SR stress in locomotor muscles of patients with chronic obstructive pulmonary disease

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Abstract

Background

The potential contribution of chronic dysregulation of sarcoplasmic reticulum (SR) protein homeostasis (a condition called SR stress) to skeletal muscle loss is poorly understood. We investigated the degree of activation of SR stress in locomotor muscles of patients with chronic obstructive pulmonary disease (COPD), a respiratory disease with systemic manifestations.

Methods

We analyzed the markers of SR stress and associated pathologies in vastus lateralis muscles of 60-65 years old male healthy controls and patients with mild (COPD stages 1 & 2) and advanced (COPD stages 3 & 4) COPD (N = 6-8 / group).

Results

Skeletal muscle proteins expressions of GRP94, BiP, CHOP and ATF were significantly elevated in advanced COPD (≈53%, ≈3.6 fold, ≈3.5 fold and ≈3.2 fold, respectively) compared with healthy controls. The expression of downstream markers of SR stress including apoptosis, inflammation and autophagy was increased, while the maximal activity of SR Ca2+ ATPase (SERCA) enzyme was significantly reduced in advanced COPD (≈41%) than healthy controls. Single muscle fiber diameter and cytoplasmic domain per myonucleus were significantly smaller (≈14% and 13%, respectively) in patients with advanced COPD than healthy controls. These changes in SR dysfunction were accompanied by substantially elevated levels of global oxidative stress including lipid peroxidation and mitochondrial ROS production.

Conclusion

Taken together, our data suggests that the muscle weakness in advanced COPD is in part driven by elevated SR stress and its pathological consequences. The data provided can lead to potential therapeutic interventions of SR dysfunction for muscle detriment in COPD.

Background

Chronic obstructive pulmonary diseases (COPD) are frequently associated with systemic manifestations in addition to well characterized progressive decline in the lung function (1). The loss of muscle mass and strength in these patients is due to multiple factors including hypoxemia, systemic inflammation, low testosterones levels, oxidative stress and sedentary lifestyle (2, 3). Both the muscle aerobic capacity and the strength are indicators of generalized health as the reduced muscle strength is a strong predictor of mortality in the patients with COPD (4) and other diseases (5). Handgrip strength (HGS) is a simple measure of upper limb muscles force and various respiratory & cardiovascular co-morbidities are associated with reduced HGS (6). Normative values of HGS have been proposed based on centile values

for more than 200,000 adults between 39 and 73 years of age, and normalized for age, sex, gender, height and the measurement side (7).

The molecular mechanism(s) underlying impaired muscle function associated with COPD are not well understood. The muscle wasting may partly be due to increased activity of ubiquitin-proteasome pathway leading to an increase in protein degradation (8). Furthermore, the muscle biopsies from patients with COPD show an increased apoptosis and reduced regenerating capacity, which likely contribute to muscle wasting in patients with COPD (2). However, the exact molecular mechanism(s) contributing to muscle loss in COPD remain elusive.

Skeletal muscle is not a highly secretory tissue; however it has highly specialized networks of endo/sarcoplasmic reticulum (ER/SR) for protein homeostasis, in addition to their better known function of Ca^{2+} homeostasis. The protein folding capacity of SR is disrupted in various physiological and pathological conditions in skeletal muscle such as disuse, generalized inflammation, Ca^{2+} dysregulation, high-fat diet and oxidative stress (9). SR responds to stress by increasing the expression of SR chaperons and activating downstream pathways called unfolded protein response (UPR) pathways. UPR is a protective pathway that tries to alleviate the stress by shutting protein translation, increasing the expression of SR chaperons and degrading misfolded proteins. These responses are associated with skeletal muscle remodeling in conditions such as exhaustive exercise and high-fat diet (10, 11). However, accumulating evidence suggests that chronic unmitigated SR stress in skeletal muscle has pathological consequences. Thus, when the capacity of UPR to alleviate cell stress is exceeded, downstream pathological processes are activated resulting in increase in apoptosis, inflammation and anabolic resistance which contribute to muscle wasting. Various muscle diseases including genetic & inflammatory myopathies, cancer cachexia and sarcopenia result in prolonged elevation of SR stress and its downstream effects (12). Furthermore, a direct causality between SR stress and muscle detriment has also been proposed since mitigating SR stress can partially restore muscle mass and/or strength in experimental animal models (13, 14).We have previously shown that the muscle weakness in COPD is associated with reduction in serum sialic acid (15), a monosaccharide with various biological functions. Sialic acid has involvement in SR stress and apoptosis (16) while deficiency of it leads to oxidative stress and atrophy in skeletal muscle (17). However, the contribution(s) of SR stress to muscle detriment in the patients with COPD is not known.

The goal of this study is to evaluate the activation and potential contribution of SR stress and UPR in skeletal muscle in COPD. Further, we have investigated the downstream consequences of chronic heightened SR stress in skeletal muscle in COPD. We also looked at the potential interface between proteostasis and Ca^{2+} regulation functions of SR and their coupling with mitochondrial function and cellular redox environment. We hypothesized that SR stress is activated in skeletal muscle of patients with COPD, which contributes to loss of muscle mass and strength.

Method

Study design & participants

This study is a cross-sectional analysis of the baseline data collected from the selected participants with or without COPD at the Kohat University of Science and technology, Kohat and the Gomal Medical College, Dera Ismail Khan. The regional ethical committees at both the universities approved the study. The study population was divided into non-COPD controls (N = 123) and patients with COPD (N = 179). Based on Global Initiative for Obstructive Lung Disease (GOLD) classification, the COPD group was further subdivided into two subgroups of mild (GOLD stages 1 & 2; N = 114) and advanced (GOLD stages 3 & 4; N = 65). COPD was defined as the FEV_1% / forced vital capacity (FVC) < 0.7 according to the GOLD guidelines (18). All participants were male, 60–65 years of age, with complete data from clinical examination, laboratory investigation, spirometry and HGS measurements. All participants provided written informed consents before participating in the study. The study was conducted in accordance with the declaration of Helsinki (19).

HGS and pedometer

HGS was measured by a digital handgrip dynamometer (CAMRY, South El Monte, CA, USA). The participants were instructed to sit down with their elbows flexed at an angle of 90◦ and the dynamometer in hand in supine position. Three attempts were performed with each hand with a 60-seconds rest between each attempt and the highest value was recorded for analysis. The reference values for the general population were taken from two different meta-analysis (20, 21). The Fitbit one, a commercially available accelerometer-based activity tracker, was used for steps counting for preceding one month before HGS was measured. The participants were asked to wear Fitbit tracker throughout the month and average steps count per day was calculated.

Spirometry

The FEV1, FVC and peak expiratory flow rate (PEFR) were measured using a portable spirometer (Contec SP10, China), according to standards set by American Thoracic Society (22). The participants were instructed to inhale maximally until the lungs were full, followed by forceful exhalation into the spirometer until no air could be exhaled (23). This was done for a minimum of three times and the severity grading was based on FEV1% of predicted values according to the global initiative for chronic obstructive lung disease (GOLD) document, GOLD 1–4 (24).

Measurement of serum biomarkers

Blood samples were drawn in the morning after 10–12 hours of fasting to determine serum metabolic parameters including blood glucose and the high-density lipoproteins—cholesterol (HDL-C) using CardioCheck® equipment (Maxglobal SA, Parsippany, NJ, USA).For analysis of serum biomarkers, 14–18 participants from the non-COPD and each of the two subgroups of COPD participants were selected and

the blood samples were drawn in the morning after 10–12 hours of fasting. Serum was assayed using ELISA kits for total sialic acid (abcam, cat # ab83375) and α–1 acid glycoprotein (abcam, cat # ab108852) according to manufacturer's instructions.

Muscle biopsies

The muscle biopsies were taken from vastus lateralis muscles of selected participants (N = 6—8 per group) using conchotome biopsy method as described previously (25). One part of biopsy specimen was snap frozen and stored at –80◦C while remaining parts were processed for immunostaining and mitochondrial assays.

Protein preparation and western blot

Muscle tissues were homogenized in RIPA buffer containing 50mM Tris (pH = 7.4), 150mM NaCl and protease inhibitors. Proteins were quantified using the Bio-Rad kit (Sigma-Aldrich, Poole, UK) and transferred to nitrocellulose membrane after electrophoresis using 8–15% polyacrylamide gels. Membranes were probed using primary antibodies at 1:1000 dilution each and secondary antibodies at 1:10,000 (HRP-linked anti-rabbit IgG; Cat # 7074S, cell signaling) or 1:25,000 (HRP-linked anti-mouse IgG Cat # 7076S, cell signaling).Primary antibodies used were GRP94 (Cat # 20292T, cell signaling), BIP (Cat # 3177T, cell signaling), CHOP (Cat # 2895T, cell signaling), ATF6 (Cat # 65880, cell signaling), p-NFkB(Cat # 3033, cell signaling), t-NFkB (Cat # 8242, cell signaling), LC3B (Cat # 2775, cell signaling), MyoD (Cat # 13812, cell signaling), p62 (Cat # 5114, cell signaling), p-AkT (Cat # 9271, cell signaling), t-AkT (Cat # 9272, cell signaling), p-P70S6k (Cat # 9205, cell signaling), t-P70S6k (Cat # 9202, cell signaling), p–4EBP1 (Cat # 2855, cell signaling), t–4EBP1 (Cat # 9644, cell signaling), SERCA2 (Cat # 9580, cell signaling), calsequestrin (Cat # ab3516, abcam), DHPR1a (Cat # MA3–920, Thermo Scientific). All image intensities were normalized to protein intensity based on ponceau stain.

Analysis of single fiber diameter, nuclei count and myonuclear domain size

The bundles of ≈80–100 muscle fibers were dissected from fresh tissues and chemically skinned in a solution containing 50% (v/v) glycerol and phosphate buffer saline (PBS) for 24 hours at 4◦C and stored at –80◦C before use, as described previously (26).On the day of experiment, single fiber segments were carefully dissected from the bundle and gently placed on the glass slide. After a brief permeabilization with 0.1% triton X–100 in PBS, fibers were stained with rhodamine-phalloidin (1:200, Molecular Probes Inc, Eugene, OR, USA) for 35 minutes and DAPI (Invitrogen, Molecular Probes, OR, USA) for 5 minutes before final wash. Linear fiber segments of ≈400 µm were chosen for imaging. All images were scanned using LSM 510 Meta confocal microscope and imported into Image J software (Wayne Rasband, National Institute of Health, Bethesda, MD) for analysis.

Sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) activity assay

The measurement of SERCA ATPase activity was performed in the muscle homogenates at 37◦C using a spectrophotometric assay as described previously (27, 28). Briefly, ≈20mg of muscle samples were diluted 1:10 (w/v) and manually homogenized in ice-cold homogenizing buffer (pH = 7.5) containing (in mM) 250 sucrose, 5 HEPES, 0.2 PMSF and 0.2% NaN_3. Ca^{2+} -dependent ATPase activity was measured in the assay buffer (pH = 7) containing (in mM) 100 KCl, 20 HEPES, 10 $MgCl_2$, 10 NaN_3, 10 phosphoenolpyruvate, 1 EGTA, 5 ATP 1 Ca^{2+} ionophore A–23187 (C–7522, Sigma) and 18 U/ml of both lactate dehydrogenase and pyruvate kinase. The reaction was carried out at 10 different Ca^{2+} concentrations, ranging between 7.6 and 5 *p*Ca units and was started by adding 0.3 mM NADH. Basal activity was determined in the presence of 40uM of the Ca^{2+}-ATPase inhibitor cyclopiazonic acid (C–1530, Sigma) in dimethyl sulfoxide.

Real-time PCR

Total RNA was extracted from the muscle tissues using TRI reagent (Life technologies, Grand Island, NY, USA). RNA purity and yield were determined by measuring the absorbance at 260 and 280nm. cDNA was prepared from 1mg of the total RNA using iScriptTM cDNA synthesis kit (Bio-Rad, Hercules, CA, USA); 2.5 ng of cDNA samples were amplified using primers for the markers of elevated SR stress (GRP94, BIP& PID), activation of IRE–1α pathway (spliced *vs.* unspliced XBP1, Deril1, Pdia6), activation of Perk pathway (Perk, ATF4 & CHOP), activation of ATF6 pathway (ATF6α, Hyou1 & Erolib) and 18S along with fast SYBR green master mix (Applied Biosystems, Grand Island, NY, USA). The data was analyzed using the ΔΔCt method.

Myosin heavy chain (MHC) quantification

The MHC contents were quantified using a high salt buffer as described previously (28). Briefly, 0.6 ug protein was loaded/lane into a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and run at 200 V for 50 min at 4◦C. The gel was stained with coomassie blue, and the optical densities of the bands corresponding to MHC and actin were quantified.

Analysis of F_2-isoprostanes

Muscle F_2-isoprostane content was measured using thin layer chromatography and mass spectrometry as described before (29). Briefly, ≈150 mg of muscle tissue was homogenized in 10 ml of ice-cold Folch solution (CHC3:MeOH; 2:1) containing butylated hydroxytoluene. Following 30 min incubation at room temperature, 2 ml of 0.9% NaCl was added. The homogenate was centrifuged at 4000g for 5 min at 4◦C. The aqueous layer was discarded while the organic layer was secured for the measurement of esterified F_2-isoprostane.

Mitochondrial function

Freshly isolated mitochondria were used for the H_2O_2 production assay using the Amplex red-HRP method, as described previously (27). The H_2O_2 dependent oxidation of nonfluorescent Amplex red to fluorescent resorufin was quantified as a measure of mitochondrial H_2O_2 production. All assays were performed at 37◦C in 96-well plate.

Statistical analysis

The anthropometric characteristics of the participants were presented using mean and standard error of mean as data met the assumption for normality. Student's *t*-test or analysis of variance was used to compare continuous variables and the comparison between different groups. A p-value < 0.05was considered to be statistically significant.

Results

Characteristics of the participants& expressions of serum biomarkers

The basic characteristics of the study population are summarized in table 1. Overall, subjects with COPD had reduced muscle strength and walking speed than the control subjects. The reduction in HGS was more prominent in participants with COPD−3 & 4 (≈19.5%, $p < 0.05$) than participants with COPD−1 & 2 (≈7.7%, $p < 0.05$), when compared to control group. The association of serum biomarkers with HGS in COPD has been reported before (30). Here, we investigated the serum association of serum AGP and TSA with HGS in healthy controls and COPD participants. Serum AGP did not show significant association with HGS ($r^2 = 0.74$, $p = 0.069$) in the healthy control and COPD participants. On the other hand, serum TSA levels were significant associated with HGS ($r^2 = 0.132$, $p < 0.05$) in the study cohort.

Markers of SR stress

The reduction in muscle force-generating capacity implicates potential alterations in the expressions of genes associated with SR stress (31). To determine whether muscle weakness in COPD can induce activation of SR stress, we measured the protein and mRNA expressions levels of markers of SR stress and its downstream UPR pathways. We hypothesized that the muscle weakness in COPD will be associated with increased SR stress. As shown in *Figure 2A,* there was an increase in protein expressions of SR chaperons GRP94 (≈53%, $p < 0.05$) and BiP (≈ 3.6 fold, $p < 0.05$) as well as their downstream targets CHOP (≈ 3.5 fold, $p < 0.05$) and ATF6 (≈ 3.2 fold, $p < 0.05$) in the COPD group, when compared to the control group. However, these changes were mainly restricted to participants with advanced COPD stages 3 & 4. On the other hand, participants with COPD−1 & 2 showed significant increase only in the expression of CHOP (≈56%, $p < 0.05$), when compared to control group. We also found significant upregulation in the mRNA expression of PERK, CHOP, BiP, ATF6 and GADD34 ($p < 0.05$) in muscle

biopsies of all participants with COPD *(Figure 2B).* Furthermore, participants with COPD–3 & 4 also showed marked upregulation in mRNA expressions of IRE–1a, XBP–1 and GRP94 ($p < 0.05$), when compared to control group *(Figure 2B).*

Markers of downstream targets of SR stress

Activation of SR stress and the subsequent UPR affect cellular inflammation, autophagy and apoptotic pathways, which can contribute to the loss of muscle mass and strength. We hypothesized that the muscles from COPD participants will show increased apoptosis, inflammation and autophagy. In order to test this hypothesis, we measured the protein and mRNA expressions of the markers of inflammation, autophagy and apoptosis. The relative protein expressions of p-NFkB was significantly increased in muscle biopsies of COPD participants with stages 1 & 2 (≈ 2.5 fold, $p < 0.05$) and stages 3 & 4 (≈ 3.7 fold, $p < 0.05$), when compared to control group. In addition to increased inflammation, the muscle biopsies from COPD participants also had increased autophagic flux as measured by increased LC3B-II/I ratio (COPD–1 & 2 ≈ 86%, $p < 0.05$; COPD–3 & 4 ≈ 3.5 fold, $p < 0.05$) and reduction in p62 content (COPD–3 & 4 ≈68%, $p < 0.05$), when compared to control group. Additionally, several markers of apoptosis were upregulated in muscles of COPD participants than control group. On the other hand, COPD had no effect on the levels of MyoD and MyoG in muscle biopsies. Collectively, these findings show that the activation of SR stress and UPR in muscle biopsies in COPD resulted in downstream consequences including increase in inflammation and apoptosis and inhibition of autophagy.

Markers of muscle protein synthesis

Since anabolic resistance is a potential consequence of SR stress in muscle cells (32), we measured expression of markers of protein synthesis in the study participants. Participants with COPD stages 3 & 4 showed reduced phosphorylation of AkT (≈ 19%, $p < 0.05$) and 4EBP–1 (≈ 36%, $p < 0.05$) than control group *(Figure 4A).* Phosphorylation of 4EBP–1 was also repressed in participants with COPD stages 1 & 2 (≈ 25%, $p < 0.05$) *(Figure 4A).* On the other hand, we found no significant change in the phosphorylation status of p70S6k in the COPD group. Preferential loss of myosin, the primary molecular motor of muscle cells is associated with muscle atrophy and weakness (28). We found selective loss of myosin in COPD participants with stages 3 & 4 (≈ 16%, $p < 0.05$) and a slight, albeit not statistically significant loss in participants with COPD stages 1 & 2 (≈ 8.2%, $p = 0.061$) *(Figure 4B).*

Muscle fibers size and myonuclei content

Because SR stress and its downstream effects can contribute to muscle atrophy, we measured single fiber diameters from control and COPD participants. When compared to control group, single fibers from COPD participants showed higher number of fibers with smaller diameter *(Figure 5A).* However, a significant reduction in single fiber diameter was only reported in participants with COPD stages 3 & 4 (≈ 14.4%, $p < 0.05$) but not with stages 1 & 2 (≈ 8.3%, $p = 0.067$) *(Figure 5B).* In compliance with our

previous findings of smaller MND size in atrophied muscle fibers (26), we observed reduced MND size in muscle fibers of participants with COPD stages–3 & 4 (≈ 13.1%, $p < 0.05$) *(Figure 5C).* On the other hand, the slight reduction in MND size in muscle fibers of stages–1 & 2 participants failed to reach statistical significance (≈ 5.8%, $p = 0.73$) *(Figure 5C).* We found no difference in the number of myonuclei per unit length of muscle fibers between the three groups *(Figure 5D).*

SERCA ATPase activity and calcium related proteins

The coupling between calcium regulation and protein folding functions of muscle SR has been proposed recently (33). Accordingly, we hypothesized that the COPD participants will show reduced SERCA activity than control group. We have previously reported reduced activity of SERCA ATPase during aging and in conditions of increased oxidative stress (27, 28). Measurement of SERCA pump activity was performed by the decrease in NADH absorbance at 340nm in muscle homogenates from control and COPD participants. Only the COPD participants with stages 3 & 4 showed significant reduction in maximal rate of SERCA activity (≈ 41.7%, $p < 0.05$), when compared to control group *(Figure 6A).* The reduced SERCA activity was not due to reduction in SERCA protein content since because we did not observe any reduction in the expression of SERCA2, the major SERCA isoform in human skeletal muscle, in COPD participants *(Figure 6B).* The reduction in SERCA pump activity implies potential changes in the expressions of proteins involved in calcium handling and voltage sensing. As shown in *Figure 6B,* we observed a significant upregulation of calsequestrin (≈ 34%, $p < 0.05$) that binds calcium in SR lumen, in COPD group–3 & 4. Moreover, the protein levels of voltage sensor protein, DHPR–1a was downregulated in participants with COPD stages 3 & 4 (≈ 17%, $p < 0.05$) *(Figure 6B).* These changes do not rule out compromise in proteins quality induced by oxidative damage.

Markers of oxidative damage

We next assessed the markers of oxidative damage in control and COPD participants. We have previously shown that reduced SERCA activity is associated with mitochondrial dysfunction (27) and increase in markers of lipid peroxidation (28). We measured mitochondrial ROS production as peroxide emission using isolated mitochondria from muscles biopsies. Mitochondria from COPD participants with stages 3 & 4 showed significantly higher peroxide production during state–1 (mitochondrial respiration without addition of external substrate) (≈ 2.5 fold, $p < 0.05$) *(Figure 7A).* Mitochondrial peroxide production was still significantly higher (≈ 1.8 fold, $p < 0.05$) when respiratory substrates glutamate/malate were added to isolated mitochondria to stimulate electron flow *(Figure 7A).* On the other hand, mitochondrial peroxide production was unchanged in the participants with COPD stages 1 & 2, when compared to control group. The levels of F_2-isoprostanes were significantly increased in participants with COPD stages 3 & 4 (≈ 72%, $p < 0.05$) but not in stages 1 & 2, when compared to control group *(Figure 7B).*

Discussion

The activation of muscle SR stress and UPR has been studied in multiple atrophy conditions. However, there is scarcity of data from human clinical studies. To our knowledge, this is the first study characterizing muscle SR stress in COPD. Muscle weakness and atrophy in COPD have been described before; however the underlying molecular mechanisms are poorly characterized. Here, we show that heightened SR stress and UPR can be potential contributors to muscle detriment in COPD. We report that the activated UPR results in downstream consequences of increased inflammation and apoptosis and inhibition of autophagy, which are associated with muscle atrophy and weakness. We also show potential coupling between protein folding and calcium handling functions of SR, as shown by compromised SERCA pump activity in muscles with SR stress. Moreover, in agreement with structural and functional communication between SR and mitochondria, we report mitochondrial dysfunction and increased oxidative stress in muscle biopsies with SR stress.

A number of factors can contribute to muscle wasting in COPD, including disuse, systemic inflammation, hypoxia, steroids and oxidative stress (2, 34). However, the intrinsic molecular mechanisms in skeletal muscle leading to atrophy and weakness remain elusive. SR stress has emerged as a potential contributor to skeletal muscle detriment in myopathies, dystrophies and other catabolic conditions (9). Here, we confirm and extend these findings by reporting heightened SR stress in muscle wasting in COPD. Although we report activation of SR stress and UPR in muscle weakness and atrophy, it remains unknown whether activation of SR stress is a common occurrence in all muscle catabolic conditions. Upregulation of markers of SR stress seems to preserves muscle mass in short-term (35). However, prolonged, unresolved SR stress can have pathological consequences on skeletal muscle and likely contributes to muscle weakness and atrophy as in myositis and chronic myopathies (36, 37). Considering the chronic nature of COPD, it seems likely that the upregulation of SR stress and UPR is pathological. This is evident by the increased expression of downstream consequences of SR stress including autophagy, apoptosis and inflammation. While autophagy at basal level removes defunct organelles from cell, excessive activation of autophagy contributes to muscle wasting in multiple catabolic conditions (38, 39). Moreover, there is evidence that the proapoptotic enzymes including caspases and calpains are activated in muscle wasting conditions (39, 40). Furthermore, inflammatory cytokines modulate the activity of various catabolic enzymes in muscle atrophy (39, 41). These findings are in agreement with previous findings of higher expression of markers of autophagy (42), apoptosis (43) and inflammatory cytokines (44) in skeletal muscle from COPD patients. While these findings do not establish direct causality between SR stress and its downstream consequences, they do suggest SR stress as a potential contributor to muscle detriment in COPD. In support of this, chronic treatment with SR stress-inhibitors attenuates muscle atrophy and weakness in myopathies and chronic muscle diseases (31).

The SR in skeletal muscle are specialized for storing and regulating Ca^{2+} homeostasis which is required for contraction. Accumulating evidence suggests that the Ca^{2+} handling function of SR is also disrupted in SR stress. Thus, the myopathy related to type 1 ryanodine receptors/SR Ca^{2+} release channels results in constant Ca^{2+} leak leading to muscle weakness, atrophy and defective excitation-contraction coupling (13). These mice show activation of SR stress and UPR in skeletal muscle. More importantly, treatment

with 4-PBA, a chemical chaperon reduced SR stress and improve Ca^{2+} homeostasis and muscle function in these mice. Moreover, thapsigargin, which is routinely used to induce SR stress, is also a potent SERCA inhibitor. These findings elaborate the coupling between protein folding and Ca^{2+} handling functions of SR. Our observation of reduced SERCA function in COPD is in agreement with these findings. The association between SERCA dysfunction and ER/SR stress has been described in other cell types (45); however to our knowledge this is the first study dissecting this relation in skeletal muscle. The SERCA dysfunction cannot be explained by change in SERCA protein content since the amount of SERCA2, the major SERCA isoform in humans was relatively unchanged in COPD, which is in line with previous reports (46). However, SERCA is susceptible to oxidative damage due to its long half-life of ≈14–17 days (47). Thus it is possible that increased oxidative stress in COPD has contributed to reduced SERCA activity in COPD. Our findings of increased oxidative stress and SERCA dysfunction in COPD are consistent with our earlier findings in aging mice (27, 28).

Mitochondria are the major source of reactive oxygen species in the skeletal muscle and maintain structural and functional networks with SR, which are essential to regulate cellular homeostasis. Among the molecular players of SR-mitochondria interface is a variant of SERCA isoform which is activated upon SR Ca^{2+} depletion, amplifies SR stress response through upregulation of UPR and mediate Ca^{2+} transfer to mitochondria (48). The resultant Ca^{2+} overload of mitochondria leads to increased ROS generation and activation of proapoptotic pathways (49). Thus, the perturbation of Ca^{2+} and protein homeostasis by SR can deleteriously affect mitochondrial function, resulting in muscle detriment. Our observations of SERCA dysfunction, activation of SR stress and UPR and increased ROS generation by mitochondria are in agreement with these findings. Moreover, the resultant oxidative stress can further exacerbate SR and mitochondrial compromise by further activating the UPR and affecting the proteins involved in SR Ca^{2+} regulation and mitochondrial homeostasis.

We have previously reported the relative maintenance of myonuclei content in the settings of muscle atrophy in aging (26) and oxidative stress (29). In line with those findings, we show that the myonuclei count is not decreased in atrophic muscle in COPD. However, a disproportional decrease in fiber size resulted in smaller MNDs in advanced stages of COPD. This may be an adaptive strategy by the atrophied muscle so that it is ready to respond to hypertrophic stimuli to initiate recovery process. However, these findings do not rule out reduced synthetic capacity of existing myonuclei and their spatial reorganization, which can potentially contribute to loss of muscle fiber size and strength in COPD. Interestingly, we have shown that the muscle weakness and atrophy in aging (26) is associated with myonuclear disorganization without a loss of myonuclei. Furthermore, damaged mitochondria and oxidative stress are not found homogenously along the length of muscle fiber (50) which make it susceptible to atrophy and weakness despite a maintenance of myonuclear count.

Increased levels of serum AGP are probably a manifest of systemic inflammation associated with COPD. However, AGP has anti-fatigue action on skeletal muscle (51) and is also shown to suppress SR stress as loss of AGP increases SR stress related response (52). Thus, the small, albeit non-significant decrease in

serum AGP in advanced COPD complies with muscle weakness and SR stress in these participants. On the other hand, sialic acid has protective effects against inflammation, muscle loss (17) and SR stress (53). Thus, the reduction in serum sialic acid in advanced COPD is in agreement with the loss of its protective effect against muscle detriment and SR stress.

While we did not investigate respiratory muscles in this study, functional impairment of locomotor muscles has many similarities with respiratory muscles dysfunction in COPD (54). Numerous studies have shown atrophy and weakness of quadriceps muscles in COPD (54–56). However, not every patient with advanced COPD develops locomotor muscles dysfunction (57). On the other hand, patients with mild to moderate COPD can develop significant dysfunction of locomotor muscles (57). This heterogeneity among COPD individuals shows that lung function is perhaps not the only driving factor of muscle decline in COPD and other factors including sedentary lifestyle also have a contribution. Thus, it is very likely that the reduced physical activity in the COPD participants further exacerbates SR stress and muscle detriment. In support of this, prolonged inactivity of skeletal muscle can induce upregulation of the markers of SR stress in muscle (58). Moreover, mild to moderate physical activity improves functional status and reduces hospital admissions in COPD (59). Thus, training seems to be a good intervention to muscle loss with COPD since it at least partially reverse some of the functional impairment in COPD (60). However, physical exercises have low compliance in COPD. Moreover, some of the abnormalities such as muscle atrophy and defects in bioenergetics persist despite training, warranting the necessity of pharmacological intervention (54). SR stress-inhibition seems an attractive target to boost muscle mass and strength in COPD, due to its preservative effects in myopathies and other muscle diseases (31). We did not dissect the activations of individual UPR arms in muscle decline in COPD. However, emerging evidence suggests that some component of UPR may have protective effect against muscle decline in chronic mitigating conditions (12). Further investigations using muscle-specific knock-in and knock-out approaches are required to rigorously characterize the role(s) of individual UPR arms in muscle atrophy and weakness.

Conclusion

In conclusion, both the local and systemic factors contribute to muscle dysfunction in COPD. Among the local factors, chronic maladaptive SR stress and UPR are able to modify local microenvironment in muscle which leads to mitochondrial dysfunction, anabolic resistance and calcium dysregulation, subsequently causing loss of muscle mass and strength. These findings suggest that modulation of SR stress and UPR can have therapeutic advantages for skeletal muscle in COPD.

List Of Abbreviations

COPD: chronic obstructive pulmonary disease (COPD),

SR: sarcoplasmic reticulum

SERCA: SR Ca2+ ATPase

ROS: Reactive oxygen species

UPR: Unfolded protein response

GOLD: Global Initiative for Obstructive Lung Disease

HGS: Handgrip strength

FEV1: Forced expiratory volume

FVC: Forced vital capacity

PEFR: Peak expiratory flow rate

HDL - C: High density lipoprotein – cholesterol

AGP: α-1 acid glycoprotein

TSA: Total sialic acid

GRP94: Glucose regulated protein 94

BiP: Binding immunoglobulin protein

CHOP: CCAAT-enhancer-binding *protein* homologous *protein*

ATF6: *Activating transcription factor 6*

NFkB: nuclear factor kappa-light-chain-enhancer of activated B cells

P70S6K: p70 S6 kinase

4EBP1: Eukaryotic translation initiation factor 4E-binding protein 1

DHPR: dihydropyridine receptor

SDH: Succinate dehydrogenase

MyoD: Myoblast determination protein

MyoG: Myogenin

MND: Myonuclear domain

Declarations

Ethics approval and consent to participate: This study was conducted at the Kohat University of Science and technology, Kohat and the Gomal Medical College, Dera Ismail Khan. The regional ethical committees at both the universities approved the study. All participants provided written informed consents before participating in the study. The study was conducted in accordance with the declaration of Helsinki (19).

Availability of data and material: Data used in this study is available from corresponding author upon request.

Conflict of Interest: The authors declare that they have no conflict of interest.

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Authors' contribution: R.Q planned the experiments. T.M provided access to patients. R.Q, M.Q and T.M performed experiments. R.Q analyzed the data and wrote the manuscript.

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Figures

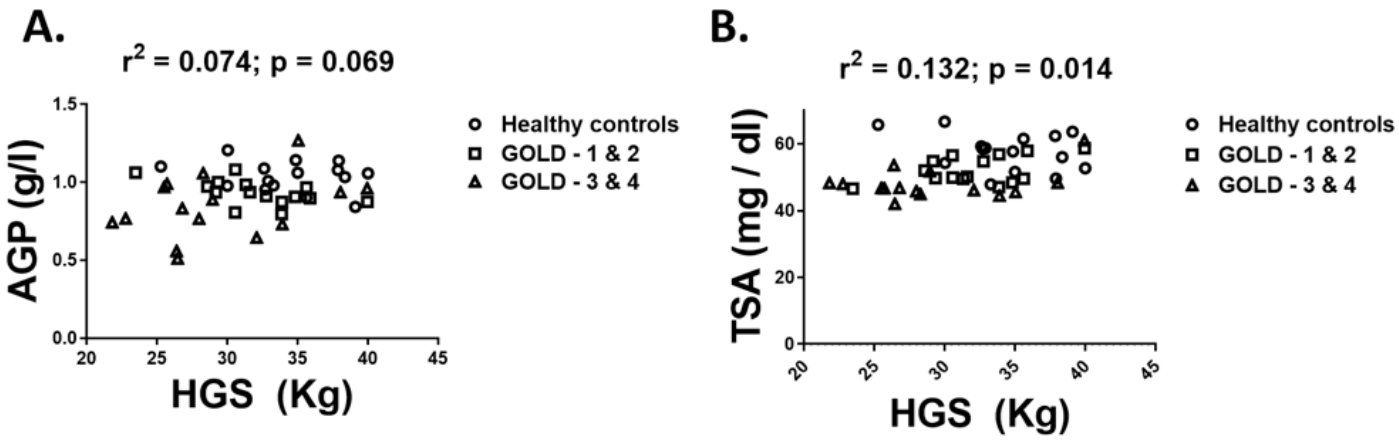

Figure 1

Relation of Serum biomarkers with HGS. Linear regression graphs of the relation between serum AGP and TSA with HGS in healthy controls and COPD patients with GOLD stage 1 & 2 and stages 3 & 4. TSA

showed significant correlation with HGS in the study population (N = 12 – 15 / group).

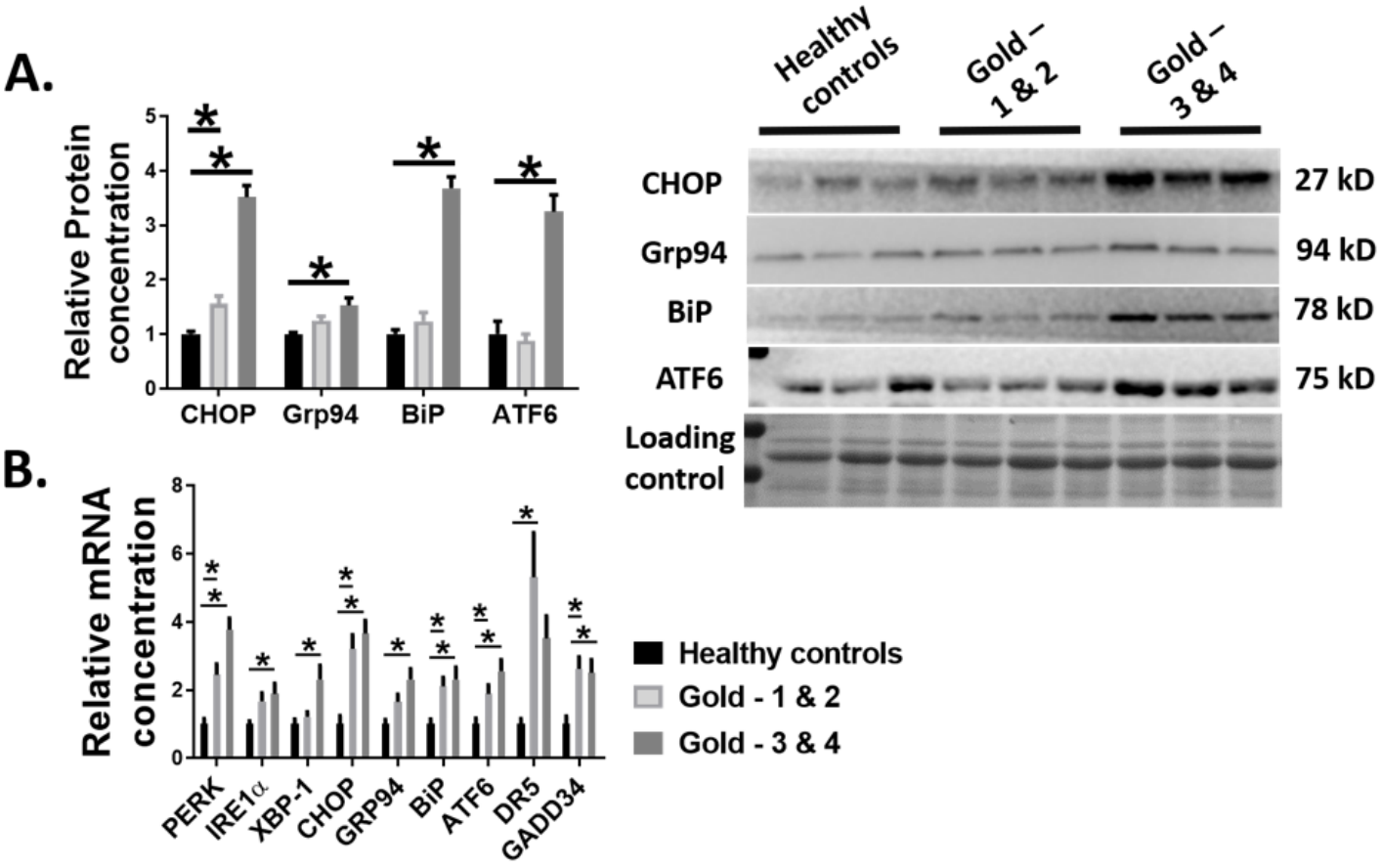

Figure 2

Markers of ER stress. Protein and mRNA expressions of the markers of SR stress in the vastus lateralis muscles of healthy controls and COPD patients with GOLD stage 1 & 2 and stages 3 & 4. Values are expressed as mean ± SEM (N = 6 – 9 / group); one-way analysis of variance, *P ≤ 0.05.

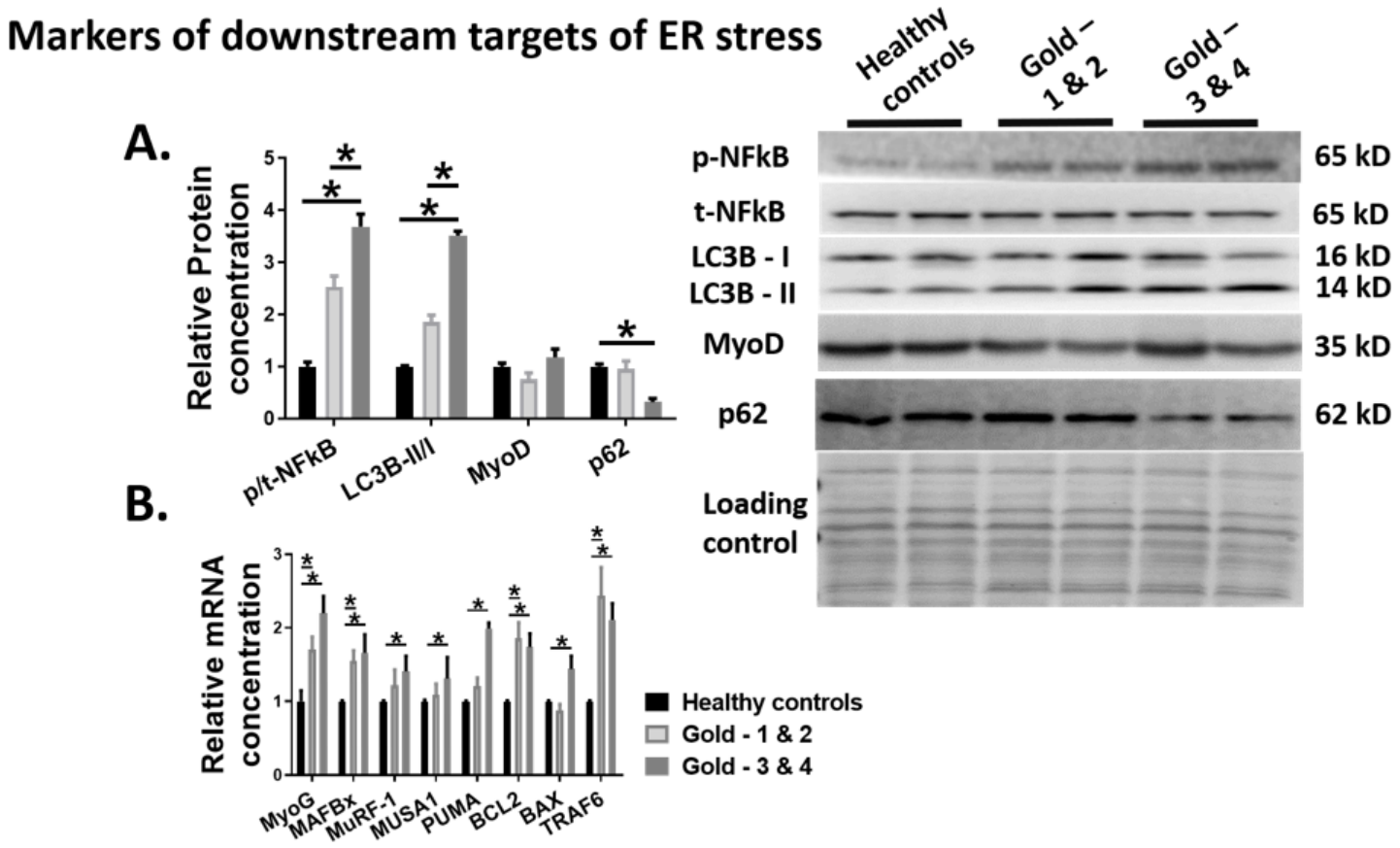

Figure 3

Markers of downstream targets of ER stress. Protein and mRNA expressions of the markers of downstream targets of SR stress in the vastus lateralis muscles of healthy controls and COPD patients with GOLD stage 1 & 2 and stages 3 & 4. Values are expressed as mean ± SEM (N = 6 – 9 / group); one-way analysis of variance, *P ≤ 0.05.

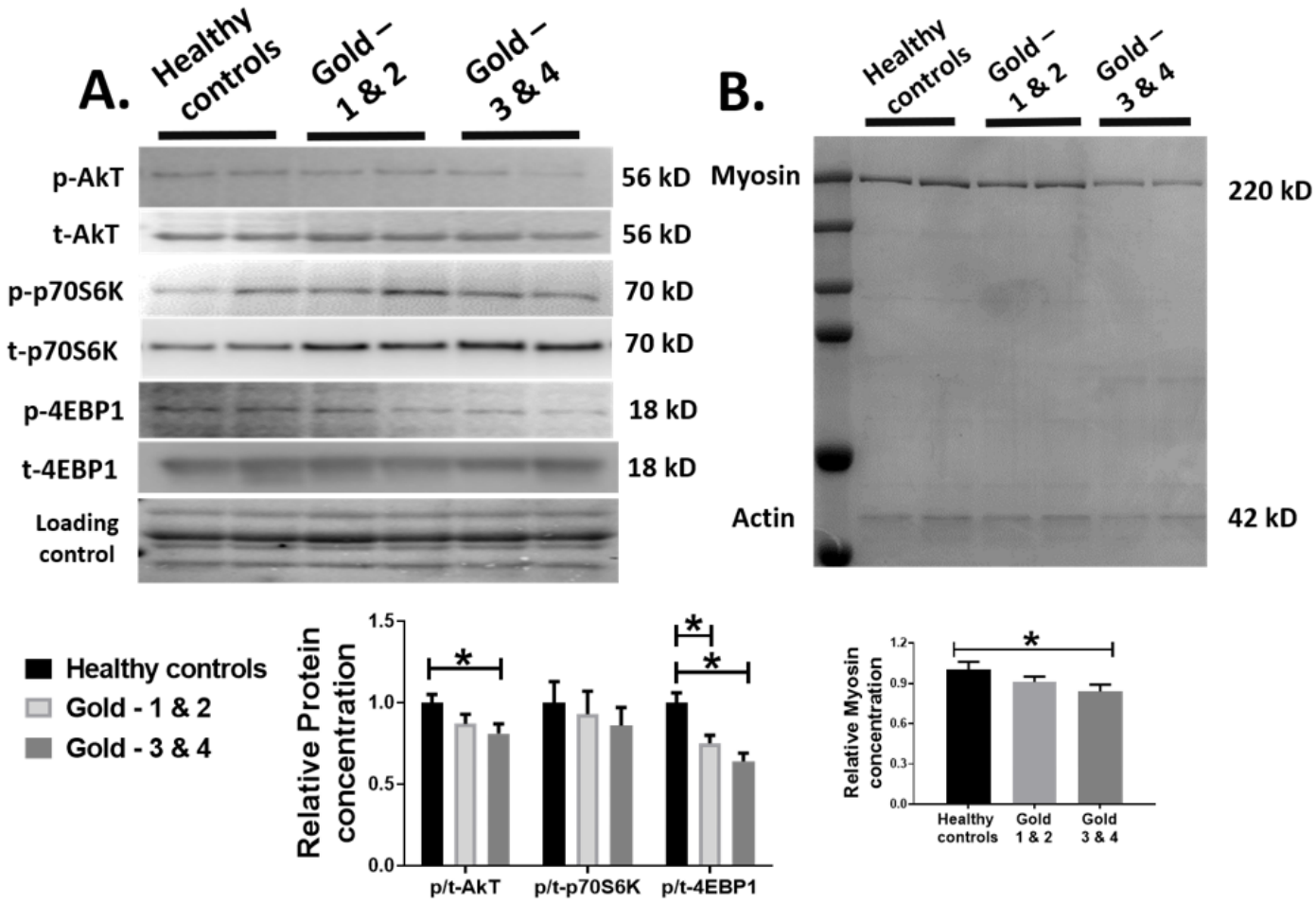

Figure 4

Markers of protein synthesis. Protein and mRNA expressions of the markers of protein synthesis and the relative myosin concentration in the vastus lateralis muscles of healthy controls and COPD patients with GOLD stage 1 & 2 and stages 3 & 4. Values are expressed as mean ± SEM (N = 6 – 9 / group); one-way analysis of variance, *P ≤ 0.05.

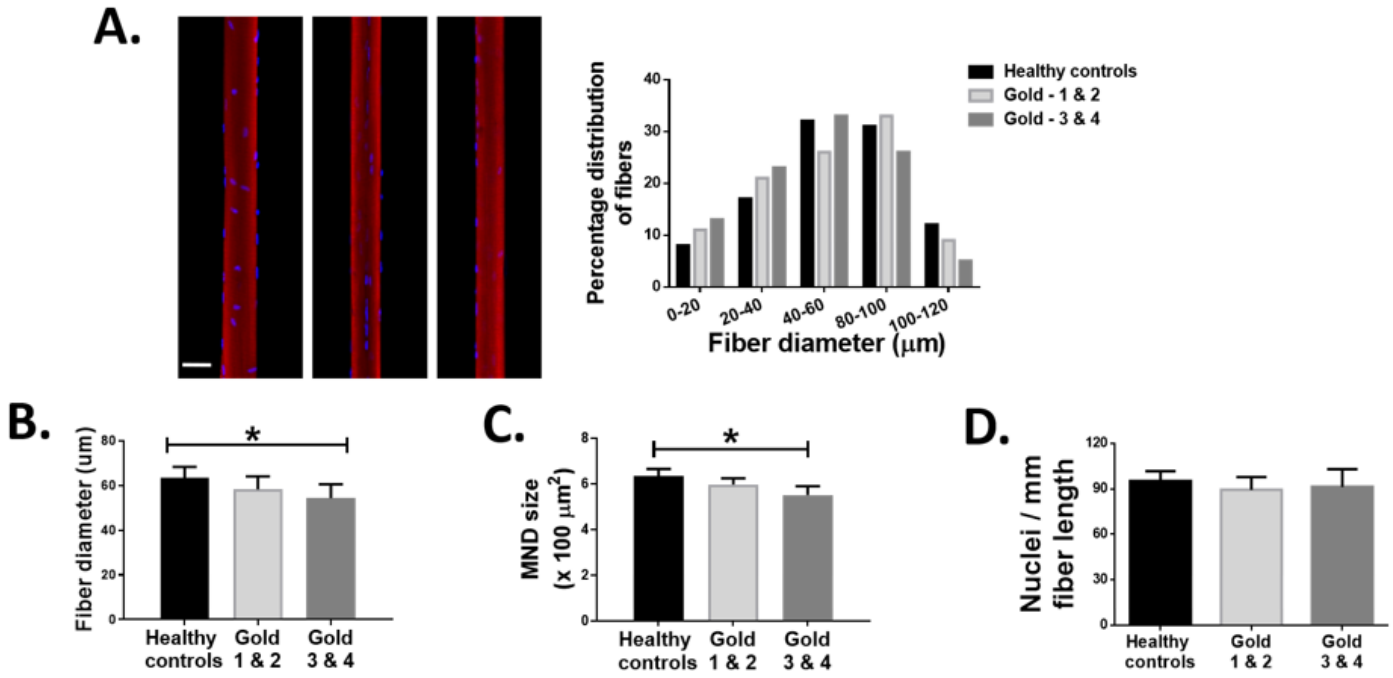

Figure 5

Single fiber CSA and myonuclei. Fiber diameters, MND size, number of nuclei per mm and the percentage distribution of fibers based on diameter in the vastus lateralis muscles of healthy controls and COPD patients with GOLD stage 1 & 2 and stages 3 & 4. Rhodamine-phalloidin labeled actin is shown in red and nuclei visualized by DAPI in blue. Scale bar denotes 50 μm. Values are expressed as mean ± SEM (N = 6 – 9 / group); one-way analysis of variance, *P ≤ 0.05.

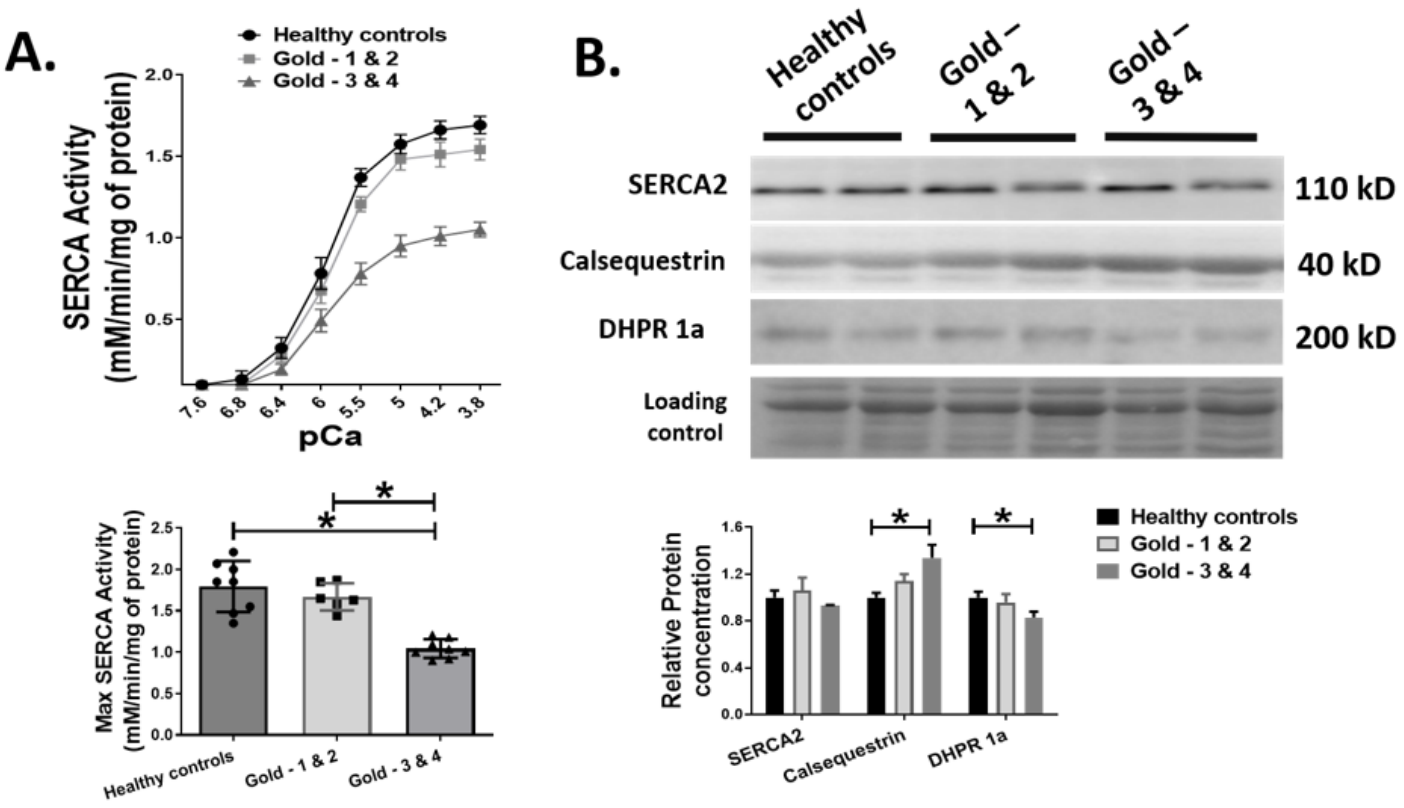

Figure 6

SERCA activity & calcium related proteins. SERCA Ca+2 dependent ATPase activity, the maximum ATPase activity (Vmax) and the relative expression of the Ca2+ related proteins in the vastus lateralis muscles of healthy controls and COPD patients with GOLD stage 1 & 2 and stages 3 & 4. Values are expressed as mean ± SEM (N = 6 – 9 / group); one-way analysis of variance, *P ≤ 0.05.

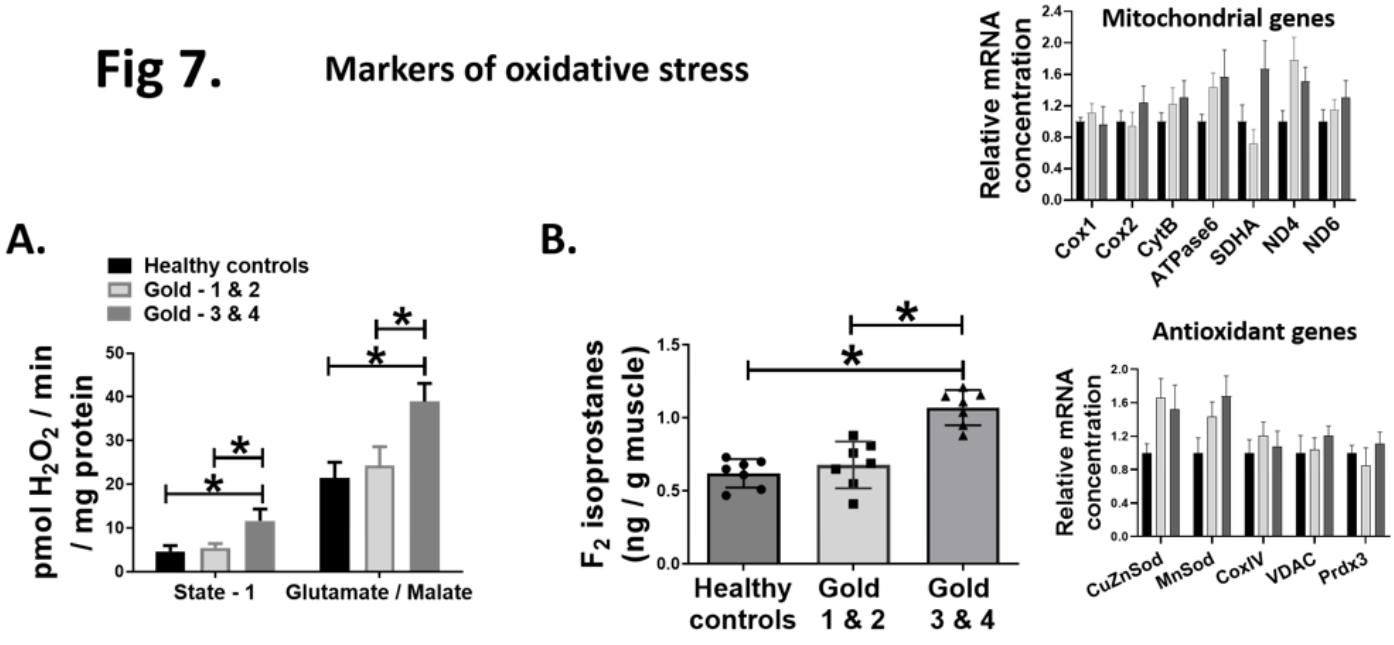

Figure 7

Markers of oxidative stress. Levels of mitochondrial state-1 and state-2, complex-1 linked (glutamate malate) peroxide production (A), the F2-isoprostanes as the markers of lipid peroxidation (B) and the mRNA expression of mitochondrial (C) and antioxidant (D) genes in the vastus lateralis muscles of healthy controls and COPD patients with GOLD stage 1 & 2 and stages 3 & 4. Values are expressed as mean ± SEM (N = 6 – 9 / group); one-way analysis of variance, *P ≤ 0.05.