Mitochondrial DNA Deletions Correspond to High Levels of Oxidative DNA Damage in Skeletal Muscle of Patients with Chronic Obstructive Pulmonary Disease

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ABSTRACT

Chronic Obstructive Pulmonary Disease (COPD) is characterized by severe lung inflammation and progressive airflow limitation. Systemic manifestations of COPD, including exercise intolerance and skeletal muscle dysfunction, contribute to disease severity and mortality. Chronic oxidative stress has been implicated as a major contributor to the development of these co-morbid conditions. Oxidized proteins and lipids have been described in COPD skeletal muscle, however oxidatively damaged DNA has not been previously investigated.

To assess the integrity of nuclear (nDNA) and mitochondrial DNA (mtDNA), we obtained muscle biopsies from 29 moderate-to-severe COPD patients (FEV₁ = 48 \pm 15% predicted) and 19 age-matched, healthy controls.

Using a competitive ELISA assay, we found that COPD patients have significantly higher levels of oxidized DNA ($387 \pm 41 \text{ pg/mL}$) compared to controls ($258 \pm 21 \text{ pg/mL}$, p = 0.03). We also detected a higher prevalence of mtDNA deletions (74% in COPD and 15% in controls, p < 0.01) and incidence of Electron Transport Chain (ETC) abnormalities ($8.0 \pm 2.1\%$ in COPD and $1.5 \pm 0.42\%$ in controls, p < 0.05). COPD patients with mtDNA deletions had significantly higher levels of oxidized DNA ($457 \pm 46 \text{ pg/mL}$) than patients with wild-type mtDNA ($197 \pm 29 \text{ pg/mL}$, p < 0.05). These results implicate ROS-mediated formation of mtDNA deletions and ETC dysfunction as novel systemic manifestations of COPD.

RESUMÉ

La maladie pulmonaire obstructive chronique (MPOC) est caractérisée par une inflammation sévère des poumons et une obstruction progressive des voies respiratoires. Les manifestations systémiques de la MPOC, incluant l'intolérance à l'effort et le dysfonctionnement des muscles squelettiques, contribuent à la gravité de la maladie et au risque de mortalité. Le stress oxydatif chronique a été identifié comme contributeur majeur au développement de ces comorbidités. Les processus d'oxydation des protéines et des lipides dans le muscle squelettique chez les personnes atteints de MPOC ont été décrits dans la littérature, néanmoins les dommages oxydatifs causé à l'ADN n'ont jamais été préalablement étudiés.

Pour évaluer l'intégrité de l'ADN nucléaire (ADNn) et de l'ADN mitochondrial (ADNmt), nous avons collecté des échantillons de biopsies musculaires chez 29 patients atteints de MPOC de niveau modérée à sévère (VEMS $1 = 48 \pm 15\%$ des valeurs prédites) et 19 sujets témoins sains du même âge.

Les résultats du test ELISA concurrent nous ont permis de constaté que les patients atteints de MPOC présentaient des niveaux significativement plus élevés d'ADN oxydée (387 \pm 41 pg / mL) par rapport aux sujets témoins (258 \pm 21 pg / ml, p = 0.03). Nous avons également détecté une prévalence plus élevée de délétions dans l'ADN mitochondriale (74% chez les patients MPOC vs. 15% chez les sujets témoins, p < 0.01) et une incidence plus élevée d'anomalie dans la chaîne de transport des électrons (CTE) $(8,0 \pm 2,1\%)$ chez les patients MPOC vs. $1.5 \pm 0.42\%$ chez les témoins, p <0.05). Les patients atteints de MPOC ayant des l'ADN mitochondriale présentaient délétions dans une concentration significativement plus élevée d'ADN oxydée ($457 \pm 46 \text{ pg} / \text{mL}$) comparativement aux patients ayant un ADN mitochondrial intacts $(197 \pm 29 \text{ pg} / \text{ml}, \text{p} < 0.05)$.

Nos résultats suggèrent la formation de stress oxydatif, induite par la présence de délétions dans l'ADN mitochondriale et l'augmentation de l'incidence d'anomalie dans la CTE, comme nouvelle manifestation systémiques de la MPOC.

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PART I: REVIEW OF LITERATURE

1. COPD: Introduction

1.1. Prevalence and Symptomology

Chronic obstructive pulmonary disease (COPD) is a lung disease characterized by irreversible airway obstruction. Affecting over 750,000 adults, COPD is the fourth leading cause of death in Canada(1). The airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lung to noxious particles or gases. This airway obstruction may result from a combination of chronic bronchitis and emphysema(2).

Chronic bronchitis is frequent inflammation of the medium size airways (bronchi) in the lungs(3). This chronic inflammation results in increased mucous production, impaired mucociliary clearance, and thickening of the bronchial walls. The main symptom of chronic bronchitis is a persistent cough that produces sputum in response to air-borne toxins(4). In addition to chronic bronchitis, COPD often involves emphysema(5).

Emphysema refers to progressive destruction of parenchyma, the specialized tissue necessary to maintain shape and function of the lungs(6). This condition is characterized by shortness of breath and wheezing(3). The severity of COPD is based on the degree of airway obstruction, from chronic bronchitis and/or emphysema(2).

2. Muscle Dysfunction in COPD

2.1 Etiology

Exercise intolerance and peripheral muscle abnormalities are important secondary features of COPD. Muscle dysfunction in COPD is associated with greater utilization of healthcare, reduced quality of life and shorter life expectancy. Specifically, low muscle mass and muscle weakness are significant predictors of increased mortality in COPD patients (7). However, the pathophysiology of muscle abnormalities in COPD remains unclear. On one hand, it is established that increased breathlessness during exercise, that is intrinsic to COPD, precipitates chronically low physical activity levels that lead to muscle deconditioning. Indeed, many of the reported muscle alterations are also reported with muscle disuse and detraining. These overlapping characteristics include fiber atrophy, decreased proportions of the aerobic, type I fibers, reductions in the activity of oxidative enzymes and capillary density, as well as the presence of oxidative stress and early lactate release during exercise(8). However, these abnormal changes in muscle structure and function are also reported in COPD patients with mild airway obstruction(9, 10), that is, independent of ventilatory limitation, suggesting that intrinsic muscular alteration are also involved(11, 12). Therefore, it is important to consider additional factors contributing, independently or in combination, to the muscle dysfunction phenotype in COPD patients. These clinically relevant complications include systemic inflammation,

oxidative and nitrositive stress, chronic hypoxia, hypercapnia, comorbidities, and the use of corticosteroid drugs.

2.2 Skeletal Muscle Abnormalities in COPD

2.2.1 Myofiber Atrophy

Skeletal muscle atrophy is a critical phenomenon in individuals with COPD(13). Loss of muscle mass is associated with increased fatigue and poor disease prognosis(10, 13). At the cellular level, loss of skeletal muscle mass results from decreased muscle protein synthesis and increased proteolysis, and this may be enhanced by decreased regenerative capacity of the muscle(14). As such, key regulators of proteolytic cascades, including myostatin and ubiquitin ligase, are up-regulated in COPD peripheral muscle(15, 16). Ubiquitin ligases are the critical enzymes that link ubiquitin moieties to the protein targeted for degradation, enhancing the ubiquitin-proteasome pathway, the predominant thereby coordinator of protein breakdown(16, 17). This engagement of muscle atrophy signaling networks results in the concurrent down-regulation of the signaling pathways that induce muscle hypertrophy(18). Overall, increased activity of protein breakdown pathways and inhibition of muscle hypertrophy is associated with myofiber atrophy and muscle weakness in patients with COPD(16, 19, 20).

Importantly, a sedentary lifestyle is a common characteristic in COPD with similar effects on peripheral muscle mass(21). Indeed, Shrikrishna and colleagues have identified physical inactivity in mild COPD to be independently associated with

muscle wasting(22). In addition, both decreased protein synthesis and increased proteolysis are reported in skeletal muscle during disuse atrophy (23). However, some differences exist between the proteolytic pathways reported with inactivity and in COPD.

For example, in limb immobilization experiments, increased ubiquitin conjugates and MuRF1 and atrogin-1 mRNA transcript levels were observed in both young and aged healthy adults 48 hours post intervention (24, 25). However, Brocca and colleagues report that atrogin-1 and MuRF1 expression levels were not increased following 24 days of bed rest (26). As such, the proteolytic pathways reported with inactivity appear to be more transient than the sustained increase in proteolytic rates reported in COPD. It is therefore suggested that disease-related triggers co-occur with inactivity to contribute to limb muscle atrophy in COPD (27).

2.2.2. Changes in Oxidative Phenotype

In addition to muscle atrophy, it is well established that skeletal muscle oxidative phenotype is disturbed in patients with COPD(9, 11). This includes a fiber-type shift toward a more glycolytic phenotype and reduced activity levels of enzymes involved in oxidative energy metabolism(28, 29). In patients with severe to very severe COPD, the reduction of the oxidative type I fibers in peripheral muscle corresponds to disease severity and low FEV₁ measurements (11). A meta-analysis of published muscle data reveals a 20% reduction of type I fibers in severe COPD

patients, compared to age-matched controls (11). Interestingly, a 20% reduction of the type I fibers is also observed in mild-to-moderate COPD patients(9). Furthermore, loss of the quadriceps oxidative phenotype correlated with decreased quadriceps endurance even in the absence of muscle wasting(9).

Fiber type transition is known to result in specific physiological adaptations. These alterations include a reduction in oxidative capacity and capillary density, as well as increased net production of ROS (29, 30). These characteristics of primarily glycolytic muscle, normally associated with disuse, are exaggerated in COPD patients and contribute to the COPD muscle phenotype(11, 31).

Additional morphological abnormalities exist in skeletal muscle of COPD subjects. Histology analyses reveal an abnormal accumulation of connective tissue and fat cell replacement compared to healthy controls(31). Higher numbers of TUNEL-positive nuclei are also reported, indicative of DNA fragmentation, resulting from continuous repair/remodeling processes. Interestingly, Barreiro and colleagues report a negative correlation between these TUNEL-positive nuclei and quadriceps muscle force (32).

2.3 Mitochondrial Dysfunction in COPD

Mitochondria are the main energy source for muscle cells. Specifically, the production of ATP depends on the quantity of oxidative phosphorylation enzymes within the mitochondrial inner-membrane space. Mitochondria are also involved in the production of reactive oxygen species (ROS) and in apoptosis. Normal

mitochondrial content and function is needed to permit normal muscle function(33). However, numerous aspects of mitochondrial function have been found to be altered in the skeletal muscle of COPD patients. These alterations include decreased mitochondrial density and biogenesis, impaired mitochondrial respiration and coupling, as well as increased mitochondrial ROS production and possibly increased apoptosis. Importantly, many of these impairments are associated with specific components of muscle dysfunction in COPD. For example, reduced mitochondrial oxidative phosphorylation and coupling have been associated with reduced muscle mass and endurance(30, 34). In addition, reduced citrate synthase (CS) activity is correlated with muscle time to fatigue (35). Finally, it has been suggested that COPD skeletal muscle is less sensitive to apoptotic events due to altered permeability transition pore kinetics and enhanced cytochrome c release (36). However, these alterations can be partially explained with the greater type 2 fiber abundance (37). Overall, it is thought that irregular mitochondrial function is implicated in skeletal muscle dysfunction in COPD (36, 38).

3. Systemic Inflammation and Muscle Dysfunction in COPD

Chronic inflammation of the pulmonary tissue in COPD is associated with a down stream systemic inflammatory response(39). This systemic inflammation has been implicated in the onset of weight loss, muscle wasting and increased mortality(40, 41).

3.1 Systemic Inflammation and Intracellular Pathways

The systemic response is characterized by the stimulation of the bone marrow to release leukocytes and platelets, as well as a release of inflammatory mediators in the circulation(39, 42). Several of these inflammatory factors, including the Creactive protein (CRP), interleukin-6 (IL-6), and pulmonary and activationregulated chemokine (PARC) have been observed to be independent predictors of mortality(43). Furthermore, some evidence suggests that chronic elevation of these inflammatory biomarkers is associated with activation of different cellular pathways that contribute to increased oxidative stress, apoptosis and autophagy (44). For example, circulating tumor necrosis factor alpha (TNF- α) can reduce intracellular levels of glutathione, an important physiological antioxidant (45). In addition, the TNF receptor family is thought to disrupt myonuclear turnover (46). As such, Almendro and co-workers have prevented DNA fragmentation, indicative of apoptosis, in septic rats by administration of a TNF- α antagonist (47). While similar results were observed in cultured myotubes, the ultimate relevance of these pathways to skeletal muscle dysfunction remains unclear due to the multinucleated nature of myofibers (48).

Furthermore, certain pro-inflammatory cytokines can activate specific catabolic systems, such as the ubiquitin-proteasome pathway, resulting in muscle atrophy (49, 50). For example, the ubiquitin 26S-proteosome system (UPS) can be stimulated to breakdown muscle proteins by circulating TNF- α and interleukin-1-beta (IL-1 β) (51). Specifically, elevated levels of TNF- α or IL-1 β can induce the

activation of NF-k β , a transcription factor within skeletal muscle that is known to up-regulate MuRF1. Since MuRF1 is one of the rate-limiting enzyme of the UPS, increases in MuRF1 will stimulate protein breakdown in skeletal muscle. Not surprisingly, elevated levels of TNF- α , IL-1 β , NF-k β and MuRF1 are reported in COPD subjects. In addition, Ling and colleagues have demonstrated TNF- α stimulated muscle proteolysis in rats and cultured myocytes (52, 53).

Finally, certain pro-inflammatory cytokines can directly compromise muscle contraction (54). These cytokines, especially TNF- α and interferon-gamma (IFN- γ), stimulate proteolysis and inhibit messenger RNA expression for myosin heavy chain, leading to decreased muscle protein synthesis(51). Overall, persistent systemic inflammation in COPD subjects contributes to multiple complications, including progressive weight loss and skeletal muscle dysfunction.

3.2 Systemic Inflammation and Co-morbidities in COPD

In addition to skeletal muscle dysfunction, systemic inflammation is implicated in the development of other co-morbidities, impacting many organ systems (55). These extra-pulmonary conditions are common and significant in COPD. Two key examples of these inflammation-related co-morbidities are cardiovascular disease and lung cancer and these are the leading causes of morbidity and mortality in patients with COPD (56). In addition to these more severe inflammation-related diseases, osteoporosis, hypertension and diabetes are some of the most prevalent co-morbidities associated with COPD (57). The etiology of these conditions is associated with high levels of inflammatory factors. Indeed, a recent study following over 8,000 COPD patients for a period of 5 years has shown that COPD patients with elevated cytokines are at a two-to-fourfold increased risk of developing major comorbidities within the follow-up period (57). This evidence supports a causative role for systemic inflammation in the development of comorbidities in COPD patients. Interestingly, most of the prevalent conditions, including chronic cardiac failure and diabetes, are also associated with muscle wasting and dysfunction (58, 59).

Finally, systemic inflammation is also correlated with oxidative stress. Free radicals can signal for the expression of inflammatory mediators, and cytokines (along with other factors such as a reduced blood flow, hypoxia and contractile activity) can modulate the level of ROS production and therefore of oxidative stress (43, 60).

4. Oxidative and Nitrosative Stress in COPD

4.1 Definitions

Reactive oxygen (ROS) and nitrogen species (RNS) are constantly generated within cells at low concentrations. At low levels, oxidants induce regulatory functions controlling cell division, migration, and contraction. Antioxidant enzymes neutralize these oxidant species, such that the quantity of the enzymes fluctuates to accommodate changes in oxidant concentration. Oxidative stress describes a physiological imbalance between the production of oxidant species and the total antioxidant capacity of a system(61). Prolonged periods of oxidative stress will result in irreversible, structural modification of cellular components, including proteins, lipids and DNA(62). Accumulation of these oxidatively damaged products will result in tissue damage and dysfunction(61).

4.2 Oxidized structural components in COPD

Proteins are major targets of oxidative stress. Direct oxidation of proteins by ROS yields highly reactive carbonyl derivatives, resulting either from oxidation of amino residues in the side chains or from the cleavage of peptide bonds (63). Depending on the extent of oxidative stress, oxidized proteins are either repaired, removed by proteolytic degradation or accumulate as damaged or unfolded proteins(64). Under chronic or high intensity oxidative stress, proteins become heavily oxidized and cross-linked, and act to inhibit the proteasome and therefore accumulate in the cytosol. Extensive accumulation of these dysfunctional protein aggregates is reported in COPD lung and skeletal muscle tissue(65, 66). Furthermore, prevalence of carbonylated proteins is correlated with increased fatigability and decreased maximal voluntary contraction of the quadriceps(65, 67). This relationship is further demonstrated by Kuwahara and colleagues who studied a transgenic mouse deficient in a muscle specific antioxidant, superoxide dismutase (68). This deficient mouse developed severe exercise intolerance and contractile dysfunction in the absence of atrophic change. These results suggest that mitochondrial ROS are implicated in the progression of exercise intolerance.

Reactive oxygen and nitrogen species can also degrade polyunsaturated lipids, forming cytotoxic products such as malondialdehyde (MDA) and 4- hydroxy-2nonenal (HNE)(69). Furthermore, both MDA and HNE are carbonyl-containing lipids that are capable of promoting structural damage within the cell. These oxidized lipids can, through adduct formation reactions, modify and disrupt protein structure and produce advanced lipoxidation end-products (ALEs) (70). The formation of ALEs has been linked to the pathogenesis and progression of numerous diseases, including diabetes, atherosclerosis and neurological disorders(71). Compared with healthy individuals, there are significantly higher levels of MDA and HNE molecules in COPD skeletal muscle(72, 73). Oxidatively damaged proteins and lipids are important biomarkers of oxidative stress, reflecting overall cellular damage induced by multiple forms of ROS (64, 74)

In addition to damaging structural and enzymatic components, oxidative stress can damage DNA. Although all DNA bases are susceptible to damage, guanine is most prone to oxidative modification, resulting in the formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG) (75). Oxidatively damaged DNA can result in induction of mutations, impaired DNA repair, chromosomal aberrations, and altered gene expression (76, 77). Oxidized DNA has been reported in blood, sputum and lung tissue in COPD (78, 79). Furthermore, increased levels of damaged DNA are associated with apoptosis, cell senescence, and pro-inflammatory phenotypic changes in COPD lung tissue(80).

Overall, it is thought that structural modification of intracellular components by chronically elevated ROS and RNS contributes to myofiber damage and dysfunction in COPD.

4.3 Redox signaling pathways and muscle atrophy

In addition to damaging myofiber components, chronic oxidative stress induces several muscle atrophy pathways. First, altered redox signaling has been shown to up-regulate gene expression of key components necessary for autophagy and proteasome systems of proteolysis(81). Second, increased ROS can activate major intracellular proteolytic systems, such as caspase-3 and calpain proteases(82). In addition, oxidized proteins themselves are more susceptible to proteolytic processing, thereby reducing overall protein content(83). Finally, ROS can inhibit protein synthesis by obstructing mRNA translation (62). Specifically, oxidants inhibit mammalian target of rapamycin (mTOR), a vital protein kinase necessary for cell growth and protein synthesis(84). ROS interferes with mTOR by reducing phosphorylation of mTOR substrates, thereby inhibiting the initiation step of mRNA translation.

4.4 Sources of Oxidative Stress in COPD

Oxidative stress may result from a combination of factors leading to greater generation of reactive species and/or insufficient quantities of antioxidants. There are multiple sources of oxidative stress in skeletal muscle of COPD patients. First, chronic systemic oxidative stress and inflammation is associated with enhanced oxidative stress within myofibers, via redox signaling and formation of reactive carbonyl species(85). Secondly, the mitochondrial electron transport chain in these myofibers is also characterized by increased ROS production, as compared with healthy controls(37). Together, these sources of increased ROS production overpower the antioxidant capabilities, resulting in the accumulation of oxidatively damaged myofibers in COPD(85, 86).

In summary, chronic oxidative stress results in oxidatively damaged proteins, lipids and DNA. Accumulation of these oxidized molecules results in tissue damage and correlates with disease severity in multiple chronic disorders. In COPD patients, high levels of oxidized products have been reported systemically, in blood, sputum, exhaled breath condensate and within lung and skeletal muscle cells. Furthermore, oxidative damage in COPD skeletal muscle is associated with muscle loss and dysfunction, thereby contributing to overall disease severity.

5. Mitochondrial DNA

5.1 Structure and Function

As previously mentioned, mitochondria are involved in several intracellular functions including calcium regulation, ROS generation, and apoptosis. Most importantly, mitochondria carry out oxidative phosphorylation, the major energyproducing pathway in skeletal muscle. Oxidative phosphorylation occurs in the inner membrane of the mitochondria and comprises of the ATP synthase and four complexes within the electron transport chain (ETC). The ETC complexes are each comprised of multiple peptides, 13 of which are encoded by mitochondrial DNA (mtDNA)(87). For this reason, damage to mtDNA can adversely impact ETC function.

The mtDNA molecule is a closed double-stranded loop that is located in the mitochondrial matrix, in close proximity to the ETC(88). The proximity to ROS-generating ETC, increased replication rates and limited repair mechanisms of the mitochondrial genome are thought to explain its greater susceptibility to structural damage and mutations than nuclear DNA (nDNA)(89). Similar to nDNA, mutations in the mtDNA can be inherited genetically or develop in response to intracellular stress such as excessive ROS (90).

5.2 Mutations in mtDNA

Mutations in mtDNA can lead to defective production of protein subunits necessary for ETC complexes and eventually ETC dysfunction(91). Progressive ETC dysfunction in turn, can lead to deficient energy production within individual cells (92). Furthermore, ETC dysfunction within skeletal muscle is associated with muscle weakness and fatigue. However the relationship between mtDNA mutations and ETC dysfunction is not straightforward.

Since each mitochondrion contains multiple copies of mtDNA, the relationship between individual mtDNA mutation events and a detectable phenotypic change remains unclear. Furthermore, each mitochondrion may contain a mixture of healthy and mutation-containing mtDNA, termed heteroplasmy (93). This heterogeneity of mtDNA can be protective, whereby healthy mtDNA molecules compensate for the mutated mtDNA. That is, a single mitochondria with multiple copies of damaged or mutated mtDNA molecules, can still function normally, in terms of OXPHOS and intracellular signaling, provided sufficient healthy mtDNA copies remain (93).

Therefore, phenotypic change of a mitochondria or individual fibers occurs only when the ratio of mutated mtDNA, relative to wild-type mtDNA, has reached an unmanageable threshold(94). Although the specific mutation threshold necessary to yield aberrant mitochondrial phenotypes remains a point of study (and may vary between tissues), it is established that mutated mtDNA can accumulate beyond this threshold through clonal expansion, whereby the mutated mtDNA undergoes preferential replication(95). This process can be accelerated by low or declining numbers of total mtDNA molecules in a mitochondria, termed mtDNA copy number(95).

Progressive accumulation of mtDNA mutations and ETC dysfunction are key features of inheritable mitochondrial myopathies. However, acquisition of mtDNA mutations has also been implicated in age-related muscle loss (92). Life-long exposure to ROS is implicated as one of the contributors to mtDNA mutations and ETC dysfunction in aging muscle and certain neurodegenerative diseases (92).

5.3 COPD and mtDNA

Mitochondrial DNA integrity has not been investigated in COPD. However, one study has examined the ratio of mtDNA to nDNA (mtDNA/nDNA) in these patients (96). The authors report a decreased mtDNA/nDNA ratio in COPD patients, compared to healthy controls, indicative of lower mtDNA content. Since oxidative stress is associated with decreases in mtDNA copy number (97), these results support a mechanistic role for ROS in mitochondrial dysfunction in COPD.

Purpose

Given these previous reports of oxidative stress in COPD skeletal muscle we hypothesized that COPD patients would have elevated levels of oxidatively damaged DNA and altered mtDNA integrity. The aim of this investigation was to examine mtDNA integrity in COPD skeletal muscle for the presence of oxidatively damaged DNA as well as deletion-containing mtDNA. COXdeficiency was also assessed to determine the existence of ETC abnormalities.

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PART II: Experimental Article

Oxidative DNA damage and mtDNA deletions in COPD patient muscle: evidence of accelerated aging?

INTRODUCTION

Chronic Obstructive Pulmonary Disease (COPD) is characterized by an excessive inflammatory response in the lungs leading to progressive airflow limitation (1). COPD is further characterized by the presence of significant extrapulmonary conditions, including exercise intolerance and muscle wasting (2, 3). These systemic manifestations contribute to disease severity and mortality. Indeed, those patients who develop skeletal muscle dysfunction in COPD have greater utilization of healthcare, reduced quality of life and shorter life expectancy (4, 5). Systemic and local oxidative stress has been identified as a major contributor to this progressive muscle dysfunction and wasting in COPD(6, 7), but the full implications of this for muscle pathophysiology in COPD remains unclear.

Oxidative stress refers to a physiological imbalance where the production of reactive oxygen species (ROS) overwhelms the antioxidant capacity of the system. The posited clinical significance of oxidative stress in COPD is that elevated ROS generation and/or depleted antioxidant defenses in skeletal muscle are often associated with stimulation of muscle atrophy pathways and activation of proteolytic machinery(8).

In addition to driving muscle atrophy, chronic oxidative stress will result in structural modification of cellular components(9). Accumulation of these oxidatively damaged products contributes to tissue damage and dysfunction(10). Indeed, elevated levels of oxidatively damaged proteins and lipids have been reported in COPD skeletal muscle, relative to age-matched healthy individuals(11). Furthermore, the prevalence of these oxidized products correlates with increased fatigability and decreased maximal voluntary contraction of the quadriceps(12).

In addition to damaging proteins, chronic oxidative stress can also oxidize nuclear bases of DNA molecules. While all four DNA bases are subject to ROS-mediated modification, guanosine is the most susceptible, whereby 8-hydroxy-2'deoxyguanosine (8-OHdG) is commonly formed(13). Nuclear DNA (nDNA) integrity is well protected from these modifications by histones and sophisticated repair mechanisms(14). In contrast, mitochondrial DNA (mtDNA) is particularly exposed to oxidative damage(15).

Mitochondrial DNA is a closed double-stranded loop that encodes crucial components of the complexes that comprise the electron transport chain (ETC) (16). The ETC complexes are essential for oxidative phosphorylation, the principal source of energy in muscle cells. However, the ETC complexes are also major

producers of intracellular ROS(17). Given its proximity to the ROS-generating ETC, its greater replication rates and limited repair mechanisms compared to nDNA, mtDNA is more susceptible to structural damage and mutations than nDNA (18).

In addition, this risk of oxidatively damaged mtDNA is likely further increased in COPD skeletal muscle given the elevated levels of mitochondrial ROS production and systemic oxidative stress reported in these patients (6, 19). Accumulation of mtDNA mutations, particularly in the form of deletions, can lead to defective production of protein subunits necessary for ETC complexes (18). Complex IV (cytochrome c oxidase, COX) is most at risk, leading to COX-deficiency, a type of ETC dysfunction (20). Progressive ETC dysfunction in skeletal muscle corresponds to deficient energy production and fiber atrophy(21). At the whole body level, ETC dysfunction is associated with muscle weakness and fatigue (22). However, neither mtDNA integrity, nor its potential manifestation as severe mitochondrial dysfunction, have been previously investigated in COPD skeletal muscle.

The aim of this investigation was to examine mtDNA integrity in COPD skeletal muscle for the presence of oxidatively damaged DNA as well as deletioncontaining mtDNA. COX-deficiency was also assessed to determine the existence of ETC abnormalities. Given the previous reports of oxidative stress in COPD skeletal muscle we hypothesized that COPD patients would have elevated levels of oxidatively damaged DNA and altered mtDNA integrity.

METHODS

Table 1. Subject characteristics

Characteristic	Control (n = 19)	COPD (n = 29)
Age	68 ± 6	66 ± 5
Weight, kg	79 ± 14	71 ± 17
Height, cm	172 ± 4	170 ± 8
BMI	26.8 ± 3.8	28.8 ± 11.9
FEV ₁ (% pred)	109.5 ± 19.7	41.8 ± 14.3*
FEV ₁ /FVC (% pred)	78.4 ± 7.1	53.3 ± 14.7*
Peak VO ₂ (mL/min/kg)	28.3 ± 6.1	11.9 ± 4.9*
Peak Work (W)	188 ± 37	58 ± 28*

* Significant differences between groups, p<0.05. Values reported as means \pm SD. BMI – body mass index. FEV₁ - volume that has been exhaled at the end of the first second of forced expiration. FVC – forced vital capacity.

Human Biopsy

Muscle biopsies were obtained from the mid-portion of the vastus *lateralis* muscle from 29 moderate-to-severe COPD patients and 19 age-matched control subjects following standard procedure (23). Briefly, local anesthetic was applied subcutaneously and a Bergstrom percutaneous biopsy needle under suction was used to extract approximately 200mg of muscle tissue. Muscle was rapidly removed from the needle and a portion was snap frozen in liquid nitrogen for biochemical and genetic analyses. For histochemical analyses, a portion of the muscle biopsy was mounted in a cross-sectional orientation on 10% Tragacanth gum (Sigma-Aldrich, G1128) and frozen in melting isopentane, precooled in liquid nitrogen. Samples were stored at -80C. COPD patients were recruited as part of a randomized control trial at the CHEST Hospital in Montreal, QC.

Quantification of 8OHdG

DNA Extraction and Digestion: DNA was extracted from collected muscle samples according to kit protocol (QIAgen DNeasy Blood and Tissue Kit; QIAgen, Valencia, CA) and quantified with a spectrophotometer (Nanodrop ND-1000; Nanodrop Technologies, Wilmington, DE).

Prior to digestion, extracted DNA samples were concentrated to 20g/mL (using standard DNA precipitation techniques. Briefly, a mixture of 3M Na-Acetate, pH 5.5 (1/10 volume of DNA sample), pre-chilled 100% Ethanol (EtOH, 2x volume of DNA sample) was added to each DNA sample. Samples were vortexed and kept at -20°C for 2 hours. Precipitated DNA was recovered by centrifugation at 19,000g for 15 min, EtOH was poured off and DNA pellet was air-dried at 4°C for 2 hours. DNA was reconstituted to 20g/mL, for a total of 3ug DNA by adding 150ul of digest mix. (Note: 3ug DNA was required to allow 3 equal triplicates of 1ug of each sample.)

Digest Mix (enough for one hundred 1µg samples) was prepared by adding 250 Units Benzonase, 300 mUnits phosphdiesterase I (Sigma P-3243) and 200 Units alkaline phosphatase (Sigma P-7923) to 5 mL Tris-HCl buffer (20mM, pH 7.9) containing 100 mM NaCl and 20 mM MgCl₂ (24). DNA samples (3µg) in 150µL Digest Mix were incubated at 37°C for 6 hours. 80HdG Assay: Levels of oxidatively damaged DNA in the digested DNA samples were measured using a competitive ELISA kit (DNA/RNA Oxidative Damage EIA Kit, Cayman Chemicals, Ann Arbor, Michigan) following the manufacturer's instructions. Briefly, samples were run in triplicates, with negative controls and 8 standards (10pg/ml – 3000pg/ml) on the same plate. The concentration of 80HdG (pg/ul) was standardized per total DNA content (1µg per well).

Cryostat Sections

Muscle cryostat sections were cut from frozen tissue using a Leica CM-3050-S cryostat. Tissue was orientated transversely and sections of 12µm thickness were cut and mounted onto glass slides. For laser capture microscopy (LCM), sections of 10µm thickness were cut and mounted on membrane slides. Sections were left to air dry for one hour and then stored at -80oC until required.

Dual Cytochrome *c* Oxidase (COX) and Succinate Dehydrogenase (SDH) Histochemistry

Muscle sections were defrosted air-dried for one hour at room temperature. All staining and incubations were carried out in a humidified chamber. The COX incubation medium was prepared by adding 200 μ l of 500 μ M cytochrome *c* to 800 μ l of 5mM 3.3'diaminobenzidine (DAB) with approximately 20 μ g of catalase enzyme. The solution was then vortexed and 200 μ l per section was added to the slides. Slides were incubated for 45 minutes at 37°C and then immersed in PBS three times to wash.

The SDH incubation medium was prepared by adding 100µl of 1.3M sodium succinate, 100µl of 2mM phenazine methosulphate (PMS) and 10µl of 100mM

sodium azide to 800µl of 1.875mM NitroBlue tetrazolium (NBT). The mixture was vortexed and 200µl was added to the sections. Again, the sections were incubated for 45 minutes at 37°C, before being immersed three times in PBS to wash. The sections were dehydrated by dipping the slides through a graded ethanol series (75%, 95%, 2 x 100%), being left in the final 100% solution for ten minutes. The slides were then cleared through two xylene baths, mounted using DPX mountant and a cover slip was applied.

Slides were imaged on a Scanscope XT digital slide scanner (Aperio, Vista, CA, USA). ImageJ software was then used to manually analyze the presence or absence of COX-deficiency in every individual fiber from all subjects, whereby blue-colouring indicated deficiency. Percent COX-deficiency for each subject was calculated based on the prevalence of COX-deficient myofibers over the total number of fibers counted on the histological section of that subject.

Detection of mtDNA Deletions

Long Range PCR: The major arc region of mtDNA, ~10.8kbp fragment, known to incur the majority of mutations was amplified using primer pairs (forward: nt.5855-nt.5875, 5'-AGA TTT ACA GTC CAA TGC TTC-3' and reverse: nt.129-nt.110, 5'-AGA TAC TGC GAC ATA GGG TG-3'). The master mix consisted of 1 μ l of DNA template, 5 μ l reaction buffer 3, 8.75 μ l 0.35mM dNTPs, 0.7 μ l Expand Taq (polymerase enzyme), 1.5 μ l of both forward and reverse primers 10pmol/ μ l, and 21.55 μ l dH2O, to give a 50 μ l reaction mixture (TaKaRa LA taq enzyme kit).

Reactions were performed on a thermal cycler under the following conditions: 3 minutes at 93°C; 10 cycles of 93°C for 30 seconds, 58°C for 30 seconds, and 68°C for 12 minutes; 20 cycles of 93°C for thirty seconds, 58°C for 30 seconds, and 68°C for 12 minutes with an additional five seconds for every cycle. Then there was a final extension of 11 minutes at 68°C.

Gel Electrophoresis: Results of the long-range PCR were visualized using gel electrophoresis and a 0.7% agarose gel (0.7% agarose in 1X TAE buffer and 0.08% SYBR Safe DNA stain). The mixture was heated to dissolve the agarose and poured into a gel-caster. To each well, 15µl of the PCR product, combined with 0.5µl of loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in dH2O), was added. A 1kb+ ladder (Invitrogen) was also loaded onto the gel to allow for an estimation of the product size. The gel was run for 150 minutes at 80V in a tank containing 1xTAE buffer, and bands were visualized on a UV gel documentation system G: BOX Chemi (Syngene, Frederick, MD).

Statistical Analyses

Results are presented as means and standard deviation. Student's t-test, two-tailed with 95% confidence intervals (CI) was used for all comparisons of oxidized DNA concentrations between controls and COPD patients. Fisher's exact test, two-tailed with 95% CI, was used for comparison of deletion-containing frequencies between controls and COPD patients. Spearman's correlation coefficient was used to assess relationships among different variables within COPD patients. In all cases, results were considered significant at p < 0.05

RESULTS

Oxidatively Damaged DNA is Increased COPD Skeletal Muscle

Levels of oxidatively damaged guanosine base (80HdG) were quantified in the quadriceps muscle of 29 COPD patients and 19 age-matched controls (Figure 1). A significantly higher concentration of 80HdG was observed in COPD patients (mean 387 pg/mL) than in the control group (mean 258 pg/mL), p=0.03.

Mitochondrial DNA Deletions are More Prevalent In COPD Patients

The major arc of the mitochondrial genome was amplified using LR-PCR in 29 COPD patients and 19 age-matched controls. The amplified region, approximately a 10kb segment, encompasses the most common mutational hot-spots and was visualized using gel-electrophoresis. When mtDNA deletions are present in the major arc, the amplified region produces smaller PCR products in addition to the full 10 kb segment. These smaller deletion-containing segments appear as additional, independent DNA bands below the wild-type, 10 kb segment. Individual subjects were then identified as either containing mtDNA deletions (when smaller PCR products can be detected in addition to the 10kb segment) or containing only the full mtDNA segment. A significantly higher proportion of the COPD group (76%) had evidence of mtDNA deletions compared to age-matched controls (16%), p = 0.0003 (Figure 2). Of the 29 COPD subjects, 22 had evidence of deletion-containing mtDNA, while in the control group mtDNA deletion were detected in 3 of the 19 subjects.

Mitochondrial DNA Deletions Correspond to Higher Levels of Oxidative Damage in COPD Skeletal Muscle

COPD patients with evidence of mtDNA deletions had significantly higher levels of oxidatively damaged DNA (Figure 3), as measured by higher concentrations of 80HdG (mean 457 \pm 7.5 SE pg/mL), compared to COPD patients who did not have mtDNA deletions (mean 197 \pm 5.4 pg/mL), p=0.0016.

COX Deficiency More Prevalent in COPD Skeletal Muscle

Cryosectioned muscle tissue from 21 COPD patients and 13 age-matched controls was dual stained for cytochrome *c* oxidase and succinate dehydrogenase activity. Normal COX-positive fibers stain brown, whereas COX-deficient fibers are stained blue (Figure 4A) by the SDH counter stain as a result of impaired complex IV activity. The proportion of fibers exhibiting mitochondrial respiratory chain defect is higher in COPD patients (mean 8.0 ± 2.1 %), than in age-matched controls (mean 1.5 ± 0.42 %, p=0.024; Figure 4B).

Oxidative Damage and Physiological Characteristics

There was no relationship observed in COPD patients between levels of oxidatively damaged DNA and (i) age (Figure 5), (ii) muscle content as measured by the Fat Free Mass Index (Figure 6) or (iii) severity of airflow obstruction (Figure 7). In addition, there was no significant correlation between oxidative DNA damage and COX-deficiency in these patients (Figure 8).



Figure 1 The concentration of oxidatively damaged guanasine base (pg of 80HdG per mL of total DNA) is significantly higher in COPD patients (mean 387 pg/mL) than in age-matched controls (mean 258 pg/mL), p=0.03. Bars denote means for each group.

Figure 2. Higher prevalence of mtDNA deletions in COPD patients than controls



Figure 2: Total incidence of mtDNA deletions is higher in COPD patients compared to age-matched controls (p = 0.0003, Fisher's exact test).

Figure 3. Mitochondrial DNA deletions correspond to high levels of oxidative DNA damage in COPD skeletal muscle



Figure 3 The concentration of oxidatively damaged guanasine base (80HdG) is significantly higher in COPD patients with mtDNA deletions (mean 457 pg/mL), compared to COPD patients with preserved, full-length (wild-type) mtDNA only (mean 197 pg/mL), p=0.0016. Bars denote means for each group.

Figure 4. Higher incidence of COX-deficient fibers in COPD patients than controls



Figure 4A: Cross-sectional images of skeletal muscle from COPD and control subjects following a COX/SDH double stain. Red arrow highlights a COX-deficient myofiber.



B

Figure 4B: Proportion of COX-deficient myofibers (% of total fibers, per subject) is higher in COPD patients (mean 8.0 ± 2.1 %) than in age-matched controls (mean 1.5 ± 0.42 %). Bars denote means for each group



Figure 5: The concentration of oxidatively damaged guanosine base (8OHdG) does not correlate with age in COPD patients.



Figure 6: Weak correlation (r = 0.29) between Fat Free Mass Index and concentration of oxidatively damaged guanosine base (8OHdG) in patients with COPD.



Figure 7: Correlations between concentration of oxidatively damaged guanosine base (8OHdG) and percent predicted values of FEV₁ (panel A) and FEV₁/FVC (panel B) in patients with COPD.



Figure 8: Correlation between COX-deficiency and oxidatively damaged DNA (80HdG).

DISCUSSION

Several studies have implicated high levels of systemic ROS in the pathogenesis of COPD co-morbidities, including skeletal muscle dysfunction. Although previous studies have reported increased levels of ROS-modified proteins and lipids (12, 85, 98), oxidatively damaged DNA has not been previously investigated in COPD skeletal muscle. The purpose of this study was to determine if the previous reports of elevated oxidative stress in COPD skeletal muscle has affected DNA integrity. Furthermore, we aimed to examine any relationship between mtDNA integrity and ETC dysfunction that may contribute to the abnormal muscle phenotype observed in COPD patients.

ROS-dependent DNA damage can be measured using a competitive ELISA assay, that detects a common DNA oxidation product 8OHdG. This technique was applied to skeletal muscle tissue from COPD patients and age-matched controls. Significantly higher levels of oxidized DNA, 8OHdG, were observed in the COPD patients (Figure 1). Given the much higher levels of nDNA relative to mtDNA within myofibers this finding likely represents oxidatively damaged nDNA in COPD patients.

Extensive studies have demonstrated age-related increases in oxidative stress and concentrations of oxidized cellular components(25-27). However, given the difference in oxidized DNA levels between the COPD group and the age-matched

controls (Figure 1) and lack of correlation between oxidized DNA and age (Figure6), we are likely measuring the COPD-dependent increases in oxidative damage.

Complex repair mechanisms exist to maintain structural integrity of nDNA, thereby reducing the frequency of mutations. However, these mechanisms are limited in mtDNA and therefore, ROS-dependent mutations occur more frequently (18). Specifically, Yakes and colleagues have suggested that mtDNA is about 30-fold more sensitive to exogenous oxidants than nuclear DNA(28).

Mutations in mtDNA, in the form of deletions, can be detected by long range PCR and gel electrophoresis. Our analyses revealed a significantly higher prevalence of mtDNA deletions in COPD patients, 72% than in control subjects 16% (Figure 2). The evidence of DNA damage and the presence of mtDNA deletions support the concept that chronic oxidative stress, that is intrinsic to COPD, damages intracellular components, including nuclear and mitochondrial genomes within skeletal muscle.

Furthermore, we found that COPD patients with evidence of mtDNA deletions have significantly higher levels of oxidized DNA than patients with intact mtDNA (Figure 3), supporting the idea that ROS may be important in driving the development of both nDNA oxidative damage and mtDNA mutations.

The presence and accumulation of mtDNA mutations is known to disrupt

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transcription of the genes encoding the respiratory chain subunits and may lead to abnormal electron transport chain (ETC) activity (29). Respiratory chain dysfunction, in the form of complex IV deficiency (cytochrome oxidase c, COX) activity, was detected using a histochemical assay, as used previously in the context of aging (30) and mtDNA disease patients (31). Significantly higher prevalence of the blue, COX-deficient fibers (Figure 4) was detected in COPD patients (8%) than in the control subjects (1.5%). This finding provides evidence to link prolonged oxidative stress and mtDNA deletions to ETC alterations in COPD skeletal muscle.

Whilst mtDNA damage may lead to respiratory chain dysfunction or muscle abnormalities, the presence of COX-negative fibers alone does not definitively prove that mtDNA damage is the underlying cause of the phenotypic observation. Specifically, since each mitochondrion contains multiple copies of mtDNA, the phenotypic presentation of a mitochondrial genetic defect depends on the ratio between mutated mtDNA and preserved, wild-type mtDNA. As such, respiratory chain abnormalities occur only when a threshold level of mutated mtDNA is breached.

In addition, the relationship between COX-deficiency and whole muscle dysfunction is also ambiguous. For example, patients with inherited mtDNA mutations experience exercise intolerance and muscle weakness, but such patients represent an extreme involvement of mtDNA (32) that may not apply to COPD

patients. Understandably, COX-deficient myofibers reported in these patients, are much more prevalent than the 8% observed in this study (33, 34).

As such, the clinical relevance of the 8% COX-deficiency we measured in the COPD patients is unclear. While the 1.5% COX-deficiency in the control group is consistent with previous reports of 1-2% deficiency in healthy adults over the age of 65 y (33).

CONCLUSIONS

Overall, although the findings of this study support the existence of ROS-mediated ETC dysfunction in COPD skeletal muscle, the prevalence of COX-deficient myofibers is relatively low. As such, we did not find any relationship between oxidatively damaged DNA and severity of lung obstruction (Figure 8) or muscle loss (Figure 7). Therefore, it is unlikely that the observed ROS-dependent mtDNA mutations and ETC abnormalities are paramount in the development of the muscle alterations and bioenergetics defects reported in COPD patients (3). However, our findings of DNA damage do suggest that mtDNA and ETC complexes are probable targets of chronically elevated systemic ROS in COPD. Furthermore, since COX-deficiency and mtDNA damage are reported to increase with normal aging (99), the higher levels observed in the COPD patients could represent an exacerbation of normal age-related oxidative stress, and thus, represent useful biomarkers of muscle involvement. Furthermore, it seems reasonable to postulate that this accelerated rate of mtDNA damage and thus, mitochondrial dysfunction, in COPD could eventually reach clinically relevant thresholds and become an important contributor to muscle dysfunction as COPD patients age.

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