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RESEARCH ARTICLE

Functional responses of uremic single skeletal muscle fibers to redox imbalances

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Abstract

Background: The exact causes of skeletal muscle weakness in chronic kidney disease (CKD) remain unknown with uremic toxicity and redox imbalances being implicated. To understand whether uremic muscle has acquired any sensitivity to acute redox changes we examined the effects of redox disturbances on force generation capacity.

Methods: Permeabilized single psoas fibers (N =37) from surgically induced CKD (UREM) and sham-operated (CON) rabbits were exposed to an oxidizing (10 mM Hydrogen Peroxide, H_2O_2) and/or a reducing [10 mM Dithiothreitol (DTT)] agent, in a blind design, in two sets of experiments examining: A) the acute effect of the addition of H_2O_2 on maximal (pCa 4.4) isometric force of actively contracting fibers and the effect of incubation in DTT on subsequent re-activation and force recovery (N =9 CON; N =9 UREM fibers); B) the effect of incubation in H_2O_2 on both submaximal (pCa 6.2) and maximal (pCa 4.4) calcium activated isometric force generation (N =9 CON; N =10 UREM fibers).

Results: Based on cross-sectional area (CSA) calculations, a 14 % atrophy in UREM fibers was revealed; thus forces were evaluated in absolute values and corrected for CSA (specific force) values. A) Addition of H_2O_2 during activation did not significantly affect force generation in any group or the pool of fibers. Incubation in DTT did not affect the CON fibers but caused a 12 % maximal isometric force decrease in UREM fibers (both in absolute force p =0.024, and specific force, p =0.027). B) Incubation in H_2O_2 during relaxation lowered subsequent maximal (but not submaximal) isometric forces in the Pool of fibers by 3.5 % (for absolute force p =0.033, for specific force p =0.019) but not in the fiber groups separately.

Conclusions: Force generation capacity of CON and UREM fibers is affected by oxidation similarly. However, DTT significantly lowered force in UREM muscle fibers. This may indicate that at baseline UREM muscle could have already been at a more reduced redox state than physiological. This observation warrants further investigation as it could be linked to disease-induced effects. HIPPOKRATIA 2017, 21(1): 3-7.

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

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Introduction

CKD is accompanied by a variety of skeletal muscle abnormalities such as skeletal muscle atrophy, muscle weakness, and limited endurance, collectively described as uremic myopathy[1,2]. The mechanisms underlying uremic myopathy and specifically muscle weakness remain poorly defined. Many factors may be implicated including a low antioxidant capacity[3] and the generation of excess reactive oxygen species (ROS)[2].

A well-studied ROS is hydrogen peroxide (H_2O_2) which can diffuse through cell membranes and contribute to the oxidation of thiol groups of proteins[4]. In vitro studies using single fiber preparations have shown that long exposure to H_2O_2 or excessive amounts of H_2O_2 can cause a shift to the oxidizing side of the redox status-force relationship[5]. This can lead to significant functional deficits, as exposure to H_2O_2 causes oxidation of multiple sites of the myosin heavy and light chains (e.g., Prochniewicz et al[6]). These functional deficits are fully or partially reversible by using antioxidant/reducing molecules such as dithiothreitol (DTT)[7–9]. However, exposure to DTT may, in turn, cause a shift to the reduced side of the redox status-force relationship also leading to functional deficits[10]. Preliminary results from our group indicated that fast muscle fibers from a CKD animal model present with lower maximally activated force[11] and a disturbed redox

profile, as reflected by increased protein carbonylation in muscle homogenates[12]. It is not however known whether CKD causes the sarcomeric functional unit to respond differently to acute redox changes.

The aim of the study was to examine, for the first time, whether the force- generating capacity of uremic fast-twitch skeletal muscle responds to acute redox changes similarly to control muscle. To avoid a variety of confounding factors present in human patient studies (i.e., years in dialysis, comorbidities, pharmaceuticals) we assessed isometric force generation in single muscle fibers with permeabilized sarcolemma, deriving from a surgically induced CKD animal model[13]. This approach[14,15] allows the study of sarcomeric function per se by acutely excluding systemic factors.

Materials and methods

Animal model

All animal procedures, including surgery and euthanasia, were in accordance to the national directives for the care and use of laboratory animals (Directive 2010/63/EU) and 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).

New Zealand young adult white female rabbits (N =4) with a body weight (BW) of ~3200 g were housed in a controlled environment (22–24 °C, 12:12-hour light-dark cycle) and acclimatized for 48 hours. The rabbits consumed a special rabbit chow containing low levels of protein, potassium, calcium, phosphorus, and sodium (Research Diets, Inc., New Brunswick, NJ, USA) and water ad libitum. After acclimatization, CKD was induced surgically using a modified protocol from Gotloib et al[13]. Surgical procedures were performed under anesthesia and maintaining body temperature. Two animals underwent removal of the left kidney after careful ligation of the left renal artery and vein; and partial nephrectomy (¾) of the right kidney (UREM group). Two age-matched animals underwent sham operation (CON group). Twelve weeks after surgery (June 2015), the animals were sacrificed by injection of sodium pentobarbital solution (50 mg/ml in a dosage of 100 mg/Kg BW) followed by bilateral thoracotomy. After cardiac arrest, blood samples were collected for subsequent serum urea and creatinine determination using standard photometric protocols.

Muscle Samples

Psoas muscle samples were fast excised and permeabilized as previously described[16]. Briefly, thin bundles of rabbit psoas muscle (~2 mm diameter) were dissected, tied to wooden sticks using surgical thread and placed in falcon tubes containing the permeabilization buffer solution [120 mM potassium acetate (KAc), 50 mM 3-(N-Morpholino) propanesulfonic acid (MOPS), 5 mM magnesium acetate ($MgAc_2$), 4 mM ethylene glycol tetraacetic acid (EGTA), and 50 % glycerol volume per volume; pH 7 at 0 °C] with the addition of 100 μl/50ml end volume, of a protease inhibitor cocktail (SIGMA-P8340). Samples were gently shaken on a vibrating platform shaker (Heidolph-Titramax 100, Thermo Fisher Scientific, Boston, MA, USA) for 24 hours at 0° C. The permeabilization solution was then replaced with fresh solution and muscle samples were stored at -20° C until mechanical assessments. Chemicals were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA).

Experimental setup for single fiber mechanics

Single fibers were dissected from a muscle bundle on a cold stage under a stereomicroscope and the fiber ends were attached between two tissue mounts of a muscle micro-dynamometer (SI Heidelberg/WPI, Heidelberg, Germany), connected to a force transducer (0.4 μN-4 N) and a motor arm (used as a fixed end for isometric assessments). The fiber was then immersed in 'baths' containing experimental solutions (see below). Data were continuously recorded and were later exported for further analysis. The micro-dynamometer consisted of a horizontally translocating platform with five independent baths and a He-Ne laser allowed for sarcomere length measurements based on a Karatzaferi & Cooke design. The temperature of the baths was adjusted to 10° C using a cooling/heating water circulator (Thermo Electron Haake WKL26, Thermo Fisher Scientific, Boston, MA, USA).

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 adenosine triphosphate (ATP) in the basic buffer. Relaxing solution with reducing agent: addition of 10 mM dithiothreitol (DTT) in the standard relaxing solution; Relaxing solution with oxidizing agent: addition of 10 mM H_2O_2 in the standard relaxing solution. Activating solutions. Submaximal: addition of 0.53 mM calcium chloride ($CaCl_2$) in the standard relaxing solution (pCa 6.2). Maximal: addition of 1.1 mM $CaCl_2$ in the standard relaxing solution (pCa 4.4). Maximal activating solution with oxidizing agent: addition of 10 mM H_2O_2 in the maximal activating solution. The ionic strength of the solutions was ~0.2 M[8,17].

Isometric tension measurements

Force generation capacity was assessed in a total of 37 single muscle fibers at 10 °C, pH 7. Each fiber was first immersed for one minute in a bath containing rigor solution (to wash out excess glycerol), and then it was transferred and equilibrated for two minutes in a bath containing the standard relaxing solution. The average diameter was determined assuming a cylindrical shape. Consequently, the fibers underwent a full assessment in one of the two protocols described below. All assessments and initial data reductions 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, 10 mM H_2O_2

were added and forces were recorded for a further five minutes. After a wash-out, the fibers were returned to a relaxing condition. They were then exposed to 10 mM DTT for ten minutes. Following washing out, a final maximal activation in standard solutions was performed[8].

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 five minutes to 10 mM H_2O_2. After a wash-out, submaximal and maximal isometric forces were reassessed in fresh standard solutions[8].

Data reduction: Force data were recorded in both absolute (μN) and specific values [mN/mm^2, i.e., corrected for the calculated cross-sectional area (CSA)]. In addition, effects of H_2O_2 or DTT on isometric force were evaluated per CON and UREM groups but also for the Pool of fibers (both CON and UREM 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 ± standard error of the mean. Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) statistical software, version 15.0 (SPSS Inc., Chicago, IL, USA) and the significance level was set at $p < 0.05$.

Results

Surgical procedures were well-tolerated and animals presented with an uneventful postoperative recovery in either group. At the end of the twelve-week postoperative period, BW ranged between 3,000-3,245 (3,123 ± 123 gr) for UREM and 3,500-3,850 for CON (3,675 ± 175 gr) animals. Renal insufficiency in experimental animals, compared to CONs, was reflected in raised serum creatinine (UREM 2.22 ± 0.33 vs CON 1.46 ± 0.00 mg/dl) and urea levels (UREM 44.0 ± 2.0 vs CON 38.0 ± 0.0 mg/dl).

Morphological characteristics of single fibers

Average fiber diameters differed significantly between groups (UREM 72.3 ± 1.9 vs CON 77.9 ± 2.0 μm, p =0.049). Likewise, calculated CSAs of UREM fibers were significantly lower compared to CONs (4,150 ± 220 vs 4,817 ± 237 μm^2, p =0.046), 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 ± 17 μN vs CON 410 ±22 μN, p =0.002). Moreover, specific forces of UREM fibers were lower by ~9 % than CONs (UREM 78.2 ± 4.4 vs CON 86.0 ± 3.4 mN/mm^2) but not significantly (p =0.172). Similarly, in a subset of fibers (N =19, 9 CON, 10 UREM) also assessed at standard submaximal calcium activation (pCa 6.2), the absolute submaximal force of UREM fibers was significantly lower compared to CONs by ~33 % (UREM 165 ± 17 vs CON 248 ± 15 μN, p =0.002). However, while the submaximal specific force in UREM fibers was lower by ~15 % than CONs, this difference was not statistically significant (UREM 40.1 ± 3.3 vs CON 46.7 ± 2.7 mN/mm^2, p =0.153).

Experimental set A: Addition of 10 mM H_2O_2 during activation (N =18, 9 UREM, 9 CON) did not affect maximal isometric forces in either group (UREM: Absolute force p =1.000, Specific force p =1.000; CON Absolute force p =1.000, Specific force p =1.000) nor in the Pool of fibers (-0.9 ± 3.0 %, Absolute force p =1.000, Specific force p =1.000). Moreover, the magnitude of the effect of H_2O_2 on the isometric force was not significantly different between groups (p =0.978). Exposure to 10 mM DTT during relaxation, however, caused a significant force decline (Absolute force p =0.024, Specific force p =0.027) only in UREM fibers compared to baseline values (by -12 %) in subsequent maximal activation (with the Pool of fibers -10.7 ± 2.5 %, Absolute force p =0.001, Specific force p =0.001). However, the magnitude of the effect of DTT did not differ significantly between fiber groups (p =0.601). To facilitate presentation, effects of H_2O_2 and DTT on the maximal isometric force are presented as percent changes from baseline (standard) forces (Figure 1).

Experimental set B: In another subset of fibers (N =19, 9 CON, 10 UREM) we examined the effect of incubation with 10 mM H_2O_2 during relaxation on submaximal (pCa 6.2) and maximal (pCa 4.4) force production. Pre-incubation with 10 mM H_2O_2 did not cause any significant decrease in subsequent submaximal isometric forces compared to standard conditions in either of the groups (UREM -0.1 ± 2.5 %, Absolute force p =1.000, Specific force p =1.000; CON -4.7 ± 4.3 %, Absolute force p =1.000, Specific force p =1.000) nor in the Pool of fibers (-2.3 ± 2.4 %, Absolute force p =1.000, Specific force p =1.000). Nevertheless, the maximal isometric tension was significantly lowered in the Pool of fibers (-3.5 ± 1.2 %) (Absolute force p =0.033, Specific force p =0.019) but not in groups separately (UREM: Absolute force p =0.468, Specific force p =0.356; CON: Absolute force p =0.272, Specific force p =0.204) after exposure to H_2O_2. Lastly, the magnitude of the effect of H_2O_2 on the isometric force was not significantly different between groups neither in submaximal (p =0.354) nor in maximal calcium activation (p =0.766). 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 2).

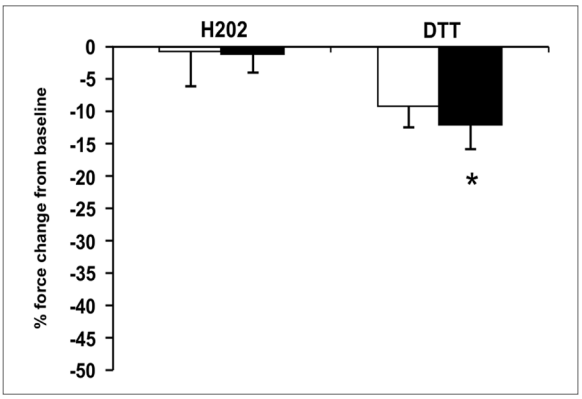

Figure 1: Force values expressed as % force change from baseline following exposure to 10 mM Hydrogen Peroxide (H_2O_2) during activation and 10 mM Dithiothreitol (DTT) during relaxation. CON fibers are shown as open bars, and UREM fibers as filled bars. "*" indicates a significant difference ($p < 0.05$) from corresponding baseline force (here only UREM).
H2O2: Hydrogen Peroxide, DTT: Dithiothreitol.

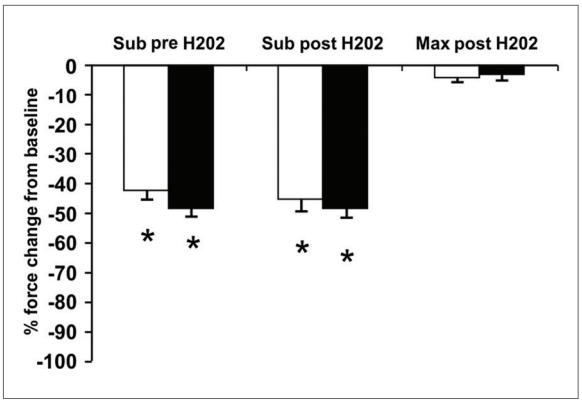

Figure 2: Force values expressed as % force change from maximal baseline force following submaximal activation before (Sub pre H2O2) and after exposure to H_2O_2 (Sub post H2O2) as well maximal activation after exposure to H_2O_2 (Max post H2O2) during relaxation. CON fibers are shown as open bars, and UREM fibers as filled bars. "*" indicates a significant difference ($p < 0.05$) from corresponding baseline force (here for both CON and UREM fibers).
Sub pre H2O2: submaximal activation before exposure to H_2O_2, Sub post H2O2: submaximal activation after exposure to H_2O_2, Max post H2O2: maximal activation after exposure to H_2O_2.

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 a reducing rather than an oxidizing agent, which is likely to indicate that their redox status at baseline may have shifted to the reduced side of the redox status-force relationship, away from the optimal redox balance.

Regarding the effects of an oxidizing agent on the maximal isometric force, addition of 10 mM H_2O_2 during activation did not cause any appreciable change in maximal isometric forces of UREM nor of CON fibers. Our finding is consistent with the study of Lamb et al[8] who examined skinned fibers from the rat extensor digitorum longus muscle using a similar H_2O_2 treatment. However, there are reports that even 5 mM H_2O_2 may cause a significant force decline in skinned psoas fibers (by ~31 %)[6]. Moreover, the effect of oxidizing agents can be time dependent[8,9] and the difference in time exposure can explain study differences.

In a subset of fibers, we examined the effects of preincubation 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 10 mM H_2O_2 during relaxation for five minutes caused a significant decrease in absolute maximal isometric forces for both CON and UREM fibers (overall by ~3.5 % in the Pool of fibers). This is in agreement with Lamb et al[8] 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 deterioration in maximal isometric force[6,18]. It has been shown that the negative effects of H_2O_2 on maximal isometric force are due to myosin head modifications that affect force generation per cross bridge[9]. 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. The magnitude of the effect of H_2O_2 on force did not significantly differ between fiber groups. Thus, it could be surmised that, in the present study, either the exposure had to be longer to reveal any differences, or that the chronic uremia had not caused the skeletal muscle to acquire any significant 'sensitivity' to H_2O_2 exposure. Moreover, the observation of Lamb et al[8], suggesting that oxidation may cause an increase in calcium sensitivity, which at submaximal activation could prevent force decline in the presence of H_2O_2, may explain the lack of an appreciable effect of the oxidizing agent on the submaximal force.

A ten-minute exposure to 10 mM DTT during relaxation did not significantly affect maximal forces of CON fibers, in agreement to past reports[8], however, it significantly lowered maximal isometric force in UREM fibers by ~12 %. This was an unexpected and novel result. DTT is a strong reductant and the lack of an effect on maximal forces of CON skinned fibers may indicate that those fibers were in a balanced redox state[8] (considering the force-redox relationship as an inverted U with a plateau for optimal force generation, with either excess oxidation or excess reduction inhibiting force, see also Andrade et al[10] and Powers et al[5]). Thus given observations that UREM muscle presents with a disturbed redox status[12], and the fact that the force decline due to DTT was significant in UREM fibers, it could not be excluded that UREM muscles' sarcomeric proteins may have acquired a reduced redox state. Therefore, the addition of DTT may have led to further reduction which could help

explain the observed lower force. Moreover, a concurrent difference in susceptibility to irreversible (or slow to reverse) oxidation-induced changes on myosin (which the current DTT exposure may not be enough to reverse) might be a possibility. Future experiments including fluorescent monitoring or other sensitive methods to track redox changes (e.g., as in Prochniewicz et al[6]) would be needed to further clarify the prevailing mechanism.

The current study also revealed a significant level of atrophy in UREM fibers by ~14 % versus CONs. This finding is consistent with human[19,20] but also with animal studies[21] examining fast-twitch muscle properties in advanced kidney disease. Still, moderate atrophy can only partially explain the lower force generation capacity in UREM muscle[20].

In conclusion, while the functional response of uremic fibers to H_2O_2 exposure did not differ compared to control fibers, DTT inhibited force only in uremic fibers. This indicated that the initial redox status of uremic muscle might be more in the reduced rather than in the oxidized side of the redox-force relationship. This finding, taken together with the presentation of a 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 the dose-response relationships between isometric force and key redox constituents. Moreover, future studies should determine which sarcomeric proteins are mostly affected, as well as delineate the possible interaction of redox imbalances with fatigue or toxicity factors.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

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