ANTIOXIDANTS & REDOX SIGNALING
Volume 12, Number 3, 2010

DOI: 10.1089/ars.2009.2808

Protein Carbonylation in Skeletal Muscles: Impact on Function

Esther Barreiro[1,2,3] and Sabah N.A. Hussain[4]

Abstract

Relatively low levels of reactive oxygen species (ROS) produced inside resting skeletal muscles play important functions in cell signaling. When ROS production increases to levels beyond the buffering capacity of muscle antioxidant systems, a state of oxidative stress develops, which leads to skeletal muscle contractile dysfunction. A clear association between oxidative stress and depressed skeletal muscle performance has been described in several acute and chronic conditions, such as systemic inflammation and chronic obstructive lung diseases. The observation that the levels of oxidant-derived posttranslational protein modifications, including protein carbonylation, are elevated inside skeletal muscle fibers when oxidative stress develops suggest that these modifications play important roles in regulating muscle function. This proposal is supported by recent studies that unveiled that several myofilament (myosin heavy chain and actin), mitochondrial (aconitase, creatine kinase), and cytosolic (enolase, aldolase and glyceraldehyde 3-phosphate dehydrogenase and carbonic anhydrase III) proteins are carbonylated inside skeletal muscle fibers in many animal models of muscle dysfunction, and in humans with impaired skeletal muscle contractility. However, the functional importance of carbonylation in determining the function of muscle-specific proteins and the precise contribution of carbonylation-induced dysfunction of these proteins to overall muscle contractile deficit in various pathologies remain to be determined. *Antioxid. Redox Signal.* 12, 417–429.

Introduction

In recent years, it has become known that proteins can be modified in many ways by reactive oxygen species (ROS), which include oxygen free radicals and peroxides. Oxygen free radicals are highly reactive because of the presence of unpaired valence shell electrons and form as a natural by-product of the normal metabolism of oxygen. ROS are known to have important roles in cell signaling; however, during times of cellular stress, they culminate into a state known as oxidative stress, in which ROS levels increase so dramatically that significant structural damage may result. A number of potentially adverse reactions, commonly known as redox reactions, involve posttranslational alterations of proteins and have been specifically linked to the pathology of several physiological disorders, including degenerative diseases, aging, atherosclerosis, and skeletal muscle dysfunction (12). Typically, a redox reaction involves a parent molecule, such as the superoxide anion ($O_2^{-\bullet}$), and includes hydroxyl radicals (OH^-), hydroperoxyl radicals (HOO^-), and hydrogen peroxide (H_2O_2). Protein carbonylation is a particular type of redox reaction characterized by the addition of carbonyl groups, such as aldehydes or ketones, to proteins, and is the consequence of a cascade of several oxidative reactions. Protein carbonylation is a primary marker for oxidative stress, which is the physiological manifestation of an imbalance between an organism's production of ROS and its ability to detoxify, efficiently and effectively, the reactive intermediates that are produced, or to repair easily the resulting molecular damage.

Protein Carbonylation

Protein carbonylation is the subject of great interest, primarily because it is both irreversible and results in irreparable damage. Protein carbonylation occurs when proteins directly

[1]Pulmonology Department and Muscle Research and Respiratory System Unit, IMIM-Hospital del Mar, Parc de Recerca Biomèdica de Barcelona (PRBB); and [2]Health and Experimental Sciences Department (CEXS), Universitat Pompeu Fabra, PRBB, Barcelona, Catalonia, Spain.
[3]Centro de Investigación en Red de Enfermedades Respiratorias (CIBERES), Instituto de Salud Carlos III (ISCIII), Bunyola, Majorca, Balearic Islands, Spain.
[4]Critical Care and Respiratory Divisions, McGill University Health Centre and Meakins-Christie Laboratories, McGill University, Montreal, Quebec, Canada.

react with ROS, leading to the formation of protein derivatives or peptide fragments containing highly reactive carbonyl groups, such as aldehydes and ketones. In addition, secondary reactions of primary amino groups of lysine residues with reducing sugars or their oxidation products, known as glycation or glycoxidation reactions or both, also generate reactive carbonyls in proteins (78). Although it has long been recognized that exposure of proteins, nucleic acids, and lipids to high-energy radiation induces biologic damage, and that modifications in the side-chains of proteins, the formation of protein aggregates *via* cross-linking reactions, and the cleavage of peptide bonds have been produced by radiolysis-induced free radical generation (77), radiolysis *per se* is not a major source of ROS production *in vivo*. Relatively large levels of ROS, however, are generated *in vivo* in response to high O_2 tension, during ischemia/reperfusion, and in response to proinflammatory stimuli (79). Protein oxidation of this sort is usually mediated through metal ion–catalyzed reactions of the Fenton and Haber–Weiss types.

Oxidation of the side chains of lysine, arginine, proline, and threonine residues have also been shown to yield carbonyl derivatives, whereas histidine residues are converted to 2-oxo-histidine. In the case of lysine, the chelate complex formed by the binding of Fe(II) to the amino group of lysine can react with hydrogen peroxide to produce a hydroxyl radical that causes the conversion of the lysine moiety to a 2-aminoadipic-semialdehyde residue. Similar reactions of Fe(II) with other amino acid targets also lead to the formation of carbonyl derivatives. Observation of the inhibition achieved by catalase of metal-catalyzed reactions further supports this site-specific mechanism of protein oxidation. However, hydroxyl scavengers do not inhibit such reactions, probably because they cannot compete with reactions of hydroxyl radicals with amino acids at the metal-binding site (78). The importance of this site-specific mechanism of amino acid oxidation has been underscored in several studies, in which oxidation of arginine, proline, and lysine residues to aldehyde derivatives have been shown to account for all of the protein carbonyl groups formed in the oxidation of glutamine synthetase by metal-catalyzed systems *in vitro*, and for ~50–60% of the carbonyl groups detected in proteins from rat liver extracts (65).

Protein carbonylation can also be the result of Michael addition reactions of lysine, cysteine, or histidine residues with α,β-unsaturated reactive aldehydes generated during the peroxidation of polyunsaturated fatty acids (78). For instance, 4-hydroxy-2-nonenal (HNE) reacts with lysine, cysteine, and histidine residues of proteins to form Michael adducts that can be stabilized and further detected by using selective antibodies (78). Moreover, malondialdehyde (MDA) reacts with lysine residues to form Schiff base adducts that can also be detected in tissues with antibodies (66). Carbonylation arising from covalent attachment by HNE and MDA is designated, in this review, as reactive carbonyl species (RCS)-derived protein carbonylation. Other forms of protein oxidation include the oxidation of aromatic amino acid residues, cyclic oxidation and reduction of methionine, protein–protein cross linkage, chlorination reactions, oxidation of free amino acids, and modifications of proteins induced by reactive nitrogen species (RNS).

In recent decades, several methods have been developed to measure oxidative stress-induced cellular damage in tissues. As protein carbonyl levels are considerably greater than are other products of oxidative modification of proteins, the carbonyl assay is the most commonly used, simple, and reliable method of quantification (67). By using several techniques, such as enzyme-linked immunosorbent assay (ELISA), immunoblotting, and immunohistochemistry, the carbonyl assay enables the detection of reactive carbonyl groups after their reaction with 2,4-dinitrophenylhydrazine (DNPH) to form 2,4-dinitrophenylhydrazone (DNP) moieties. Although other oxidative modifications may also occur in tissue or cell proteins, the simplicity, reliability, and convenience of the carbonyl assay makes it extremely useful and meaningful as an index of total protein oxidation in biologic systems. Differences in the protein carbonyl content between several samples reflect the level of resistance or susceptibility to oxidative stress.

ROS Production Within Skeletal Muscle Fibers

Reactive oxygen species (ROS) are normally produced at relatively low levels inside skeletal muscle fibers. ROS are derived from the superoxide anion (O_2^-). In normal skeletal muscle fibers, ROS play a positive role in maintaining muscle contractility and in regulating various signaling pathways (64). However, increases in the production of ROS to levels that are significantly greater than those that can be neutralized by intracellular antioxidant defenses lead to the development of a state of oxidative stress, which has profound effects on action-potential conduction, excitation–contraction coupling, contractile proteins, and mitochondrial respiration (64). Elevated levels of ROS generated inside skeletal muscle fibers in response to proinflammatory conditions have also been shown to cause severe depression of muscle contractile performance (74). It should be emphasized that skeletal muscle fibers possess strong antioxidant systems that protect the myocytes from potential ROS-induced deleterious effects. For instance, the antioxidants CuZn-superoxide dismutase (SOD), catalase, and glutathione peroxidase are present in the sarcoplasm, whereas Mn-SOD and glutathione peroxidase are localized within the mitochondrial matrix (Fig. 1). Other thiol-based antioxidant proteins, such as thioredoxins and peroxiredoxins, are also abundantly expressed inside skeletal muscle fibers (28). Moreover, nonenzymatic antioxidant-system components such as vitamin E, carotenes, ubiquinol, ascorbic acid, urate, lipoate, and glutathione, the most abundant nonprotein thiol, also contribute to antioxidant defenses inside muscle fibers (28) (Fig. 1).

The main sources of ROS generation inside skeletal muscle fibers are the mitochondria, xanthine oxidase and NADPH oxidases. The mitochondria have long been considered to be a major source of ROS generation inside skeletal muscle fibers, as 5% of molecular O_2 is reduced to O_2^- as a result of electron leaks from the respiratory chain. However, recent estimates of the electron flow that gives rise to ROS generation by the mitochondria has been shown to be <10% of the original minimal estimate, suggesting that mitochondria do not produce considerable amounts of ROS under physiologic conditions (76). This notion is also supported by the fact that ROS generation by skeletal muscle mitochondria is regulated by intrinsic control mechanisms that involve several uncoupling proteins, especially uncoupling protein-3 (UCP-3) (16). However, strong evidence indicates that ROS generation by

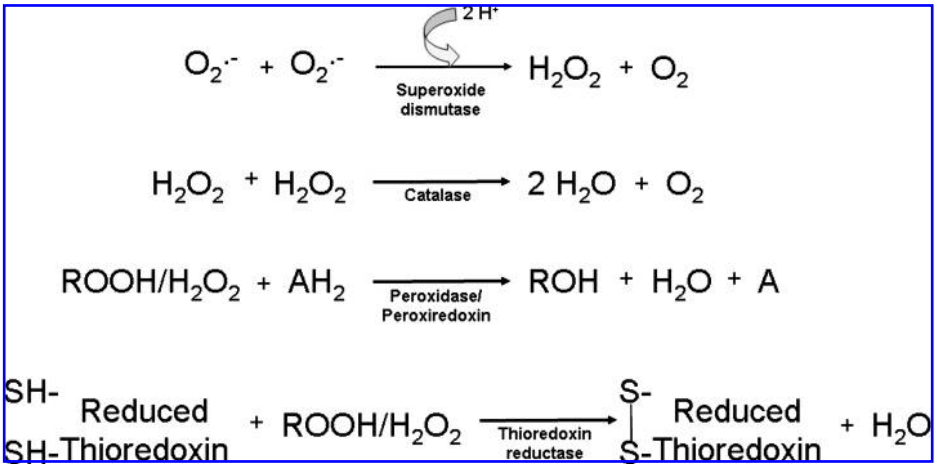

FIG. 1. Schematic of the antioxidant systems in skeletal muscles.

the mitochondria is elevated inside skeletal muscle fibers in several pathologic conditions and that mitochondrial proteins might be targeted for oxidation by ROS.

Another important source of ROS generation inside skeletal muscle fiber is xanthine oxidase. Under physiologic conditions, hypoxanthine and xanthine are oxidized to uric acid by xanthine dehydrogenase. However, under ischemic or hypoxic conditions, xanthine dehydrogenase is converted to xanthine oxidase, which preferentially reduces molecular O_2 to O_2^- and H_2O_2 (26). During exercise, both hypoxia and increased levels of xanthine and hypoxanthine cause significant elevation of xanthine oxidase activity, which may contribute to protein oxidation and lipid peroxidation. Moreover, several recent studies have confirmed that xanthine oxidase–derived ROS contribute significantly to oxidative protein modifications, including carbonyl formation inside skeletal muscle fibers (36, 37, 85).

NADPH oxidase is an enzyme complex that was first described in phagocytes, where it consists of four essential subunits ($p22^{phox}$, $gp91^{phox}$ (Nox2), $p47^{phox}$, and $p67^{phox}$) and two additional subunits ($p40^{phox}$ and Rac2). NADPH oxidase is also expressed in nonphagocytes, in which it produces ROS under basal conditions. However, on stimulation, ROS are produced intracellularly at much lower levels than they are in phagocytes. Recent studies have confirmed the presence of $p22^{phox}$, Nox2, $p47^{phox}$, and $p67^{phox}$ subunits in skeletal muscle samples and have implicated these subunits in the regulation of Ca^{2+} influx and insulin signaling (32, 35). These studies clearly suggest that NADPH oxidase–derived ROS play important physiologic roles in regulating skeletal muscle signaling. However, the extent to which NADPH oxidase–derived ROS contribute to protein carbonylation and other oxidative modifications of proteins inside skeletal muscle fibers, and how they relate to various pathologies, remains to be investigated.

Skeletal Muscle Activation and Protein Carbonylation

It has been well established that physical exercise triggers an imbalance between ROS levels and antioxidant systems, resulting in the development of oxidative stress and oxidative modification of proteins, including protein carbonylation. The degree of exercise-induced muscle protein carbonylation is highly dependent on the duration, intensity, and the type of exercise being performed. For instance, an early report by Reznick *et al.* (68) documented 8 and 17% increases in protein carbonylation in the white quadriceps and gastrocnemius muscles, respectively, in rats undergoing one bout of exhaustive treadmill exercise. These elevations were associated with substantial declines in muscle lipophilic antioxidants. The same authors also observed that certain limb muscles do not show an increase in protein carbonylation during exercise. For instance, red quadriceps muscles show no evidence of enhanced carbonyl formation. This phenomenon was attributed to the abundance of antioxidants in muscles rich in oxidative muscle fibers, as is the case with the red quadriceps muscle. This notion is also supported by observations that antioxidant supplementation, such as through a high–vitamin E or high–palm oil diet, is able to attenuate skeletal muscle carbonyl formation, both at rest and after exercise (68).

Other forms of physical exercise (for example, swimming) have also been shown to elicit augmentation of protein carbonylation both in the plasma and inside skeletal muscle fibers (84). In addition, in an early report, Saxton and colleagues (71) observed that acute concentric knee exercise in humans results in greater levels of skeletal muscle protein carbonyl formation, measured immediately after the cessation of exercise, than do those that are produced with eccentric exercise. Enhanced protein carbonyl formation has also been observed in the plasma of humans after several days after the cessation of exercise (75).

Exercise-induced protein carbonylation in response to acute muscle activation is not limited to the limb muscles. Strong activation of the respiratory muscles, triggered by respiratory loading of a magnitude similar to that observed in patients with chronic obstructive pulmonary disease (COPD), or to that used in respiratory training programs, may induce sarcolemmal and sarcomere damage and increase levels of ROS production (4, 47).

Measurements inside the diaphragms of dogs undergoing acute respiratory resistive loading revealed that the extent of muscle protein carbonyl formation is directly related to the intensity of respiratory muscle contraction, with a significant increase in diaphragm protein carbonylation in

animals exposed to inspiratory respiratory loads that exceed 40% of maximum (5). Administration of the antioxidant *N*-acetylcysteine (NAC) also significantly reduces diaphragm protein carbonylation, further emphasizing the importance of antioxidants in regulating the levels of muscle protein carbonylation (5).

Alterations in skeletal muscle protein carbonylation were also in the limb and respiratory muscles in response to prolonged increases in muscle activation. For instance, Witt and colleagues (87) examined protein carbonyl formation in limb muscles and livers of rats undergoing 12 weeks of exercise training. They reported a twofold increase in skeletal muscle protein carbonylation, whereas liver protein carbonyl levels remained unchanged. Similar findings have been reported by other investigators (61). In the respiratory system, Supinski *et al*. (81) described an ~2.5-fold increase in protein carbonylation, and a similar increase in lipid peroxidation, in the diaphragms of rats exposed to severe resistive loading for 4–12 days.

In yet another model of high-intensity exercise in rats, carbonylation levels of Ca^{2+}-ATPase of the sarcoplasmic reticulum (SERCA2) have been shown to be significantly greater than those in control animals and are inversely correlated with the function of this enzyme (48). Furthermore, the intensity of carbonylation of SERCA2 decreases progressively toward control levels after 1 h of recovery from exercise, indicating that exercise-induced oxidation of this enzyme is a transient phenomenon (48).

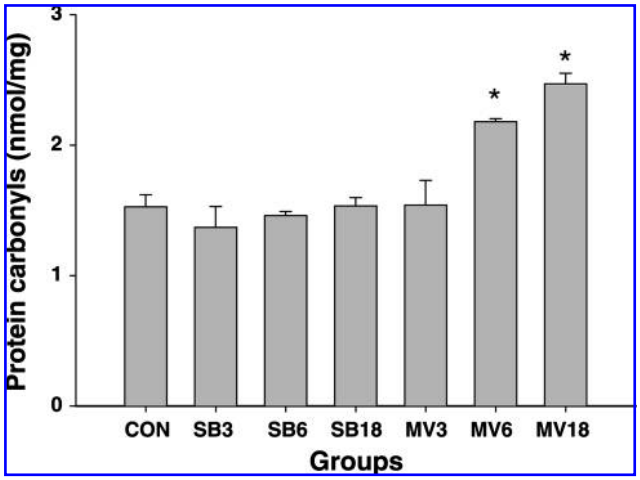

FIG. 2. Influence of mechanical ventilation on protein carbonylation in the diaphragm of rats. Spectrophotometric analysis of the accumulation of total reactive carbonyl derivatives (RCDs) in diaphragmatic insoluble proteins in all seven experimental groups. Group 1 is control. Groups 2, 3, and 4 are animals that were spontaneously breathing for 3, 6, and 18 h, respectively. Groups 5, 6, and 7 are animals that were mechanically ventilated for 3, 6, and 18 h, respectively. SB = Spontaneous breathing. MV = Mechanical ventilation. Values are expressed as mean ± SEM. *Significantly different ($p < 0.05$) from Con, SB6, and SB18. Reprinted with permission from Zergeroglu *et al*. (88).

Immobilization Muscle Dysfunction and Protein Carbonylation

It has been well established that reduced contractile activity results in muscle atrophy as a consequence of increased proteolysis and decreased protein synthesis. Clinical situations that require prolonged bed rest and respiratory or limb-muscle immobilization lead to disuse muscle atrophy. Moreover, the resulting muscle-mass loss may be worsened in special populations such as the elderly and very small children. Oxidative stress is just one among many factors that are involved in triggering the enhanced protein breakdown that leads to immobilization-induced muscle atrophy. Several early reports proposed that the imbalance between ROS generation and antioxidant defense during muscle immobilization is due to an increase in cytosolic ROS generation, primarily because of a reduction in the expression and activities of catalase and glutathione peroxidase, enhanced xanthine oxidase activity, and attenuation of antioxidant scavenging capacity (41, 43). A clear case of the association between skeletal muscle immobilization and protein carbonylation inside muscle fibers has been illustrated in the diaphragms of rats and humans undergoing mechanical ventilation. Inactivation of the diaphragm leads to contractile dysfunction, which, in turn, coincides with increased ROS generation and enhanced protein degradation, particularly by the proteasomal pathway (54).

Shanely *et al*. (73) were the first to report that 18 h of controlled mechanical ventilation in rats elicits rapid and progressive atrophy of the diaphragm due to increased protein degradation, a situation that is accompanied by 44 and 53% increases in the levels of protein carbonyls and 8-isoprostane, respectively, where 8-isoprostane is an index of lipid peroxidation. In a subsequent study by the same group, a modest but significant increase in protein carbonylation in the diaphragm was detected after only 6 h of controlled mechanical ventilation (88) (Fig. 2). The authors proposed that actin and myosin heavy chain are the main targets for carbonyl formation inside muscle fibers of mechanically ventilated diaphragms. The importance of the imbalance between ROS generation and antioxidant defense is underlined by the observation that administration of the antioxidant trolox significantly attenuated mechanical ventilation–induced proteolysis, contractile dysfunction, and the amount of myofilament protein available for the proteasome pathway in rat diaphragms (14, 50).

Whidden *et al*. (85) recently administered oxypurinol to assess the contribution of xanthine oxidase to protein carbonylation in the diaphragm of mechanically ventilated rats and reported that, although oxypurinol therapy provides protection against mechanical ventilation-induced contractile dysfunction, it fails to eliminate completely the increases in protein carbonylation, suggesting that other sources of ROS generation are likely to be involved. Indeed, recent studies confirmed that both the mitochondria and NADPH oxidase contribute to ROS generation and that a modest increase in total carbonyl formation and RCS-derived protein carbonylation can be detected in the mitochondria of mechanically ventilated diaphragms (38, 49).

Protein Carbonylation in Skeletal Muscles of Patients with Chronic Obstructive Pulmonary Disease (COPD)

It has become increasingly evident that COPD is a systemic disease in which several extrapulmonary manifestations,

including skeletal muscle dysfunction, contribute to morbidity and mortality. Skeletal muscle dysfunction in COPD patients is characterized by reductions in muscle strength and endurance, atrophy, or losses in muscle mass, fiber-type redistribution, in which proportions of oxidative fibers are decreased and glycolytic fibers are increased, with decreased oxidative metabolic capacity, and decreased capillary density (13; 86). In addition to inactivity and sedentary lifestyle, many local and systemic factors have also been implicated in skeletal muscle dysfunction in COPD patients. Local factors include the production of ROS and RNS and enhanced protein degradation inside muscle fibers. Systemic factors include systemic inflammation, malnutrition, corticosteroid use, hypoxemia, aging, and smoking (40).

Oxidative stress has been documented in the plasma, urine, and limb muscles of COPD patients, whether at rest or after exercise. At rest, the quadriceps muscles of patients with moderate to severe COPD and normal body weight have been shown to be significantly reduced in total glutathione concentrations and to have elevated concentrations of lipofuscin (a product of lipid peroxidation) (1, 6).

As a means of assessing the degree of protein carbonylation in the muscles of COPD patients, several authors used immunoblotting and observed strong carbonylation of five protein bands, with molecular masses ranging from 27 to 68 kDa, in the resting quadriceps muscles (21). Similar results were reported by Barreiro *et al*. (6). It should be emphasized that neither study found a difference in the overall intensity of protein carbonylation in muscles of COPD patients or control subjects, despite the presence of a >300% increase in RCS-derived carbonylation (HNE-protein adduct formation). This suggests that, in the absence of severe muscle wasting, membrane lipid peroxidation is selectively increased in COPD patients. However, this is not the case in patients with severe COPD and significant quadriceps muscle wasting, in whom the intensity of ROS-derived protein carbonylation, measured with selective ELISA and immunoblotting, is significantly elevated and correlates negatively with quadriceps isometric force and total exercise capacity (Vo_{2max}) (10). Enhanced protein carbonylation in atrophied quadriceps muscles of patients with severe COPD is not mediated by increased proinflammatory cytokine expression (11). Barreiro *et al*. (8) used a proteomic approach involving two-dimensional electrophoresis and mass spectrometry to identify the targets of protein carbonylation in quadriceps muscles of patients with moderate to severe COPD and normal body weight. Their results revealed that mitochondrial creatine kinase (CK) and carbonic anhydrase III (CAIII) are the main carbonylated proteins in both COPD patients and control subjects. CK catalyzes the reversible transfer of a phosphoryl group from ATP to creatine to produce ADP and phosphocreatine and is localized in the mitochondria and the cytosol. Its activity is coupled to myosin ATPase, sarcolemmal Na/K ATPase, and SERCA2. CAIII is a zinc metallo-enzyme that catalyzes the reversible hydration of CO_2, has carboxyl esterase and tyrosine phosphatase activities, and is involved in carbohydrate utilization. Barreiro *et al*. (8) revealed that total CK activity and CK protein expression, as well as the intensity of CK carbonylation, are significantly greater in quadriceps muscles of COPD patients, as compared with control subjects. In addition, they reported that the intensity of carbonylation of CK correlated negatively with its activity, suggesting that carbonylation may results in inhibition of enzyme activity (Fig. 3). In contrast, CAIII protein expression and its carbonylation intensity were similar in the two groups (8). Thus, limb muscles of COPD patients may undergo selective upregulation of CK expression; however, the functional implications of changes in ROS-derived protein carbonylation intensity and expression remain to be investigated.

In addition to examining the resting state, several investigators have addressed the influence of exercise and exercise training on the development of oxidative stress in limb muscles of patients with moderate to severe COPD. Elevated levels of ROS-induced DNA damage in peripheral blood mononuclear cells, plasma uric acid, blood oxidized glutathione, and lipid peroxides have been observed at the cessation of whole-body exercise, suggesting that these patients experience systemic oxidative stress, even during normal daily activity (31, 51). Moreover, Couillard *et al*. (21) used measurements of carbonyl formation, as detected with immunoblotting, to suggest that exercising limb muscles might be an important source of ROS products released into the plasma.

Measurements of protein-carbonylation intensity have also been used to evaluate the effects of endurance training on the redox status of limb muscles of COPD patients. Endurance training has been shown to elicit beneficial effects on skeletal muscle bioenergetic and exercise performance and has long been considered an essential element in pulmonary rehabilitation programs. However, it remains unclear as to whether exercise training at 60 and 70% of peak work rate, which is required to improve skeletal muscle oxygen transport and mitochondrial oxidative capacity, would worsen or reduce oxidative stress in the skeletal muscles of COPD patients (46, 70). Barreiro *et al*. (10) very recently addressed this issue by measuring the influence of 3 weeks of endurance training on the intensities of total and RCS-derived (HNE-protein adduct formation) protein carbonylation in the quadriceps of patients with severe COPD. Their results indicate that endurance training selectively augments RCS-derived protein carbonylation, suggesting that the elevated ROS concentrations generated during prolonged exercise selectively target membrane lipids in various muscles. A milder form of endurance training, with magnetic stimulations of the quadriceps muscles of

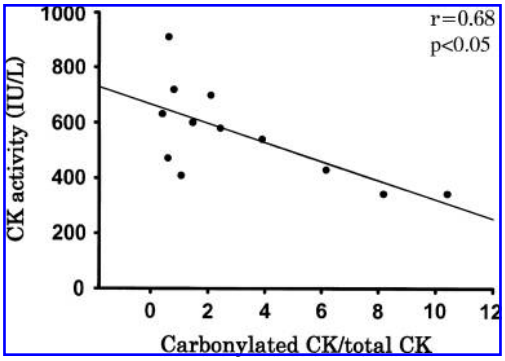

FIG. 3. Correlation analysis between creatine kinase (CK) activity and the intensity of CK carbonylation (normalized per total CK level) in the vastus lateralis muscle of patients with COPD. Reprinted with permission from Barreiro *et al*. (8).

patients with severe COPD over an 8-week training period, resulted in a significant increase in the size of oxidative (myosin heavy chain type I) fibers, but did not alter the intensity of protein carbonylation (19). This study suggests that magnetic stimulation is an appropriate and a promising tool to be used in clinical settings for improving the oxidative capacity of limb muscles of COPD patients.

It should be emphasized that skeletal muscle dysfunction in COPD differs between various muscle groups. For instance, lower-limb muscles, owing to disuse or deconditioning, are more adversely affected than are upper-limb and ventilatory muscles. Moreover, the ventilatory muscles in COPD patients exert different workloads than do limb muscles. The diaphragm, for instance, is in a chronically overloaded state because of the increased work of breathing. Such loading-pattern differences are likely behind the specific biochemical and structural adaptations that have been described in the ventilatory muscles of COPD patients, including increased proportions of type I fibers, shortening of sarcomere lengths, increased mitochondrial densities, and enhanced ATPase activity (44, 45, 56). Yet despite these adaptive changes, maximal diaphragm strength in COPD patients remains less than 50% of that measured in control subjects, and the activity of the proteasomal pathway remains significantly greater than it does in control diaphragms (57).

In contrast to the amount of research focusing on limb muscles, little information is as yet available regarding alterations in the redox status of the diaphragm in COPD patients. Barreiro *et al*. (4) reported for the first time that total carbonyl formation and RCS-derived protein carbonylation are significantly elevated in the diaphragms of patients with severe COPD, as compared with control subjects (Fig. 4). They also reported that negative correlations exist between carbonylation intensity and the degree of airway obstruction, and between the degree of RCS-derived carbonylation (HNE-protein adduct formation) and ventilatory muscle strength (4) (Fig. 4).

In a subsequent study, Marrin-Corral *et al.* (47) used a proteomic approach similar to the one described earlier to identify myosin heavy chain, sarcomeric actin, CK, and CAIII as the main carbonylated proteins in the human diaphragm. Myosin heavy-chain carbonylation increased by fivefold in the diaphragms of severe COPD patients, as compared with

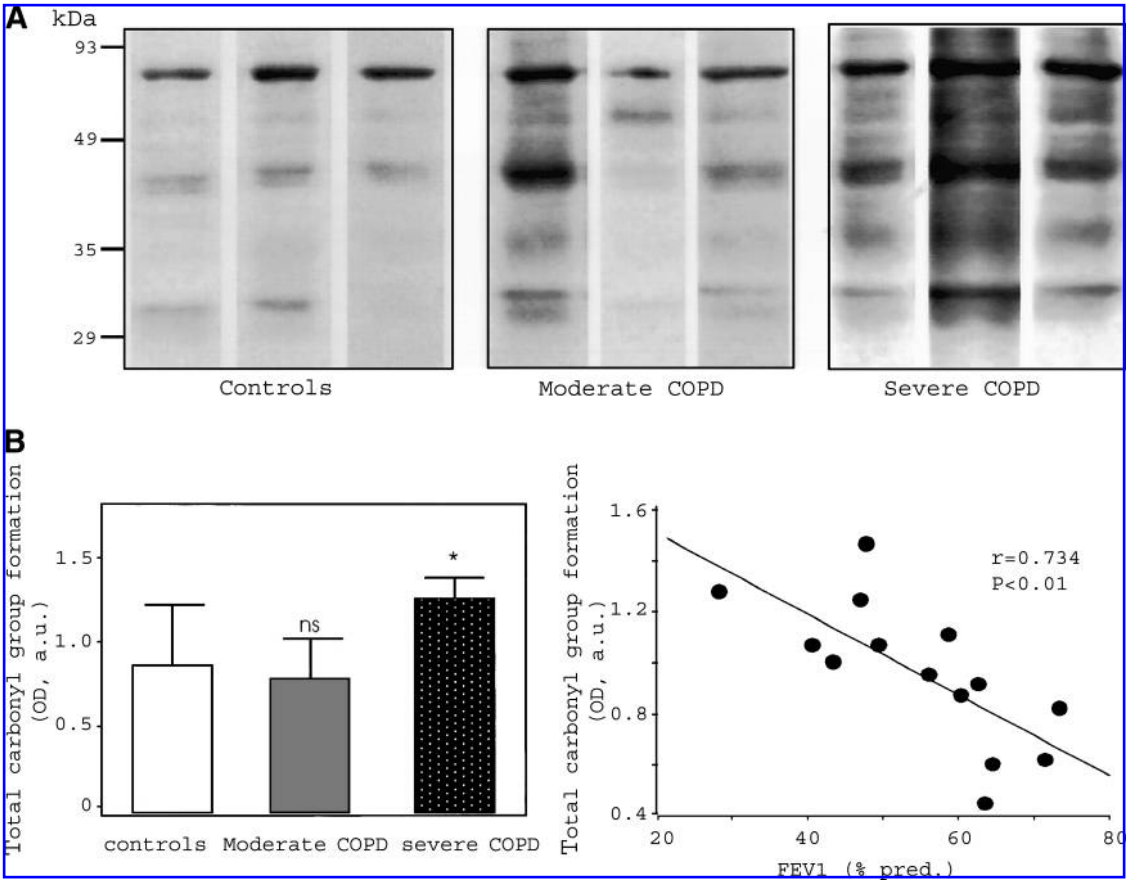

FIG. 4. (A) Representative examples of protein oxidation (total carbonyl groups) in diaphragms of control subjects and patients with moderate and severe chronic obstructive pulmonary disease (COPD). Several protein-carbonylated bands of different molecular weights (MWs) were detected. (**B**) Mean values ± SD of total carbonyl formation were higher in the muscles of patients with severe COPD compared with control muscles (*$p = 0.05$). Total diaphragmatic carbonyl formation did not differ between patients with moderate COPD and control subjects (ns = nonsignificant). Among the overall patients with COPD, optical densities of total carbonyl group formation significantly correlated with FEV_1 (percentage predicted). Note that 14 patients with COPD are depicted (two mild, six moderate, and six severe) in the correlation graph. Reprinted with permission from Barreiro *et al*. (4).

control subjects. They (47) also found that carbonylation levels of CK are elevated in the diaphragms of COPD patients, whereas carbonylation levels of sarcomeric actin and CAIII are unchanged between COPD patients and control subjects. Moreover, protein carbonylation in the diaphragms of COPD patients appears to be derived mainly by ROS generated by the mitochondrial and membrane compartments (47). These results indicate that, as in the quadriceps muscles, excessive ROS generation inside the diaphragm of patients with severe COPD results in oxidative modifications of proteins, and that specific proteins, including those involved in ATP production, myofilament contraction, and CO_2 hydration, are the main targets of ROS action. The functional significance of this increased carbonylation to the regulation of myofilament interaction and overall diaphragm contractility and endurance remains to be determined. Indirect evidence suggests that carbonylation of specific residues is associated with significant impairment of enzyme function, particularly in the case of CK.

Skeletal Muscle Protein Carbonylation in Patients with Obstructive Sleep Apnea

Obstructive sleep apnea syndrome (OSAS) is a highly prevalent condition characterized by recurrent episodes of upper airway obstruction during sleep. During apneic episodes, arterial saturation levels decrease precipitately, and the ventilatory muscles are strongly recruited. The diaphragm appears to be the primary ventilatory muscle that is recruited in some patients with OSAS, whereas the intercostal muscles are recruited in others. Repeated cycles of strong muscle contraction coupled with hypoxia/reoxygenation have been proposed as the main factors responsible for depressed inspiratory muscle endurance in these patients. The development of oxidative stress and posttranslational modifications of proteins in the ventilatory muscles and the correlation between these modifications and the severity of OSAS remain unclear.

In a recent study, Barreiro *et al.* (9) examined external muscle biopsies from OSAS patients for indices of oxidative modification and evaluated the influence of continuous positive airway pressure (CPAP) therapy on these modifications. They described a 25% increase in total protein carbonyl formation and an even larger increase in the intensity of RCS-derived (MDA and HNE) protein carbonylation in the external intercostals of patients with OSAS, as compared with control subjects. The degree of RCS-mediated protein modifications correlated negatively with inspiratory muscle endurance and positively with the severity of the disease (apnea/hypopnea index) (Fig. 5). Treatment with CPAP had no influence on oxidative modifications. This study provides the first evidence of the development of oxidative stress in the external intercostal muscles of OSAS patients and suggests that strong and repeated contractions of these muscles during numerous apneic episodes each night are largely responsible for an imbalance between ROS production and intrinsic muscle antioxidant defense. Several questions remain unanswered, however. For instance, it is unclear whether other inspiratory muscles, including the diaphragm, also develop oxidative stress. The extent to which a moderate increase in protein oxidative modification in the external intercostal muscles alters contractile performance is also unclear.

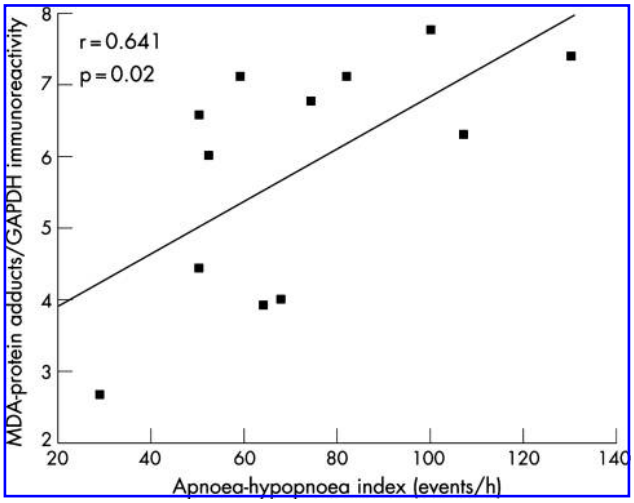

FIG. 5. The correlation between the ratios of the optical densities of total MDA-protein adducts to those of GAPDH of the external costal muscles and the apnea–hypopnea index (events/hour) in patients with obstructive sleep apnea syndrome. Reprinted with permission from Barreiro *et al.* (9).

Muscle Protein Carbonylation and Ischemia/Reperfusion Injury

Skeletal muscle ischemia develops as a complication of several pathologies, including acute arterial thrombosis or embolism, trauma-induced vascular damage, and chronic atherosclerotic vascular insufficiency. When ischemic muscles are reperfused with oxygenated blood, a cascade of events known as reperfusion injury develops. Reperfusion injury is characterized by substantial increases in ROS production that cause lipid peroxidation of cell membranes, oxidative modifications of proteins, increased proteolysis, and DNA fragmentation, factors that eventually lead to the disruption of cellular integrity and the inhibition of energy production (18).

Increased superoxide anion and hydroxyl radical production by xanthine oxidase has been suggested to play a major role in the development of reperfusion injury (18). Xanthine oxidase is usually found as xanthine dehydrogenase in normally perfused tissues. It is usually transformed into the ROS-generating form in ischemic tissues, a transformation that is dependent on muscle type and the duration of ischemia. Many reports documented significant augmentation of protein carbonyl formation in response to ischemia/reperfusion in both skeletal and cardiac muscles. In the majority of these studies, it was shown that ischemia alone is not a sufficient cause of increased protein carbonylation. A recent study by Ozyurt *et al.* (58), however, implicated increased xanthine oxidase activity in eliciting a twofold increase in limb-muscle protein carbonyl contents in response to 2-h ischemia and 2-h reperfusion of the hindlimb of rats. In another study, a shorter period of reperfusion (45 min) of ischemic hindlimbs elicited no significant alterations in skeletal muscle carbonyl formation, despite the development of oxidative stress. These results emphasize the importance that reperfusion duration has with respect to the augmentation of protein carbonyl formation in limb muscles (30).

In heart muscles and in cultured cardiac myocytes, however, significant increases in protein carbonyl formation occur in response to ischemia/reperfusion injury (39, 62). Interestingly, protein oxidation of heart muscle appears to be elicited by relatively shorter periods of reperfusion, as compared with skeletal muscle. Myofibrillar proteins appear to be the major targets of protein oxidation by ROS during ischemia/reperfusion of the heart. In isolated rat hearts, actin carbonylation increased by 80% in response to ischemia/reperfusion (62). Augmentation of actin carbonylation also was associated with significant depression of postischemic contractile function (62). Actin, desmin, and tropomyosin have also been shown to be targeted by ROS and significantly carbonylated in response to 30 min of ischemia and 3 min of reperfusion in isolated rat hearts (20). Oxidation of these myocardial myofibrillar proteins is attenuated by the administration of antioxidants (20).

In addition to targeting myofibrillar proteins, ROS also target mitochondrial proteins during ischemia/reperfusion. In isolated rat hearts, carbonyl contents of multiple unidentified mitochondrial and nonmitochondrial proteins are significantly elevated in response to global ischemia/reperfusion, and the enhanced myocardial protein carbonylation is associated with significant attenuation of mitochondrial respiration (39). It is worth noting that the development of oxidative stress and enhanced protein oxidation in response to ischemia/reperfusion is mediated by both enhanced ROS production during the perfusion period and reduced antioxidant capacity inside muscle fibers. One important antioxidant system that appears to be important in modulating muscle protein oxidation during ischemia/reperfusion is thioredoxin-1. Recently, the importance of this system was emphasized by the observations that exposure to intermittent hypoxia for a period of 2 weeks, before ischemia/reperfusion injury, results in a twofold augmentation of protein carbonylation and a threefold increase in lipid peroxidation of myocardial proteins (59). These responses were attributed, in part, to the inhibition of thioredoxin-1. Taken together, these studies on myofibrillar and mitochondrial protein targets of ROS suggest that oxidation plays a major role in the postischemic contractile dysfunction of the myocardium.

Protein Carbonylation and Muscle Dysfunction in Sepsis

Early observations and subsequent studies confirmed that skeletal muscles, in general, and the diaphragm, in particular, develop severe contractile dysfunction in response to systemic inflammatory conditions, such as severe sepsis, septic shock, peritonitis, and bacterial pneumonia (15, 34, 42). Although many sites inside muscle fibers are likely to be affected, sarcolemmal-related processes, such as conduction of action potentials and excitation/contraction coupling, and various myofilament proteins are particular targets of ROS during sepsis. Numerous studies have confirmed that sepsis-induced muscle injury is mediated largely by an increase in ROS levels. For instance, Peralta *et al*. (60) reported a doubling of limb ROS levels in septic rats, which coincides with a significant decline in antioxidant capacity. Moreover, lipid peroxidation has been reported to increase by 200% in the diaphragm of LPS-injected animals (74).

The importance of oxidative stress is underscored by several reports that have shown that administration of ROS scavengers prevents reduction in muscle-force generation in animal models of infection and systemic inflammation (74, 80). In addition, several authors have documented a significant increase in tissue markers of protein carbonylation in skeletal muscles of animals with systemic inflammation and sepsis (27, 33). Protein carbonylation appears to be a particularly sensitive index of ROS-mediated protein modifications inside the diaphragm, where it increases by more than sixfold after 24 h of administration of bacterial lipopolysaccharide in rats (2). The importance of these modifications in mediating contractile dysfunction of the ventilatory muscles is underlined by the observation that administration of ROS scavengers to animals with sepsis prevents oxidative modifications in the diaphragm in parallel with preservation of contractile function (74, 83).

Barreiro *et al.* (2) revealed that inhibition of the activity of heme oxygenases, which are enzymes that are involved in antioxidant defenses and in regulating heme metabolism, is associated with increasing total protein carbonyl formation and RCS-mediated carbonylation (HNE-protein adduct formation) in the diaphragms of septic animals. Such inhibition also worsens the contractile dysfunction of this muscle, indicating that heme oxygenases play important roles in attenuating the deleterious effects of ROS on proteins and lipids, as well as in preserving muscle function in sepsis. Barreiro *et al.* (7) also recently identified the nature of carbonylated and HNE-modified proteins inside the diaphragm of septic rats. They reported an increase in protein carbonylation inside the diaphragm of 300% within 12 h of the induction of septic shock (Fig. 6). They also identified the involvement of enolase 3β, aldolase, and glyceraldehyde 3-phosphate dehydrogenase, CK, CAIII, sarcomeric α-actin, and ubquinol-cytochrome *c* reductase in increased protein carbonylation

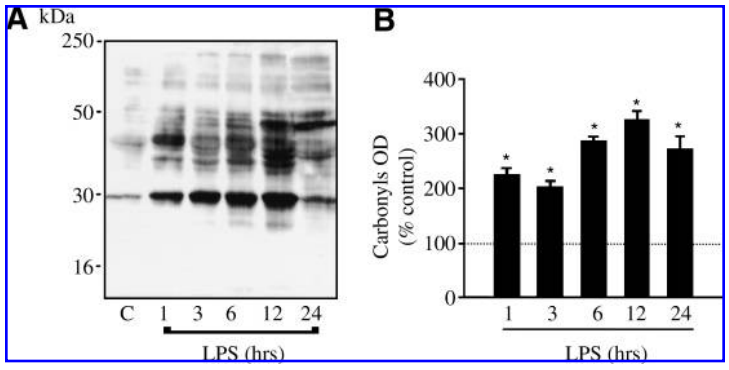

FIG. 6. (A) A representative immunoblot showing the time course of protein carbonylation in crude diaphragm homogenates detected with the Oxyblot kit during the course of sepsis in rats. C refers to control rats (injected with saline and killed 24 h later). LPS = *E. coli* lipopolysaccharide. Animals were injected IP with LPS, and the diaphragm was collected after 1, 3, 6, 12, and 24 h. (**B**) Mean values ($n = 5$ in each group) of total carbonyl OD (expressed as percentage of control rats) in crude diaphragm homogenates during the course of sepsis in rats. *$p < 0.05$ as compared with control values. Reprinted with permission from Barreiro *et al*. (7).

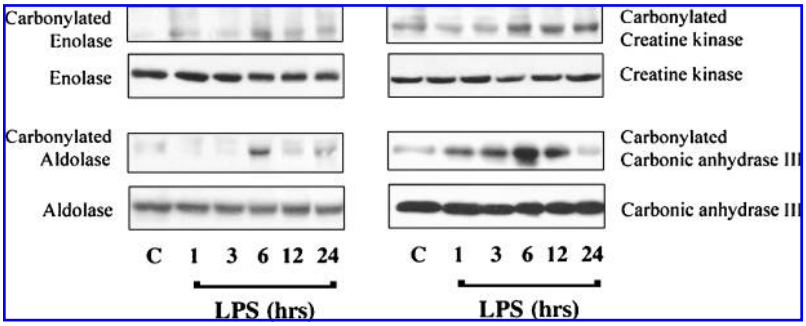

FIG. 7. Representative examples of the changes in protein carbonylation of enolase, aldolase, creatine kinase, and carbonic anyhdrase III in the cytosolic fraction of rat diaphragms during the course of sepsis. Abbreviations are identical to those in Fig. 5. Protein carbonyl levels were measured with the Oxyblot kit, whereas specific protein levels were measured with selective primary antibodies. Reprinted with permission from Barreiro *et al*. (7).

levels (7). Of interest, the expressions of these proteins were not altered during the course of septic shock, although carbonyl contents increased significantly 1 h after induction of septic shock (7) (Fig. 7). Results also revealed that the activities of CK and aldolase in septic diaphragms correlated negatively with their carbonyl contents, suggesting that this type of oxidative modification may alter enzymatic function (7).

In a second study, Hussain *et al.* (33) determined that HNE-protein adduct formation (RCS-mediated protein carbonylation) in the diaphragm of septic animals peaks within 3 h of sepsis, suggesting that enhanced lipid peroxidation occurs earlier than other sources of protein carbonyl formation. Their analysis of the nature of HNE-modified proteins in the diaphragm of septic rats reveals that, in addition to the previously described enzymes, triosephosphate isomerase 1, aconitase 2, electron-transfer flavoprotein-ß, and dihydrolipoamide dehydrogenase are modified by HNE (33). These results indicate that oxidant-derived protein modifications inside the diaphragms of septic animals involve myriad myofilament, cytosolic, and mitochondrial proteins that are involved in various critical processes, including glycolysis and ATP production. To assess the direct influence of HNE-derived protein modification on enzyme function, Hussain *et al.* measured the *in vitro* activity of purified enolase in the presence of increasing concentrations of HNE (33). Results suggest that HNE elicits a significant dose-dependent inhibition of enolase activity, confirming the widely held notion that oxidative modifications elicit deleterious effects on enzyme function in a wide range of proteins (Fig. 8).

Protein Carbonylation and Cancer Cachexia

In advanced malignant diseases, cachexia is one of the most common systemic manifestations. It often implies a poor prognosis. A clear association between oxidative stress and muscle wasting has been documented in cultured skeletal muscle cells. Exposure of these cells to ROS-generating enzymes or H_2O_2 results in downregulation of myosin expression and significant induction of protein catabolism coupled with enhanced activity of the proteasomal pathway (17, 29). Administration of the proinflammatory cytokine tumor necrosis factor alpha (TNF-α) *in vivo* also was shown to induce both significant muscle wasting and induction of ROS production, whereas administration of antioxidants attenuates both muscle wasting and the levels of ROS (17). In rats bearing the Yoshida ascites hepatoma AH-130, Barreiro *et al*. (3) investigated the association between muscle wasting and oxidative protein modification and reported a threefold increase in total and RCS-derived protein carbonylation after 7 days of tumor-cell injection. No significant alterations in the expression of the antioxidants Mn-SOD, catalase, and heme oxygenase-1 occurred, suggesting that the development of oxidant-derived protein modifications in skeletal muscles is due to an imbalance between ROS production and muscle antioxidant-enzyme levels (3).

Uncoupling proteins (UCPs) are members of a family of mitochondrial carriers located in the inner mitochondrial membrane. Their importance in regulating mitochondrial state 4 respiration, respiration uncoupling, and proton leaks is well documented (69). UCPs also participate in several other processes related to metabolic efficiency. Brand and colleagues (16) argued that the ancestral function of UCPs is to inhibit O_2^- release by the mitochondrial complexes I and III by simply inducing mild uncoupling. This new and important biologic role of UCPs was emphasized by Echtay *et al*. (25), who proposed that lipid peroxidation products, such as HNE and *trans*-retinoic acid, regulate mitochondrial ROS production by inducing the expressions of UCP1, 2, and 3 and that these proteins then inhibit mitochondrial O_2^- production by inducing uncoupling. These results suggest that lipid

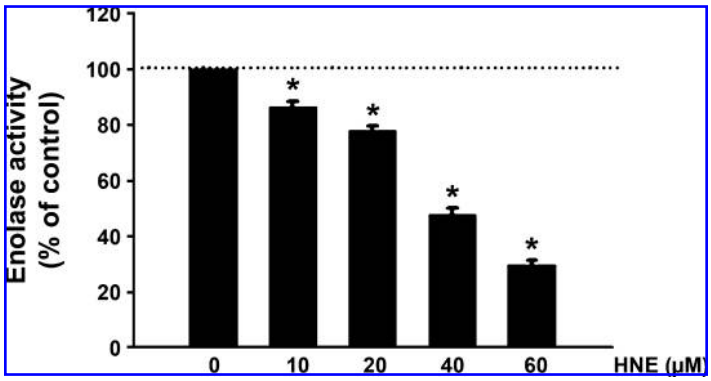

FIG. 8. Effect of increasing concentrations of HNE on enolase activity measured after 10 min of *in vitro* incubation at 37°C. Enolase activity was expressed as percentage of control (0 HNE concentration). *$p < 0.05$ compared with control values. Reprinted with permission from Hussain *et al*. (33).

peroxidation products are not merely toxic byproducts but that they also serve physiologic functions, including regulation of ROS production by the mitochondria.

Barreiro *et al*. (this issue) recently performed a detailed analysis of the intensity and molecular targets of RCS-derived protein carbonylation in skeletal muscles of rats bearing the Yoshida ascites hepatoma AH-130. Their analysis reveals that HNE- and MDA-protein adduct levels increase significantly in gastrocnemius, tibialis anterior, soleus, and heart muscles of cachectic rats. In addition, they found that enzymes involved in glycolysis, ATP production, carbon dioxide hydration, muscle contraction, and mitochondrial metabolism that have been previously described as targets of carbonylation in skeletal and ventilatory muscles of COPD patients and septic animals are also the targets of RCS (HNE and MDA)-derived carbonyl formation in limb muscles of cachetic rats. These results support the concept that cancer-induced cachexia alters redox balance in skeletal and cardiac muscles by inducing oxidative modifications of key proteins involved in muscle metabolism and contraction.

Carbonylation of Myofilament Proteins

In several pathologic conditions, including sepsis, ischemia/reperfusion, diabetes, and COPD, it has become increasingly evident that myofilament proteins in the ventilator and limb muscles are strongly carbonylated. Some of the proteins that undergo this form of oxidation are actin, myosin light and heavy chains, desmin, and tropomysin (4, 7, 55), confirming observations by Nagasawa *et al.* (53) that muscle myofibrils are highly sensitive to free radical–mediated oxidative stress. Actin appears to be a particular target for carbonylation, and several *in vitro* studies have confirmed that it is indeed susceptible to oxidative modification when isolated muscle fibers are exposed to oxidants (24, 52, 62, 72). Dalle-Donne *et al.* (23), for example, reported that exposing actin to hypochlorous acid (HOCl) causes a rapid increase in carbonylation, a result of oxidation of methionines, tryptophans, and lysines, in addition to increased dityrosine formation. They also suggested that oxidation of some methionine residues in actin results in strong inhibition of actin polymerization and a complete disruption of actin-filament organization (23). In addition, actin was recently shown to be modified by 4-hydroxy-2-nonenal (HNE) in both type I and type II fibers from rats of varying ages (82). It also was demonstrated that modification of actin by α-β unsaturated aldehydes involves adduct formation at His^{40}, His^{87}, His^{173}, and Cys^{374} and is associated with significant distortion of the ATP-binding sites (22).

Both myosin light and heavy chains also are targeted by ROS in skeletal and cardiac muscles. Oxidation of myosin light and heavy chains elicits distinct functional, structural, and chemical changes in muscle fibers. Prochniewicz and colleagues (63), for example, recently reported that exposing single skeletal muscle fibers to H_2O_2 results in dose-dependent impairments of muscle contractility and significant depression of myosin ATPase activity. These changes are associated with oxidative modifications of multiple methionine residues on the myosin light-chain and the myosin heavy-chain head. On the basis of these results, we propose that oxidative stress–induced skeletal muscle injury, along with an associated depression of muscle contractile performance, may be mediated, in part, through selective carbonylation of different intracellular proteins, including the structural myofilament proteins actin, myosin, and tropomyosin.

Concluding Remarks

It has become increasingly evident that in various physiologic and pathologic conditions, skeletal muscle fibers show significant increases in oxidant-derived posttranslational protein modifications, such as those associated with ROS-induced protein carbonylation, and that these modifications are associated with the development of oxidative stress and contractile dysfunction. Our understanding of the molecular sources of the ROS involved in protein modifications and the nature of proteins that are targeted by ROS inside muscle fibers has advanced over the past several years, yet despite this, many aspects of skeletal muscle protein oxidation remain to be explored. These aspects include the identification of possible targets of oxidant-derived modifications that are expressed at relatively low levels, more detailed analyses of intracellular distributions of oxidatively modified proteins in muscle fibers, identification of the precise residues that are targeted by ROS in various muscle proteins, and, more important, comprehensive analysis of the functional significance of protein carbonylation on skeletal muscle proteins, including those involved in regulating muscle contractile performance.

Acknowledgments

We acknowledge the support of FIS 06/1043, FIS CA06/0086, 2005-SGR01060, CIBERES (Instituto de Salud Carlos III, Ministerio de Sanidad) (Spain) and Canadian Institute of Health Research. Dr. Esther Barreiro was the recipient of the European Respiratory Society (ERS) COPD Research Award 2008.

References

1. Allaire J, Maltais F, LeBlanc P, Simard PM, Whittom F, Doyon JF, Simard C, and Jobin J. Lipofuscin accumulation in the vastus lateralis muscle in patients with chronic obstructive pulmonary disease. *Muscle Nerve* 25: 383–389, 2002.
2. Barreiro E, Comtois AS, Mohammed S, Lands LC, and Hussain SN. Role of heme oxygenases in sepsis-induced diaphragmatic contractile dysfunction and oxidative stress. *Am J Physiol Lung Cell Mol Physiol* 283: L476–L484, 2002.
3. Barreiro E, de la Puente B, Busquets S, Lopez-Soriano FJ, Gea J, and Argiles JM. Both oxidative and nitrosative stress are associated with muscle wasting in tumour-bearing rats. *FEBS Lett* 579: 1646–1652, 2005.
4. Barreiro E, de la Puente B, Minguella J, Corominas JM, Serrano S, Hussain SN, and Gea J. Oxidative stress and respiratory muscle dysfunction in severe chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 171: 1116–1124, 2005.
5. Barreiro E, Galdiz JB, Marinan M, Alvarez FJ, Hussain SN, and Gea J. Respiratory loading intensity and diaphragm oxidative stress: *N*-acetyl-cysteine effects. *J Appl Physiol* 100: 555–563, 2006.
6. Barreiro E, Gea J, Corominas JM, and Hussain SN. Nitric oxide synthases and protein oxidation in the quadriceps femoris of patients with chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol* 29: 771–778, 2003.
7. Barreiro E, Gea J, Di Falco M, Kriazhev L, James S, and Hussain SN. Protein carbonyl formation in the diaphragm. *Am J Respir Cell Mol Biol* 32: 9–17, 2005.

8. Barreiro E, Gea J, Matar G, and Hussain SN. Expression and carbonylation of creatine kinase in the quadriceps femoris muscles of patients with chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol* 33: 636–642, 2005.
9. Barreiro E, Nowinski A, Gea J, and Sliwinski P. Oxidative stress in the external intercostal muscles of patients with obstructive sleep apnoea. *Thorax* 62: 1095–1101, 2007.
10. Barreiro E, Rabinovich R, Marin-Corral J, Barbera JA, Gea J, and Roca J. Chronic endurance exercise induces quadriceps nitrosative stress in patients with severe COPD. *Thorax* 64: 13–19, 2009.
11. Barreiro E, Schols AM, Polkey MI, Galdiz JB, Gosker HR, Swallow EB, Coronell C, and Gea J. Cytokine profile in quadriceps muscles of patients with severe COPD. *Thorax* 63: 100–107, 2008.
12. Berlett BS and Stadtman ER. Protein oxidation in aging, disease, and oxidative stress. *J Biol Chem* 272: 20313–20316, 1997.
13. Bernard S, LeBlanc P, Whittom F, Carrier G, Jobin J, Belleau R, and Maltais F. Peripheral muscle weakness in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 158: 629–634, 1998.
14. Betters JL, Criswell DS, Shanely RA, Van Gammeren D, Falk D, DeRuisseau KC, Deering M, Yimlamai T, and Powers SK. Trolox attenuates mechanical ventilation-induced diaphragmatic dysfunction and proteolysis. *Am J Respir Crit Care Med* 170: 1179–1184, 2004.
15. Boczkowski J, Dureuil B, Brauger C, and Aubier M. Effects of sepsis on diaphragmatic function in rats. *Am Rev Respir Dis* 138, 260–265, 1988.
16. Brand MD, Affourtit C, Esteves TC, Green K, Lambert AJ, Miwa S, Pakay JL, and Parker N. Mitochondrial superoxide: production, biological effects, and activation of uncoupling proteins. *Free Radic Biol Med* 37: 755–767, 2004.
17. Buck M and Chojkier M. Muscle wasting and dedifferentiation induced by oxidative stress in a murine model of cachexia is prevented by inhibitors of nitric oxide synthesis and antioxidants. *EMBO J* 15: 1753–1765, 1996.
18. Bulkley GB. Pathophysiology of free radical-mediated reperfusion injury. *J Vasc Surg* 5: 512–517, 1987.
19. Bustamante V, Casanova J, Lopez dS, Mas S, Sellares J, Gea J, Galdiz JB, and Barreiro E. Redox balance following magnetic stimulation training in the quadriceps of patients with severe COPD. *Free Radic Res* 42: 939–948, 2008.
20. Canton M, Neverova I, Menabo R, Van Eyk J, and Di Lisa F. Evidence of myofibrillar protein oxidation induced by postischemic reperfusion in isolated rat hearts. *Am J Physiol Heart Circ Physiol* 286: H870–H877, 2004.
21. Couillard A, Maltais F, Saey D, Debigare R, Michaud A, Koechlin C, LeBlanc P, and Prefaut C. Exercise-induced quadriceps oxidative stress and peripheral muscle dysfunction in COPD patients. *Am J Respir Crit Care Med* 167: 1664–1669, 2003.
22. Dalle-Donne I, Carini M, Vistoli G, Gamberoni L, Giustarini D, Colombo R, Maffei FR, Rossi R, Milzani A, and Aldini G. Actin Cys374 as a nucleophilic target of alpha,beta-unsaturated aldehydes. *Free Radic Biol Med* 42: 583–598, 2007.
23. Dalle-Donne I, Rossi R, Giustarini D, Gagliano N, Lusini L, Milzani A, Di SP, and Colombo R. Actin carbonylation: from a simple marker of protein oxidation to relevant signs of severe functional impairment. *Free Radic Biol Med* 31: 1075–1083, 2001.
24. Dalle-Donne I, Milzani A, and Colombo R. H_2O_2-treated actin: assembly and polymer interactions with cross-linking proteins. *Biophys J* 69: 2710–2719, 1995.
25. Echtay KS, Esteves TC, Pakay JL, Jekabsons MB, Lambert AJ, Portero-Otin M, Pamplona R, Vidal-Puig AJ, Wang S, Roebuck SJ, and Brand MD. A signalling role for 4-hydroxy-2-nonenal in regulation of mitochondrial uncoupling. *EMBO J* 22: 4103–4110, 2003.
26. Engerson TD, McKelvey TG, Rhyne DB, Boggio EB, Snyder SJ, and Jones HP. Conversion of xanthine dehydrogenase to oxidase in ischemic rat tissues. *J Clin Invest* 79: 1564–1570, 1987.
27. Fagan JM, Ganguly M, Tiao G, Fischer JE, and Hasselgren PO. Sepsis increases oxidatively damaged proteins in skeletal muscle. *Arch Surg* 131: 1326–1332, 1996.
28. Ferreira LF and Reid MB. Muscle-derived ROS and thiol regulation in muscle fatigue. *J Appl Physiol* 104: 853–860, 2008.
29. Gomes-Marcondes MC and Tisdale MJ. Induction of protein catabolism and the ubiquitin-proteasome pathway by mild oxidative stress. *Cancer Lett* 180: 69–74, 2002.
30. Grisotto PC, dos Santos AC, Coutinho-Netto J, Cherri J, and Piccinato CE. Indicators of oxidative injury and alterations of the cell membrane in the skeletal muscle of rats submitted to ischemia and reperfusion. *J Surg Res* 92: 1–6, 2000.
31. Heunks LM, Vina J, van Herwaarden CL, Folgering HT, Gimeno A, and Dekhuijzen PN. Xanthine oxidase is involved in exercise-induced oxidative stress in chronic obstructive pulmonary disease. *Am J Physiol* 277: R1697–R1704, 1999.
32. Hidalgo C, Sanchez G, Barrientos G, and Racena-Parks P. A transverse tubule NADPH oxidase activity stimulates calcium release from isolated triads via ryanodine receptor type 1 S-glutathionylation. *J Biol Chem* 281: 26473–26482, 2006.
33. Hussain SN, Matar G, Barreiro E, Florian M, Divangahi M, and Vassilakopoulos T. Modifications of proteins by 4-hydroxy-2-nonenal in the ventilatory muscles of rats. *Am J Physiol Lung Cell Mol Physiol* 290: L996–L1003, 2006.
34. Hussain SN, Simkus G, and Roussos C. Respiratory muscle fatigue: a cause of ventilatory failure in septic shock. *J Appl Physiol* 58: 2033–2040, 1985.
35. Javesghani D, Magder SA, Barreiro E, Quinn MT, and Hussain SN. Molecular characterization of a superoxide-generating NAD(P)H oxidase in the ventilatory muscles. *Am J Respir Crit Care Med* 165: 412–418, 2002.
36. Judge AR and Dodd SL. Oxidative damage to skeletal muscle following an acute bout of contractile claudication. *Atherosclerosis* 171: 219–224, 2003.
37. Judge AR and Dodd SL. Xanthine oxidase and activated neutrophils cause oxidative damage to skeletal muscle after contractile claudication. *Am J Physiol Heart Circ Physiol* 286: H252–H256, 2004.
38. Kavazis AN, Talbert EE, Smuder AJ, Hudson MB, Nelson WB, and Powers SK. Mechanical ventilation induces diaphragmatic mitochondrial dysfunction and increased oxidant production. *Free Radic Biol Med* 46: 842–850, 2009.
39. Khaliulin I, Schneider A, Houminer E, Borman JB, and Schwalb H. Apomorphine prevents myocardial ischemia/reperfusion-induced oxidative stress in the rat heart. *Free Radic Biol Med* 37: 969–976, 2004.
40. Kim HC, Mofarrahi M, and Hussain SN. Skeletal muscle dysfunction in patients with chronic obstructive pulmonary disease. *Int J Chron Obstruct Pulmon Dis* 3: 637–658, 2008.
41. Kondo H, Nakagaki I, Sasaki S, Hori S, and Itokawa Y. Mechanism of oxidative stress in skeletal muscle atrophied by immobilization. *Am J Physiol* 265: E839–E844, 1993.
42. Lanone S, Mebazaa A, Heymes C, Henin D, Poderoso JJ, Panis Y, Zedda C, Billiar T, Payen D, Aubier M, and Boczkowski J. Muscular contractile failure in septic patients: role

of the inducible nitric oxide synthase pathway. *Am J Resp Crit Care Med* 162: 2308–2315, 2000.
43. Lawler JM, Song W, and Demaree SR. Hindlimb unloading increases oxidative stress and disrupts antioxidant capacity in skeletal muscle. *Free Radic Biol Med* 35: 9–16, 2003.
44. Levine S, Gregory C, Nguyen T, Shrager J, Kaiser L, Rubinstein N, and Dudley G. Bioenergetic adaptation of individual human diaphragmatic myofibers to severe COPD. *J Appl Physiol* 92: 1205–1213, 2002.
45. Levine S, Kaiser L, Leferovich J, and Tikunov B. Cellular adaptations in the diaphragm in chronic obstructive pulmonary disease. *N Engl J Med* 337: 1799–1806, 1997.
46. Maltais F, LeBlanc P, Simard C, Jobin J, Berube C, Bruneau J, Carrier L, and Belleau R. Skeletal muscle adaptation to endurance training in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 154: 442–447, 1996.
47. Marin-Corral J, Minguella J, Ramirez-Sarmiento AL, Hussain SNA, Gea J, and Barreiro E. Oxidised proteins and superoxide anion production in the diaphragm of severe COPD patients. *Eur Respir J* 33: 1309–1319, 2009.
48. Matsunaga S, Mishima T, Yamada T, Inashima S, and Wada M. Alterations in in vitro function and protein oxidation of rat sarcoplasmic reticulum Ca^{2+}-ATPase during recovery from high-intensity exercise. *Exp Physiol* 93: 426–433, 2008.
49. McClung JM, Van Gammeren D, Whidden MA, Falk DJ, Kavazis AN, Hudson MB, Gayan-Ramirez G, Decramer M, DeRuisseau KC, and Powers SK. Apocynin attenuates diaphragm oxidative stress and protease activation during prolonged mechanical ventilation. *Crit Care Med* 37: 1373–1379, 2009.
50. McClung JM, Whidden MA, Kavazis AN, Falk DJ, DeRuisseau KC, and Powers SK. Redox regulation of diaphragm proteolysis during mechanical ventilation. *Am J Physiol Regul Integr Comp Physiol* 294: R1608–R1617, 2008.
51. Mercken EM, Hageman GJ, Schols AM, Akkermans MA, Bast A, and Wouters EF. Rehabilitation decreases exercise-induced oxidative stress in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 172: 994–1001, 2005.
52. Milzani A, Rossi R, Di SP, Giustarini D, Colombo R, and Dalle-Donne I. The oxidation produced by hydrogen peroxide on Ca-ATP-G-actin. *Protein Sci* 9: 1774–1782, 2000.
53. Nagasawa T, Hatayama T, Watanabe Y, Tanaka M, Niisato Y, and Kitts DD. Free radical-mediated effects on skeletal muscle protein in rats treated with Fe-nitrilotriacetate. *Biochem Biophys Res Commun* 231: 37–41, 1997.
54. Nin N, Lorente JA, de Paula M, Fernandez-Segoviano P, Penuelas O, Sanchez-Ferrer A, Martinez-Caro L, and Esteban A. Aging increases the susceptibility to injurious mechanical ventilation. *Intens Care Med* 34: 923–931, 2008.
55. Oh-Ishi M, Ueno T, and Maeda T. Proteomic method detects oxidatively induced protein carbonyls in muscles of a diabetes model Otsuka Long-Evans Tokushima Fatty (OLETF) rat. *Free Radic Biol Med* 34: 11–22, 2003.
56. Orozco-Levi M, Gea J, Lloreta JL, Felez M, Minguella J, Serrano S, and Broquetas JM. Subcellular adaptation of the human diaphragm in chronic obstructive pulmonary disease. *Eur Respir J* 13: 371–378, 1999.
57. Ottenheijm CA, Heunks LM, Sieck GC, Zhan WZ, Jansen SM, Degens H, de Boo T, and Dekhuijzen PN. Diaphragm dysfunction in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 172: 200–205, 2005.
58. Ozyurt B, Iraz M, Koca K, Ozyurt H, and Sahin S. Protective effects of caffeic acid phenethyl ester on skeletal muscle ischemia-reperfusion injury in rats. *Mol Cell Biochem* 292: 197–203, 2006.
59. Park AM and Suzuki YJ. Effects of intermittent hypoxia on oxidative stress-induced myocardial damage in mice. *J Appl Physiol* 102: 1806–1814, 2007.
60. Peralta JG, Llesuy S, Evelson P, Carreras MC, Gonzalez B, and Poderoso J. Oxidative stress in skeletal muscle during sepsis in rats. *Circ Shock* 39: 153–159, 1993.
61. Pinho RA, Andrades ME, Oliveira MR, Pirola AC, Zago MS, Silveira PC, Dal Pizzol F, and Moreira JC. Imbalance in SOD/CAT activities in rat skeletal muscles submitted to treadmill training exercise. *Cell Biol Int* 30: 848–853, 2006.
62. Powell SR, Gurzenda EM, and Wahezi SE. Actin is oxidized during myocardial ischemia. *Free Radic Biol Med* 30: 1171–1176, 2001.
63. Prochniewicz E, Lowe DA, Spakowicz DJ, Higgins L, O'Conor K, Thompson LV, Ferrington DA, and Thomas DD. Functional, structural, and chemical changes in myosin associated with hydrogen peroxide treatment of skeletal muscle fibers. *Am J Physiol Cell Physiol* 294: C613–C626, 2008.
64. Reid MB. Nitric oxide, reactive oxygen species, and skeletal muscle contraction. *Med Sci Sports Exerc* 33: 371–376, 2001.
65. Requena JR, Chao CC, Levine RL, and Stadtman ER. Glutamic and aminoadipic semialdehydes are the main carbonyl products of metal-catalyzed oxidation of proteins. *Proc Natl Acad Sci U S A* 98: 69–74, 2001.
66. Requena JR, Fu MX, Ahmed MU, Jenkins AJ, Lyons TJ, and Thorpe SR. Lipoxidation products as biomarkers of oxidative damage to proteins during lipid peroxidation reactions. *Nephrol Dial Transplant* 11(suppl 5): 48–53, 1996.
67. Requena JR, Levine RL, and Stadtman ER. Recent advances in the analysis of oxidized proteins. *Amino Acids* 25: 221–226, 2003.
68. Reznick AZ, Witt E, Matsumoto M, and Packer L. Vitamin E inhibits protein oxidation in skeletal muscle of resting and exercised rats. *Biochem Biophys Res Commun* 189: 801–806, 1992.
69. Ricquier D and Bouillaud F. The uncoupling protein homologues: UCP1, UCP2, UCP3, StUCP and AtUCP. *Biochem J* 3452: 161–179, 2000.
70. Sala E, Roca J, Marrades RM, Alonso J, Gonzalez De Suso JM, Moreno A, Barbera JA, Nadal J, de Jover L, Rodriguez-Roisin R, and Wagner PD. Effects of endurance training on skeletal muscle bioenergetics in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 159: 1726–1734, 1999.
71. Saxton JM, Donnelly AE, and Roper HP. Indices of free-radical-mediated damage following maximum voluntary eccentric and concentric muscular work. *Eur J Appl Physiol Occup Physiol* 68: 189–193, 1994.
72. Schwalb H, Olivson A, Li J, Houminer E, Wahezi SE, Opie LH, Maulik D, Borman JB, and Powell SR. Nicorandil decreases postischemic actin oxidation. *Free Radic Biol Med* 31: 607–614, 2001.
73. Shanely RA, Zergeroglu MA, Lennon SL, Sugiura T, Yimlamai T, Enns D, Belcastro A, and Powers SK. Mechanical ventilation-induced diaphragmatic atrophy is associated with oxidative injury and increased proteolytic activity. *Am J Respir Crit Care Med* 166: 1369–1374, 2002.
74. Shindoh C, DiMarco A, Nethery D, and Supinski G. Effect of PEG-superoxide dismutase on the diaphragmatic response to endotoxin. *Am Rev Respir Dis* 145, 1350–1354. 1992.
75. Silva LA, Silveira PC, Pinho CA, Tuon T, Dal PF, and Pinho RA. *N*-acetylcysteine supplementation and oxidative dam-

age and inflammatory response after eccentric exercise. *Int J Sport Nutr Exerc Metab* 18: 379–388, 2008.
76. St Pierre J, Buckingham JA, Roebuck SJ, and Brand MD. Topology of superoxide production from different sites in the mitochondrial electron transport chain. *J Biol Chem* 277: 44784–44790, 2002.
77. Stadtman ER. Metal ion-catalyzed oxidation of proteins: biochemical mechanism and biological consequences. *Free Radic Biol Med* 9: 315–325, 1990.
78. Stadtman ER and Levine RL. Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. *Amino Acids* 25: 207–218, 2003.
79. Starke PE, Oliver CN, and Stadtman ER. Modification of hepatic proteins in rats exposed to high oxygen concentration. *FASEB J* 1: 36–39, 1987.
80. Supinski G, Nethery D, and DiMarco A. Effect of free radical scavengers on endotoxin-induced respiratory muscle dysfunction. *Am Rev Respir Dis* 148, 1318–1324. 1993.
81. Supinski G, Nethery D, Stofan D, Hirschfield W, and DiMarco A. Diaphragmatic lipid peroxidation in chronically loaded rats. *J Appl Physiol* 86: 651–658, 1999.
82. Thompson LV, Durand D, Fugere NA, and Ferrington DA. Myosin and actin expression and oxidation in aging muscle. *J Appl Physiol* 101: 1581–1587, 2006.
83. Van SC, Boczkowski J, Pasquier C, Du Y, Franzini E, and Aubier M. Effects of *N*-acetylcysteine on diaphragmatic function and malondialdehyde content in *Escherichia coli* endotoxemic rats. *Am Rev Respir Dis* 146: 730–734, 1992.
84. Veskoukis AS, Nikolaidis MG, Kyparos A, Kokkinos D, Nepka C, Barbanis S, and Kouretas D. Effects of xanthine oxidase inhibition on oxidative stress and swimming performance in rats. *Appl Physiol Nutr Metab* 33: 1140–1154, 2008.
85. Whidden MA, McClung JM, Falk DJ, Hudson MB, Smuder AJ, Nelson WB, and Powers SK. Xanthine oxidase contributes to mechanical ventilation-induced diaphragmatic oxidative stress and contractile dysfunction. *J Appl Physiol* 106: 385–394, 2009.
86. Whittom F, Jobin J, Simard PM, LeBlanc P, Simard C, Bernard S, Belleau R, and Maltais F. Histochemical and morphological characteristics of the vastus lateralis muscle in patients with chronic obstructive pulmonary disease. *Med Sci Sports Exerc* 30: 1467–1474, 1998.
87. Witt EH, Reznick AZ, Viguie CA, Starke-Reed P, and Packer L. Exercise, oxidative damage and effects of antioxidant manipulation. *J Nutr* 122: 766–773, 1992.
88. Zergeroglu MA, McKenzie MJ, Shanely RA, Van Gammeren D, DeRuisseau KC, and Powers SK. Mechanical ventilation-induced oxidative stress in the diaphragm. *J Appl Physiol* 95: 1116–1124, 2003.

Address correspondence to:
Dr. Sabah Hussain
Room L305
Critical Care Division
Royal Victoria Hospital
McGill University Health Centre
Montreal, Quebec, Canada

E-mail: sabah.hussain@muhc.mcgill.ca

Date of first submission to ARS Central, August 3, 2009; date of acceptance, August 15, 2009.

Abbreviations Used

COPD = chronic obstructive pulmonary disease
DNP = 2,4-dinitrophenylhydrazone
DNPH = 2,4-dinitrophenylhydrazine
FEV_1 = forced expiratory volume in 1 s
HNE = hydroxynonenal
H_2O_2 = hydrogen peroxide
HOO^- = hydroperoxyl radical
MDA = malondialdehyde
MIP = maximal inspiratory pressure
NAC = *N*-acetylcysteine
NADPH = nicotinamide adenine dinucleotide phosphate
$O_2^{-\bullet}$ = superoxide anion
OH^- = hydroxyl radical
OSAS = obstructive sleep apnea syndrome
RNS = reactive nitrogen species
ROS = reactive oxygen species
SOD = superoxide dismutase
UCP = uncoupling protein

This article has been cited by:

1. Vikas Kumar , Timothy Dean Calamaras , Dagmar Haeussler , Wilson Steven Colucci , Richard Alan Cohen , Mark Errol McComb , David Pimentel , Markus Michael Bachschmid . 2012. Cardiovascular Redox and Ox Stress Proteomics. *Antioxidants & Redox Signaling* **17**:11, 1528-1559. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
2. Anders Nedergaard, Morten A. Karsdal, Shu Sun, Kim Henriksen. 2012. Serological muscle loss biomarkers: an overview of current concepts and future possibilities. *Journal of Cachexia, Sarcopenia and Muscle* . [CrossRef]
3. Vincenzo Alterio, Anna Di Fiore, Katia D'Ambrosio, Claudiu T. Supuran, Giuseppina De Simone. 2012. Multiple Binding Modes of Inhibitors to Carbonic Anhydrases: How to Design Specific Drugs Targeting 15 Different Isoforms?. *Chemical Reviews* **112**:8, 4421-4468. [CrossRef]
4. Jessica M. Curtis, Wendy S. Hahn, Eric K. Long, Joel S. Burrill, Edgar A. Arriaga, David A. Bernlohr. 2012. Protein carbonylation and metabolic control systems. *Trends in Endocrinology & Metabolism* **23**:8, 399-406. [CrossRef]
5. Teresa A. Sherman, Sharath C. Rongali, Tori A. Matthews, Jason Pfeiffer, Keith Nehrke. 2012. Identification of a nuclear carbonic anhydrase in Caenorhabditis elegans. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* **1823**:4, 808-817. [CrossRef]
6. N. Okudan, K. Esra Nurullaho#lu-Atal#k, S. Revan, M. Belviranl#, #. S. Balc#, H. Gökbel, H. Pepe. 2012. Effects of treatment with coenzyme Q₁₀ on exercised rat aorta. *Acta Physiologica Hungarica* **99**:1, 40-50. [CrossRef]
7. Diego A. Rodriguez, Susana Kalko, Ester Puig-Vilanova, Maitane Perez-Olabarría, Francesco Falciani, Joaquim Gea, Marta Cascante, Esther Barreiro, Josep Roca. 2012. Muscle and blood redox status after exercise training in severe COPD patients. *Free Radical Biology and Medicine* **52**:1, 88-94. [CrossRef]
8. Francesca Magherini, Provvidenza Maria Abruzzo, Michele Puglia, Luca Bini, Tania Gamberi, Fabio Esposito, Arsenio Veicsteinas, Marina Marini, Claudia Fiorillo, Massimo Gulisano, Alessandra Modesti. 2012. Proteomic analysis and protein carbonylation profile in trained and untrained rat muscles. *Journal of Proteomics* **75**:3, 978-992. [CrossRef]
9. Laura A.A. Gilliam , Daret K. St. Clair . 2011. Chemotherapy-Induced Weakness and Fatigue in Skeletal Muscle: The Role of Oxidative Stress. *Antioxidants & Redox Signaling* **15**:9, 2543-2563. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
10. Tina-Tinkara Peternelj, Jeff S. Coombes. 2011. Antioxidant Supplementation during Exercise Training. *Sports Medicine* 1. [CrossRef]
11. Daniel C. Andersson, Matthew J. Betzenhauser, Steven Reiken, Albano C. Meli, Alisa Umanskaya, Wenjun Xie, Takayuki Shiomi, Ran Zalk, Alain Lacampagne, Andrew R. Marks. 2011. Ryanodine Receptor Oxidation Causes Intracellular Calcium Leak and Muscle Weakness in Aging. *Cell Metabolism* **14**:2, 196-207. [CrossRef]
12. Marina Bayeva, Hossein Ardehali. 2010. Mitochondrial Dysfunction and Oxidative Damage to Sarcomeric Proteins. *Current Hypertension Reports* **12**:6, 426-432. [CrossRef]
13. Yuichiro J. Suzuki , Marina Carini , D. Allan Butterfield . 2010. Protein Carbonylation. *Antioxidants & Redox Signaling* **12**:3, 323-325. [Citation] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]