), absolute force of UREM fibers was significantly lower compared to CONs by ~33% (UREM 165±17 vs CON 248±15μN, $P<0.05$). Moreover, submaximal

specific force tended to be lower in UREM fibers by ~15%, but not significantly (UREM 40.1±3.3 vs CON 46.7±2.7μN, P>0.05).

Experimental set A: Addition of 10mM H_2O_2 during activation (n=18, 9 UREM/ 9 CON) did not affect maximal isometric forces in neither group (P>0.05) nor in the Pool of fibers (-0.9±3.0%, P>0.05). Moreover, the magnitude of the effect of H_2O_2 on isometric force was not significantly different between groups (P>0.05). Exposure to 10mM DTT during relaxation however, caused significant (P<0.05) force reductions in UREM fibers compared to baseline values (by –12%) in subsequent maximal activation (with the Pool of fibers -10.7±2.5%, P<0.05) but not in CON fibers (P>0.05). However, the magnitude of the effect of DTT did not differ significantly between fiber groups (P>0.05). To facilitate presentation, effects of H_2O_2 and DTT on maximal isometric force are presented as percent changes from baseline (standard) forces (Figure 2).

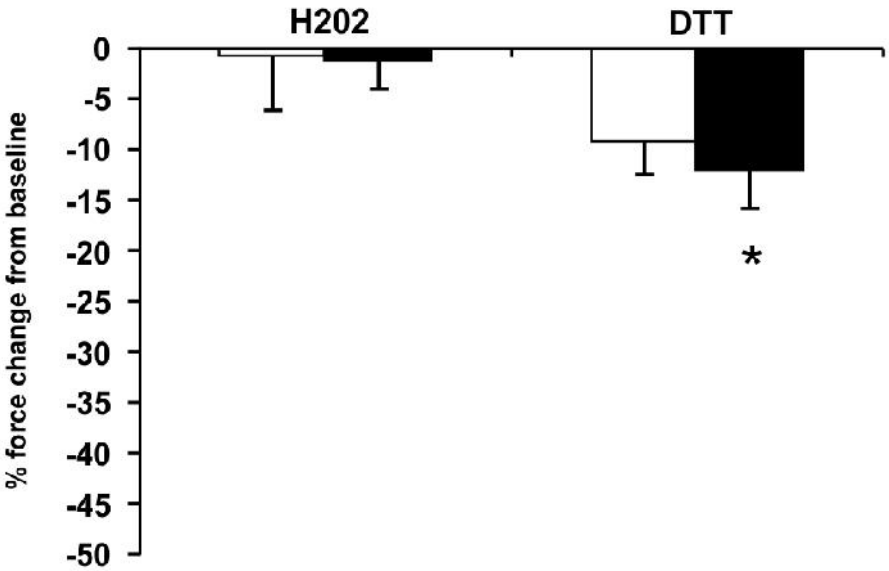

Figure 2. Force values expressed as % force change from baseline following exposure to 10mM H_2O_2 during activation (H2O2) and 10mM DTT during relaxation

(DTT). CON fibers are shown as open bars. * indicates the significant difference ($P<0.05$) from baseline force for UREM (filled bars) fibers.

Experimental set B: In another subset of fibers (n=19, 9 CON/ 10 UREM) we examined the effect of incubation with 10mM H_2O_2 during relaxation on submaximal (pCa 6.2) and maximal (pCa 4.4) force production. Pre-incubation with 10mM H_2O_2 did not cause any significant reduction in subsequent submaximal isometric forces compared to standard conditions in neither of the groups (UREM -0.1±2.5%, $P>0.05$; CON -4.7±4.3%, $P>0.05$) nor in the Pool of fibers (-2.3±2.4%**,** $P>0.05$). Nevertheless, maximal isometric tension was significantly reduced in the Pool of fibers (-3.5±1.2%, $P<0.05$) but not in groups separately (UREM $P>0.05$; CON $P>0.05$) after exposure to H_2O_2. Lastly there was no significant difference between groups in their magnitude of response to H_2O_2 ($P>0.05$). To facilitate presentation, the effects of exposure to H_2O_2 during relaxation on submaximal and maximal isometric force are presented as percent changes from baseline (Figure 3).

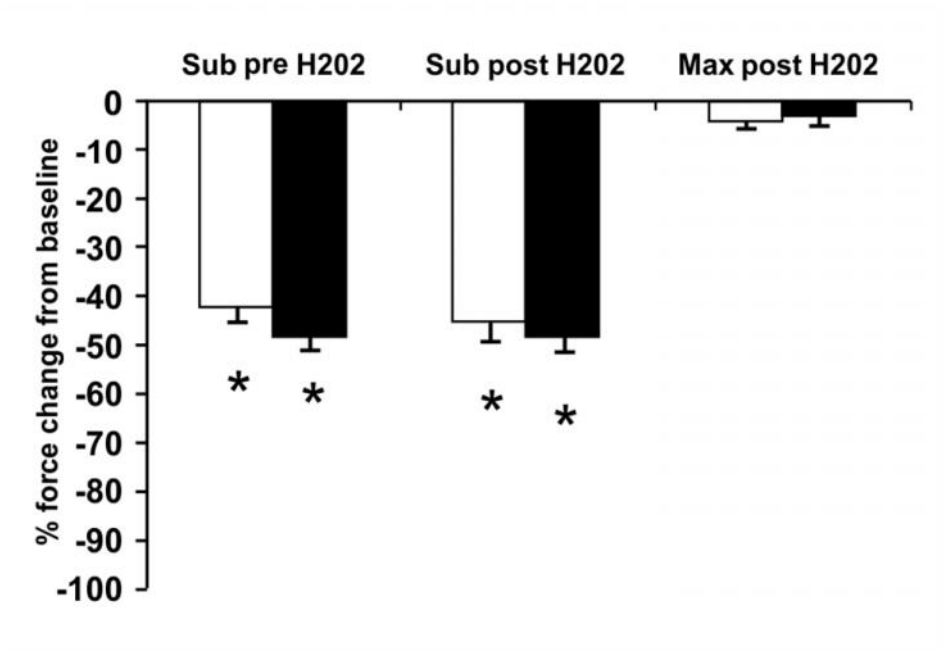

Figure 3. Force values expressed as % force change from maximal baseline force following submaximal activation before (Sub pre H2O2) and after exposure to H_2O_2 (Sub post H2O2) as well maximal activation after exposure to H_2O_2 (Max post H2O2) during relaxation. * indicates the significant difference ($P<0.05$) from baseline force for CON (open bars) and UREM (filled bars) fibers.

DISCUSSION

To our knowledge this is the first study to examine the effects of redox challenges on force generation capacity of skeletal muscle fibers from an animal model of uremia. Our results show that contractile properties of UREM fibers are inhibited by an acute load of reduction rather than oxidation, which indicates that their redox status at baseline may have shifted to reduction.

Regarding the effects of an oxidizing agent on maximal isometric force, addition of 10mM H_2O_2 during activation did not cause any appreciable change in maximal isometric forces of UREM nor of CON fibers. This is in consistent with the study of Lamb et al (Lamb & Posterino, 2003) who examined rat EDL muscle skinned fibers using a similar H_2O_2 treatment (10mM H_2O_2 for 5 minutes during activation). However, other studies using different methodology have found different results in maximal isometric forces. For example Prochniewicz et al (Prochniewicz, Lowe, et al., 2008) found that even 5mM H_2O_2 may cause significant reductions in forces of skinned psoas fibers from New Zealand rabbits by ~31% while higher concentrations of H_2O_2 may cause larger reductions (e.g. 50mM H_2O_2 lead to ~85% force reduction). However in this study fibers were incubated in H_2O_2 for 30 minutes before functional assessment and it has been clearly reported that effect of oxidized agents is time dependent (e.g. long lasting exposures lead to larger reductions in maximal force) (Lamb & Posterino, 2003; Murphy et al., 2008). Other studies have shown that extremely higher concentrations of H_2O_2 (i.e. 50mM) lead to smaller force reductions (e.g. 19%) in slow fiber types of rats (soleus slow-twitch muscle fibers) (Gilliver et al., 2010).

In a subset of fibers, we examined the effects of pre-incubation to oxidizing conditions during relaxation on subsequent submaximal and maximal force. We found

no influence on submaximal isometric forces of either group. However, exposure to 10mM H_2O_2 during relaxation for 5 minutes caused a significant reduction in absolute maximal isometric forces for both CON and UREM fibers (by ~3.5%. in the Pool of fibers). This is in agreement to Lamb et al (Lamb & Posterino, 2003) who have reported that the effect of H_2O_2 on force differs if the fiber is oxidized during relaxation or activation. Indeed, other studies have also shown that exposure to H_2O_2 during relaxation leads to significant force reductions in maximal isometric forces (Gilliver et al., 2010; Prochniewicz, Lowe, et al., 2008). It has been shown that the negative effect of H_2O_2 in maximal isometric force is due to modifications in myosin heads causing force reductions per cross bridge (Murphy et al., 2008) It can be appreciated that actin-binding sites of the myosin heads are continuously exposed during relaxation while when myosin heads are attached to actin, these same binding sites are intermittently exposed. However, since the magnitude of the effect of H_2O_2 on force did not significantly differ between fiber groups it could be surmised either that the exposure had to be longer to reveal any differences or that the chronic uremia had not caused the skeletal muscle to acquire any level of 'sensitivity' to H_2O_2 exposure. Moreover, the observation of Lamb et al (Lamb & Posterino, 2003) suggesting that oxidation may cause an increase in calcium sensitivity which at submaximal activation could prevent force reductions in the presence of H_2O_2 can explain the lack of appreciable effect of the oxidizing agent on submaximal force.

A 10-minute exposure to 10mM DTT during relaxation did not affect significantly maximal forces of control fibers, in agreement to past reports (Lamb & Posterino, 2003), however it significantly reduced force in uremic fibers by ~12%. This was an unexpected and novel result. DTT is a strong reductant and the lack of effect on maximal forces of CON skinned fibers indicate that those fibers were in

balanced redox state (Lamb & Posterino, 2003) [considering the force-redox relationship as an inverted U with a plateau, with either excess oxidation or reduction reducing force, see also (Andrade, Reid, Allen, & Westerblad, 1998) and (Powers, Ji, Kavazis, & Jackson, 2011)]. Thus given observations that uremic muscle presents with a disturbed redox status (Poulianiti et al., 2015), and the fact that force reduction due to DTT was significant only in UREM fibers it could not be excluded that UREM muscle sarcomeric proteins may have acquired a reduced state. Thus addition of DTT led to further reduction which could thus explain the observed force reduction more.

Regarding maximal baseline isometric forces, results showed that UREM fibers had significantly lower absolute forces compared to CONs. This functional limitation of UREM fibers is in consistent with our previous results (Research paper 1) but after correcting for fibers' CSA, specific forces were not significantly different between fiber groups, probably due to the smaller number of fibers. Lower absolute forces of UREM fibers can be explained from their significant atrophy manifested in smaller CSAs compared to CONs while reduced fiber CSA is associated with reduced contractile protein content and number of myosin cross bridges, leading to low force generation per fiber (Fitts et al., 1991).

Our findings regarding atrophy of UREM psoas muscle fibers is in accord with other studies indicating significant atrophy mainly in fast twitch fibers of uremic muscle (Diesel et al., 1993; Fahal et al., 1997; Sakkas, Ball, et al., 2003). It has been reported that IIX fibers appear with greater atrophy among all fast MHC isoforms in uremic muscle (Fahal et al., 1997; Sakkas, Ball, et al., 2003) and this was why we assessed psoas muscle which is a fast-twitch muscle expressing mainly IIX myosin heavy chain (MHC) isoforms [95% IIX(IId)] (Aigner et al., 1993).

Our study had a number of strengths and mainly the employment of an animal model of CKD and not of acute kidney injury. Our animal model was allowed to develop CKD for 3 months after nephrectomy which corresponds to a sufficient period considering rabbit lifespan whereas an acute model of CKD may not induce realistically relevant modifications on muscle properties. Furthermore, we used the single fiber technique, which allows forming conclusions about sarcomeric function by acutely isolating other factors such as abnormal intracellular content and excitation-contraction coupling issues. Another strength of our study was its blind design and the use of sham operated animals instead of non-surgery control animals. By this way we avoided differences in results due to the different animal handling. Our study had also some limitations that we have to address. First, despite implementing the same surgical approach, the biochemical responses were somewhat variable which perhaps explains why the UREM fibers presented with greater variability in force values than CONs. Furthermore we did not assess contractile properties at near physiological temperatures (ie. $\geq 30^{o}C$). At $30^{o}C$ where sarcomere derangement is common (Karatzaferi et al., 2008), our long and repetitive activations could lead in fibers' exhaustion. Lastly, fibers' cross sectional areas were calculated assuming a cylindrical instead of elliptical shape but this happened in a systematic way and could not affect our results.

In conclusion, although functional response of uremic fibers to H_2O_2 does not differ compared to controls, DTT inhibited force only in uremic fibers indicating that the initial redox status of uremic muscle is more in a reduced rather than oxidized state. This finding, taken together with the presentation of moderate atrophy and the tendency for lower specific forces in uremic muscle, point to possible explanations for the muscle weakness observed in patients presenting with uremic myopathy. Further

work is required to determine dose-response relationships between isometric, maximal and submaximal, force and key redox constituents, to determine which sarcomeric proteins are mostly affected, as well as to delineate the possible interaction of redox imbalances to fatigue or toxicity factors.

CHAPTER 7: GENERAL DISCUSSION

To the best of our knowledge this is the first study to examine the effect of chronic renal insufficiency on the contractile properties in combination to morphological characteristics of single psoas muscle fibers in an animal model mimicking CKD. Chronic renal insufficiency induced significant impairments in contractile properties of single muscle fibers. Specifically, maximal isometric force and calcium sensitivity were found to be lower in uremic muscle at standard and at near physiological temperatures, at rest and acidosis. Furthermore, in experiments carried out only in resting conditions (stretch activation) it was found that uremia may induce a slowing of myosin head cross-bridge kinetics. Additionally, uremic muscle found to enter experimentation in a more reduced state. Apart from functional deficits of the uremic fibers, cross sectional areas and sarcomere lengths were also found with significant impairments while CSAs were smaller and sarcomere lengths larger compared to controls. The smaller CSAs of uremic fibers indicated the presence of atrophy in uremic muscle which could however only partly explain their lower forces, as specific forces of uremic fibers were still depressed. Our results indicate significant functional deficits of uremic muscle at the single fiber level independently of fiber atrophy and further investigation is warranted to pinpoint the contributions of possible changes in sarcomeric proteins to the evident functional deficit.

A significant functional deficit for uremic muscle at the single fiber level was first revealed from their impaired capacity to generate maximal isometric force at standard conditions, as compared to control fibers. Moreover, the significant force deficit was evident also at the temperature of 30°C, an observation with high physiological relevance. Uremic fibers demonstrated a significant and moderate atrophy which could not fully account for the reduced specific forces. It is indicative

that the specific force deficit of uremic fibers was approximately -28% at 30°C. The significantly smaller CSA of uremic fibers could not thus account for the depressed specific forces of uremic fibers we observed.

The specific force deficit of single uremic muscle fibers observed in our study could indicate a reduced capacity of cross bridges to generate force or perhaps possible disturbances on how generated force is transmitted across the sarcomeric arrangements. Such changes could be the result of alterations in sarcomeric proteins such as myosin which can be a target for posttranslational modifications that affect its functional properties (Kay, 1978). An additional factor which may be implicated is maybe the oxidative stress which is one of the biochemical repercussions of renal insufficiency (Kaltsatou et al., 2015) and can act on its own or in combination with other molecules to cause muscle dysfunction at multiple levels. Oxidative stress can also affect function by promoting the formation of advanced glycation end products (AGEs) (Miyata et al., 1997), which have been shown to affect myosin structural and functional properties (Ramamurthy et al., 2001). As glycosylation is often observed in renal disease (Thornalley, 2006) along with oxidative stress (Kaltsatou et al., 2015), whether chronic oxidative stress in renal insufficiency may cause irreversible structural modifications affecting the actomyosin interaction cannot be excluded and should be investigated in the future.

Other factors, related to uremic toxicity effects may be also at play, either promoting atrophy or affecting muscle properties. As CKD progresses, uremia develops in parallel with the accumulation of related toxic waste products (Richet, 1988). These are implicated in the etiology of abnormal muscle function and fatigue intolerance experienced by renal patients (Campistol, 2002; Davis et al., 1983) with research so far indicating a multiple of candidates with a possible role (Vanholder et

al., 2003, 2009). Not much is known about their possible direct role on skeletal muscle function, however the so- called ‘uremic toxins’ (Vanholder et al., 2009) could worsen oxidative stress and inflammation and hasten muscle loss and mitochondrial dysfunction, thus contributing to the moderate atrophy observed in this study as well as to further, hitherto undefined, changes in sarcomeric protein properties.

Limitations in force generation under submaximal calcium activation can significantly reduce muscle performance in daily life, especially if one considers the functional impairment presented in CKD patients. It was found that in resting conditions uremic fibers presented with lower calcium sensitivity compared to controls at both 10°C and 30°C but the difference between fiber groups were more evident at 30°C. In acidosis, uremic fibers presented again with lower calcium sensitivity than controls. The decrease in calcium sensitivity in uremic fibers indicated that more calcium was required in order to achieve the 50% of maximal isometric force.

Calcium sensitivity depends mainly on the function of troponin-C (TNC) protein which holds the regulatory calcium binding sites. However, other thin filament proteins may also affect calcium sensitivity such as tropomyosin (TM) (Schiaffino & Reggiani, 1996) and nebulin (Lee et al., 2013). Thick filament proteins are also implicated since strong cross bridge attachment causes the stabilization of the thin filament in a state with high Ca^{2+} affinity (Schiaffino & Reggiani, 1996). Moreover, the sarcomere length can affect calcium sensitivity (Stephenson & Williams, 1982). Given that we have observed slightly longer sarcomere lengths in uremic fibers (Research paper 3), despite these being at the optimal range for isometric force production, we cannot exclude that part of our observations may also

reflect differences in elastic forces maintaining the sarcomere length during contraction. In addition to calcium sensitivity, in resting conditions, n_H values revealed a lower thin filament cooperativity for uremic fibers compared to controls which was more intense at 30°C. The cooperativity which is reflected in n_H values depends on the thin filament proteins tropomyosin, troponin complex (TNC, TNT, TNI), actin and myosin heads. All of the above need to act cooperatively for the achievement of muscle contraction (Boussouf & Geeves, 2007) and even a small degree of change in the n_H value of the force-pCa relationship could have profound implications. However it has been reported that n_H has a descriptive role and should not be overestimated (Walker et al., 2011). Thus, given present results we cannot form safe conclusions regarding the effects of uremia on cooperativity of calcium activation. From a functional point of view, loss of sensitivity to calcium as shown by the present work could make an appreciable contribution to an overall disturbed functional profile in CKD muscle.

We evaluated our samples' response to a stretch protocol. Our stretch activation measurements revealed an approx. 15% higher average t_3 value in uremic fibers compared to controls. The stretch activation protocol used in this study, in which a sudden lengthening (stretch) is imposed on a fully activated muscle cell that contracts under isometric conditions, with the time from the beginning of the stretch to the peak value of the delayed force increase shown by $t_{3,}$ has been proposed to primarily reflect cross bridge kinetics of force development (Galler et al., 1994).

Therefore our results could be explained by changes in cross bridge kinetics due to some changes in myosin isoform properties of uremic fibers, with a shift to slower myosin properties. Such change of properties could relate to either myosin heavy chain alterations or myosin light chain changes. Other changes in sarcomeric

proteins of uremic fibers could also occur. One site of interest would be at the troponin-tropomyosin regulatory level considering that the delayed force increase could be the result of problems in the displacement of tropomyosin segments (Linari, Reedy, et al., 2004). Another site of interest would be however at the properties of the giant protein, titin. Titin tends to bind to actin (Li et al., 1995) as it can occur with their unfolding during stretching (Labeit et al., 2003). The latter possibility is re-enforced by our observation of some laxity in baseline sarcomere lengths of uremic fibers. In *in vivo* conditions, an appropriate response to a sudden stretch is crucial for an efficacious and safe locomotion and one should consider the important role of fast-twitch muscle in locomotion and the loss of this type due to ageing and/or disease (Lexell, 1995). A possible impairment in the ability of a muscle to maintain its length and respond to sudden stretches in CKD [where a preferentially atrophy of type II fibers has been reported (Sakkas, Ball, et al., 2003)] could lead to injury and falls. Thus our results point to a weakened response of uremic muscle to sudden stretch which should be further explored in future work.

We also examined whether uremic skeletal muscle fibers responded to an acute load of oxidative stress (H_2O_2) and/or to reducing agent (DTT) similarly compared to control fibers. Acute exposure to H_2O_2 during activation did not affect force but DTT caused significant force reductions only in uremic fibers indicating that the initial redox status of uremic muscle is more in a reduced rather than oxidized state. On the other hand, incubation with H_2O_2 during relaxation reduced maximal isometric forces in the Pooled of fibers (both control and uremic fibers), but not submaximal force production, without differences between groups. Our results may suggest that chronic renal insufficiency has not caused such modifications in uremic muscle fibers such as to make them more susceptible to an acute exposure of an

oxidizing agent. It seems however that uremic fibers may have started off at a more reduced state than controls [considering reports on the upregulation of the Reduced form of Glutathione (GSH) in uremic tissues]. Such redox modifications and their functional consequences should be explored in the future.

In parallel with functional properties of single fibers we also assessed their morphological characteristics. Uremic psoas fibers presented with significantly smaller CSAs compared to controls by ~11%. In CKD patients, muscle weakness may be partially explained by atrophy which has been reported to be exacerbated but only partly explained by inactivity (Sakkas, Sargeant, et al., 2003a), pointing to a disease specific effect, mainly presented in type II fibers (Sakkas, Ball, et al., 2003; Sawant et al., 2011). Our results support the notion that atrophy could start earlier during the disease progress and could appreciably affect fast muscles which are normally tasked to provide high levels of muscle power.

Furthermore, we observed that uremic fibers presented with 10% larger resting sarcomere lengths compared to controls. Such alterations could reflect changes in sarcomeric elastic proteins. Thus a titin-mediated mechanism could in part be responsible for the overall repressed tension under stretch observed in uremic fibers since resting sarcomere length is mainly regulated from the giant elastic protein titin (Kontrogianni-Konstantopoulos et al., 2009).

This PhD thesis had many strengths but also some limitations that we have to address. Despite implementing the same surgical approach, the biochemical responses were somewhat variable which perhaps explains why the uremic fibers presented with greater variability in force values than controls. Also, it was not possible to evaluate all psoas fibers at 30°C since fibers often broke (or did not fulfill pre-set criteria upon return to 10°C and were thus discarded). This is not surprising as exactly due to this

known sensitivity of skinned fibers, researchers have developed such t-jump protocols (Karatzaferi et al., 2004, 2008; Pate et al., 1995), while the majority of skinned single fiber studies are performed at lower temperatures (e.g. 10°C-20°C) (Fitzsimons et al., 2001; Gilliver et al., 2010; Mollica et al., 2012; Murphy et al., 2004; Reggiani et al., 1997; Stienen et al., 1992; Sweeney et al., 1988). Additionally, fibers' cross sectional areas were calculated assuming a cylindrical and not an elliptical shape and this may be why our baseline control force values were lower compared to other studies (Coupland et al., 2005). Nevertheless, this happened in a systematic way in all single fibers and could not affect the interpretation of our results, as for example our control fibers' response to the drop of pH was similar to what observed previously (Cooke et al., 1988; Karatzaferi et al., 2003; Pate et al., 1995). Another weakness of our study was that a 'positive' bias has been inadvertently introduced, due to the blind design, as uremic fibers were in retrospect proven difficult to dissect and handle. I.e. the fibers that withstood our handling were assessed but many more that could not be handled were not assessed due to the bad quality of muscle tissue. Also, given some standard criteria that have been used [e.g. (Karatzaferi et al., 2003; Liang et al., 2008)] for force data quality, it later transpired that more uremic fibers were discarded than controls. Thus, the 'better' fibers were measured by each round of assessments.

This thesis had also some important strengths, foremost, the employment of an animal model of chronic renal disease, and not of acute kidney injury, which we consider to induce realistically disease relevant modifications on muscle properties. Moreover, as the model was representative of a pre-dialysis stage our results may hold larger clinical relevance given the extent of 'silent' kidney disease among the general population (Stenvinkel, 2010). Furthermore, we used the single fiber technique, which allows forming conclusions about sarcomeric function by acutely isolating other

factors such as muscle atrophy and excitation-contraction coupling issues. Another fact that strengthens our observations is that we examined contractile properties of samples in a blind fashion and that we used sham operated instead of purely control animals, under controlled diet, in order to avoid differences in results due to the different animal handling.

CHAPTER 8: CONCLUSIONS & FUTURE DIRECTIONS

To the best of our knowledge this is the first study that examined the effect of chronic renal insufficiency on the contractile properties of skeletal muscle. We used an animal model mimicking CKD and found that chronic renal insufficiency induced significant impairments in contractile properties of single muscle fibers.

Specifically, maximal isometric force and calcium sensitivity were found to be lower in uremic muscle at standard and at near physiological temperatures, at rest and acidosis. Furthermore, it appeared that uremia may induce a slowing of myosin head cross-bridge kinetics but uremic fibers did not differ in their response to acute exposure to an oxidizing agent and pH challenges. However the significant effect of a reducing agent only in uremic fibers indicates that uremic muscle is more in a reduced rather than oxidized state.

Overall, uremic fibers presented with atrophy which however could not fully account for the observed force deficit. Moreover, sarcomere lengths were larger in uremic muscle fibers compared to controls.

In conclusion, our results indicate significant functional deficits of uremic muscle at the single fiber level independently of fiber atrophy and further investigation is warranted to pinpoint the contributions of possible changes in sarcomeric proteins to the evident functional deficit. However, we should also consider that in the literature there is enough evidence suggesting that muscle function in this clinical population is also inhibited by other factors such as neural dysfunction (Adams & Vaziri, 2006). Therefore, considering that our study showed significant deficiencies at the single fiber level one cannot exclude that other parameters may act

alone or synergistically with the possible functional abnormalities at the sarcomeric level leading patients to even worse functional profile than predicted by our data.

Several outstanding questions were revealed with this work:

- It is not yet clear whether chronic oxidative stress in renal insufficiency may cause irreversible structural modifications affecting the actomyosin interaction. This cannot be excluded and should be investigated in the future.

- Present results do not allow us to form safe conclusions regarding the effects of uremia on cooperativity of calcium activation. From a functional point of view, loss of sensitivity to calcium as shown by the present work could make an appreciable contribution to an overall disturbed functional profile in CKD muscle. Whether regulatory proteins are to blame or other mechanisms should be investigated.

- Muscle dysfunction of the kind observed in CKD can lead to injury and falls. Our results point to a weakened response of uremic muscle to sudden stretch which should be further explored in future work.

- Lastly, it seems that uremic fibers may have a more reduced state than controls. Such redox modifications and their functional consequences should be explored in the future.

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APPENDIXES

Appendix 1: Bioethics Approval

ΠΑΝΕΠΙΣΤΗΜΙΟ ΘΕΣΣΑΛΙΑΣ
ΤΜΗΜΑ ΕΠΙΣΤΗΜΗΣ ΦΥΣΙΚΗΣ ΑΓΩΓΗΣ ΚΑΙ ΑΘΛΗΤΙΣΜΟΥ

Εσωτερική Επιτροπή Δεοντολογίας

Τρίκαλα: 10/10/2012
Αριθμ. Πρωτ.: 630

Αίτηση Εξέτασης της πρότασης για διεξαγωγή Έρευνας με τίτλο:
«Μηχανισμοί της μυϊκής λειτουργίας: Η επίδραση της ουραιμίας στην μορφολογία, τον μεταβολισμό και την ανοχή στην κόπωση»
Επιστημονικώς υπεύθυνη / επιβλέπουσα: Δρ. Χριστίνα Καρατζαφέρη
Ιδιότητα: Επίκουρη Καθηγήτρια, ΠΘ
Ίδρυμα: Πανεπιστήμιο Θεσσαλίας
Τμήμα: Τμήμα Επιστήμης Φυσικής Αγωγής και Αθλητισμού
Κύρια ερευνήτρια / φοιτήτρια: Γεωργία Μήτρου
Πρόγραμμα Σπουδών: Διδακτορικός κύκλος σπουδών
Ίδρυμα: Πανεπιστήμιο Θεσσαλίας
Τμήμα: Τμήμα Επιστήμης Φυσικής Αγωγής και Αθλητισμού

Η προτεινόμενη έρευνα θα είναι:

Ερευνητικό πρόγραμμα X Μεταπτυχιακή διατριβή ☐ Διπλωματική εργασία ☐ Διδακτορική έρευνα X

Τηλ. επικοινωνίας: 6948 176 157
Email επικοινωνίας: geomi_@hotmail.com

Η Εσωτερική Επιτροπή Δεοντολογίας του Τ.Ε.Φ.Α.Α., Πανεπιστημίου Θεσσαλίας μετά την υπ. Αριθμ. 2-1/10-10-2012 συνεδρίασή της εγκρίνει τη διεξαγωγή της προτεινόμενης έρευνας.

Ο Πρόεδρος της
Εσωτερικής Επιτροπής
Δεοντολογίας – ΤΕΦΑΑ

Τσιόκανος Αθανάσιος
Αναπληρωτής Καθηγητής

Appendix 2: Copyright Statement

Υπεύθυνη Δήλωση

Η κάτωθι υπογεγραμμένη Γεωργία Μήτρου με ΑΕΜ 17/2011, διδακτορική φοιτήτρια του Τμήματος Επιστήμης Φυσικής Αγωγής και Αθλητισμού του προγράμματος ΄Ασκηση και Υγεία’.

δηλώνω υπεύθυνα ότι αποδέχομαι τους παρακάτω όρους που αφορούν

(α) στα πνευματικά δικαιώματα της Διδακτορικής διατριβής μου με τίτλο «Μηχανισμοί της μυϊκής λειτουργίας: Η επίδραση της ουραιμίας στην μορφολογία, τον μεταβολισμό και την ανοχή στην κόπωση».

(β) στη διαχείριση των ερευνητικών δεδομένων που θα συλλέξω στην πορεία εκπόνησής της:

1. Τα πνευματικά δικαιώματα του τόμου της μεταπτυχιακής ή διδακτορικής διατριβής που θα προκύψει θα ανήκουν σε μένα. Θα ακολουθήσω τις οδηγίες συγγραφής, εκτύπωσης και κατάθεσης αντιτύπων της διατριβής στα ανάλογα αποθετήρια (σε έντυπη ή/και σε ηλεκτρονική μορφή).
2. Η διαχείριση των δεδομένων της διατριβής ανήκει από κοινού σε εμένα και στον/στην κύριο επιβλέποντα -ουσα καθηγητή -τρια.
3. Οποιαδήποτε επιστημονική δημοσίευση ή ανακοίνωση (αναρτημένη ή προφορική), ή αναφορά που προέρχεται από το υλικό/δεδομένα της εργασίας αυτής θα γίνεται με συγγραφείς εμένα τον ίδιο, τον/την κύριο-α επιβλέποντα -ουσα ή/και άλλους ερευνητές (πχ μέλη της τριμελούς συμβουλευτικής επιτροπής, συνεργάτες κλπ), ανάλογα με τη συμβολή τους στην έρευνα και στη συγγραφή των ερευνητικών εργασιών.
4. Η σειρά των ονομάτων στις επιστημονικές δημοσιεύσεις ή επιστημονικές ανακοινώσεις θα αποφασίζεται από κοινού από εμένα και τον/την κύριο -α επιβλέποντα -ουσα της εργασίας, πριν αρχίσει η εκπόνησή της. Η απόφαση αυτή θα πιστοποιηθεί εγγράφως μεταξύ εμού και του/της κύριου-ας επιβλέποντος -ουσας.

Τέλος, δηλώνω ότι γνωρίζω τους κανόνες περί δεοντολογίας και περί λογοκλοπής και πνευματικής ιδιοκτησίας και ότι θα τους τηρώ απαρέγκλιτα καθ’ όλη τη διάρκεια της φοίτησης και κάλυψης των εκπαιδευτικών υποχρεώσεων μου που προκύπτουν από το ΠΜΣ/τμήμα και καθ’ όλη τη διάρκεια των διαδικασιών δημοσίευσης που θα προκύψουν μετά την ολοκλήρωση των σπουδών μου.

20/10/2012
Η δηλούσα
Γεωργία Μήτρου

Appendix 3: Rabbit diet

D30006 and D07122101

Low Phytoestrogen Rabbit Diet and Same With Lower Protein, Potassium, Calcium,Phosphorus, and Sodium

Product #	**D30006**		**D07122101**	
	gm%	*kcal%*	gm%	*kcal%*
Protein	17.8	*22*	8.9	*11*
Carbohydrate	55.0	*68*	63.6	*79*
Fat	3.5	*10*	3.5	*10*
Total		*100*		*100*
kcal/gm	3.23		3.21	
Ingredient	**gm**	***kcal***	**gm**	***kcal***
Casein, 30 Mesh	175	*700*	87.5	*350*
DL-Methionine	3	*12*	1.5	*6*
Corn Starch	390	*1560*	459	*1836*
MaltoDextrin 10	25	*100*	25	*100*
Sucrose	125	*500*	145	*580*
Cellulose, BW200	150	*0*	150	*0*
Inulin	25	*0*	25	*0*
Soybean Oil	35	*315*	35	*315*
Mineral Mix S30003	60	*0*	0	*0*
Mineral Mix S39102	0	*0*	60	*0*
Vitamin Mix V30002	10	*40*	10	*40*
Choline Bitartrate	2	*0*	2	*0*
Sodium Bicarbonate	0	*0*	4	*0*
Total	**1000**	***3227***	**1004**	***3227***
gm%				
Potassium	1.0		0.25	
Calcium	0.8		0.4	
Phosphorus	0.5		0.126	
Sodium	0.2		0.2	
Chloride	0.3		0.2	
Iron	0.01		0.01	

Formulated by E.A. Ulman, Ph.D., Research Diets, Inc., 7/9/01.
Using purified ingredients, this diet matches Purina 5321.It is very low in phytoestrogens.