Cigarette Smoke–induced Oxidative Stress

A Role in Chronic Obstructive Pulmonary Disease Skeletal Muscle Dysfunction

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Rationale: **Inflammation and oxidative stress contribute to muscle dysfunction in patients with chronic obstructive pulmonary disease (COPD). Oxidants contained in cigarette smoke (CS) induce adverse effects on tissues through oxidative phenomena.**

Objectives: **To explore oxidative stress and inflammation in quadriceps of human smokers and in diaphragm and limb muscles of guinea pigs chronically exposed to CS.**

Methods: **Muscle function, protein oxidation and nitration, antioxidants, oxidized proteins, inflammation, creatine kinase activity, and lung and muscle structures were investigated in vastus lateralis of smokers, patients with COPD, and healthy control subjects and in diaphragm and gastrocnemius of CS-exposed guinea pigs at 3, 4, and 6 months.**

Measurements and Main Results: **Compared with control subjects, quadriceps muscle force was mildly but significantly reduced in smokers; protein oxidation levels were increased in quadriceps of smokers and patients with COPD, and in respiratory and limb muscles of CS-exposed animals; glycolytic enzymes, creatine kinase, carbonic anydrase-3, and contractile proteins were significantly more carbonylated in quadriceps of smokers and patients with COPD, and in respiratory and limb muscles of CS-exposed guinea pigs. Chronic CS exposure induced no significant rise in muscle inflammation in either smokers or rodents. Muscle creatine kinase activity was reduced only in patients with COPD and in both diaphragm and gastrocnemius of CS-exposed animals. Guinea pigs developed bronchiolar abnormalities at 4 months of exposure and thereafter.**

Conclusions: **CS exerts direct oxidative modifications on muscle proteins, without inducing any significant rise in muscle inflammation. The oxidative damage to muscle proteins, which precedes the characteristic respiratory changes, may contribute to muscle loss and dysfunction in smokers and patients with COPD.**

Keywords: cigarette smoke; guinea pigs; healthy smokers; muscle inflammation and oxidative stress; quadriceps muscle function

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Oxidative stress is a proposed contributor to chronic obstructive pulmonary disease (COPD) muscle dysfunction. Oxidants contained in cigarette smoke induce adverse effects on tissues through oxidative modifications of key biological structures. It remains to be elucidated whether chronic cigarette smoking induces direct oxidative damage in skeletal muscles.

What This Study Adds to the Field

Chronic cigarette smoking exerts direct oxidative modifications on muscle proteins, without inducing significant rise in either molecular or cellular muscle inflammation. Importantly, the oxidative damage to specific muscle proteins, which precedes the characteristic respiratory changes, may contribute to muscle mass loss and dysfunction in smokers and patients with COPD.

It is generally accepted that the large number of oxidants contained in cigarette smoke (CS) induces adverse effects on tissues through oxidative damage of key biological structures. In addition, CS-induced activation of inflammatory cells may also contribute to enhanced oxidant production in tissues. For instance, lipid peroxidation (1, 2), protein and thiol oxidation (3, 4), and oxidized DNA (5) levels were shown to be increased in the blood of smokers (1–5) and in several organs of animals chronically exposed to CS (5). Moreover, smoking is also a recognized risk factor for many chronic conditions such as dyslipidemia, glucose intolerance (6), and nutritional abnormalities characterized by anorexia, weight loss, and reduced brown and white adipose tissues (7, 8).

Highly prevalent conditions such as chronic obstructive pulmonary disease (COPD) are frequently associated with muscle loss and skeletal muscle dysfunction. These systemic manifestations have a considerable impact on the exercise tolerance and quality of life of the patients, and are also associated with increased mortality (9). Systemic and local oxidative stress, among other factors, has been suggested as a contributor to this process of muscle dysfunction and wasting in COPD (10). Moreover, the spillover of oxidants and inflammatory molecules from the lungs is another potential mechanism of muscle dysfunction in COPD. However, it could be reasoned that CS per se may also exert deleterious effects on skeletal muscles. In this regard, smokers have been shown to exhibit lower peripheral muscle fatigue resistance than nonsmokers (11). Moreover, in spontaneously hypertensive rats exposed to CS, proportions and sizes of muscle fibers were indeed altered in soleus and extensor digitorum longus (12, 13). Also, the vastus lateralis muscle of

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smokers was shown to exhibit a reduction in the content of constitutive nitric oxide synthases together with a smaller size of the slow-twitch fibers (14).

Despite this progress, it remains to be elucidated whether CS induces direct oxidative damage within skeletal muscle fiber structures. In this regard, transient and repeated bouts of reduction–oxidation (redox) imbalance induced by chronic CS exposure may oxidize key proteins involved in muscle metabolism or function, eventually contributing to the muscle dysfunction of patients with COPD. In the current investigation, two different approaches were used: (*1*) limb muscles of current smokers free of lung or cardiovascular disease were analyzed together with muscles of patients with severe COPD; and (*2*) guinea pigs, which develop lesions in their airways similar to those documented in human smokers (15–17), were exposed to chronic CS exposure. On this basis, our objectives were to selectively explore redox balance in lower limb muscles of both human smokers and patients with severe COPD, and in both diaphragm and limb muscles of guinea pigs exposed to CS for 3, 4, and 6 months. Furthermore, the nature and function of the muscle proteins exhibiting the greatest levels of oxidation as well as inflammatory events were also determined in these muscles. Some of the results of these studies have been previously reported in the form of an abstract (18, 19).

METHODS

See the online supplement for additional information.

Human Study Subjects

This is a hospital-based study in which a group of nine white, male, current smokers with normal spirometry were recruited from the smoking cessation clinic together with 10 healthy male, age-matched control subjects and 10 stable patients with severe COPD (20). Asymptomatic smokers were defined as individuals with a smoking history of more than 20 pack-years and who exhibited a postbronchodilator ratio of FEV_1 to FVC greater than 0.7 (20). The current investigation was designed in accordance with both the ethical standards on human experimentation in our institution and the World Medical Association guidelines for research on human beings. Approval was obtained from the institutional ethics committee on human investigation (Hospital de Cruces, Barakaldo, Spain). Informed written consent was obtained from all individuals.

Nutritional and Functional Assessment

Nutritional evaluation included determination of body mass index and fat-free mass index by bioelectrical impedance (21). Forced spirometry was performed according to standard procedures (22). Quadriceps strength was evaluated in smokers, patients, and control subjects by isometric maximal voluntary contraction (QMVC) as formerly described (23).

Muscle biopsies

Muscle samples of smokers, patients with COPD, and control subjects were obtained from the vastus lateralis by open muscle biopsy as previously described (24–26).

Animal Experiments

Experimental groups. Groups of seven male Hartley guinea pigs were exposed to the smoke of seven commercial cigarettes (24 h, 5 d/wk) for periods of 3, 4, and 6 months (15–17, 27). Corresponding control animals underwent the same procedures except for CS exposure. Twenty-four hours after the end of each experimental period, diaphragm, gastrocnemius, and lungs were obtained from all animals. This was a controlled study designed in accordance with the institutional ethics standards and the Helsinki Convention for the use and care of animals. All experiments were approved by the institutional Animal Research Committee at Hospital Clinic (Barcelona).

Muscle Biology Analyses

Immunoblotting of one-dimensional electrophoresis. The effects of reactive oxygen and nitrogen species (ROS and RNS, respectively) on muscle proteins were evaluated according to methodologies previously published (24, 28–31).

Identification of carbonylated and tyrosine-nitrated muscle proteins: two-dimensional electrophoresis. Carbonylated and nitrated proteins were separated and identified in the muscles as published elsewhere (25, 30, 32, 33).

Identification of carbonylated and tyrosine nitrated muscle proteins: mass spectrometry. Identification of carbonylated and nitrated proteins was conducted in the proteomics laboratory according to previously published procedures (25, 30, 32, 33).

Creatine kinase activity assay. Total muscle creatine kinase activity was measured in all muscles as previously published (25, 32, 33).

Cytokines. Protein levels of the cytokines tumor necrosis factor (TNF)-α and IL-6 were quantified in all muscles as published elsewhere (26).

Muscle inflammatory cells. As previously published, inflammatory cell counts were determined immunohistochemically in all muscles (14, 34).

Muscle fiber counts and morphometry. Morphometric analyses were conducted in all muscle as published elsewhere (24, 29, 33).

Morphometric Studies in Lung Tissue

In the guinea pigs, the number of bronchiolar goblet cells was evaluated in paraffin-embedded lung sections counterstained with hematoxylin–eosin and alcian blue. The degree of emphysema was assessed by measuring the mean distance between alveolar septa.

Statistical Analysis

Results are presented as means (SD). In each experimental model, comparisons of physiological and biological variables among the different study groups were analyzed by one-way analysis of variance. Tukey's *post hoc* analysis was used to adjust for multiple comparisons.

RESULTS

Clinical Characteristics

Human studies. As shown in Table 1, age, body mass index, and fat-free mass index did not significantly differ among the three study groups of subjects. Lung function parameters were significantly reduced in patients with COPD compared with either smokers or healthy control subjects, and all patients had severe COPD (Table 1). Interestingly, QMVC was mildly but significantly reduced in the smokers compared with the healthy control subjects. As expected, QMVC was also significantly decreased in the patients with severe COPD compared with either smokers or healthy control subjects (Table 1).

TABLE 1. ANTHROPOMETRIC CHARACTERISTICS AND RESPIRATORY AND MUSCLE FUNCTIONS OF HUMAN STUDY SUBJECTS

	Control Subjects ($n = 10$)	Smokers ($n = 9$)	COPD ($n = 10$)
Age, yr	56 (6)	53 (9)	58 (3)
BMI, kg/m^2	26.7 (4.0)	27.4 (5.1)	26.5 (4.2)
FFMI, kg/m^2	20.0 (2.4)	18.1 (2.6)	18.6 (2.9)
FEV_1, % pred	94 (13)	89 (5)	30 (6)*†
FVC, % pred	91 (11)	93 (9)	75 (11)‡§
FEV_1/FVC, %	79 (7)	76 (10)	32 (8)*†
QMVC, kg	38.50 (1.7)	36.78 (1.5)‖	28.20 (1.31)*†

Definition of abbreviations: % pred = percentage of the predicted value; BMI = body mass index; COPD = chronic obstructive pulmonary disease; FFMI = fat-free mass index; QMVC = quadriceps maximal voluntary contraction.

Values are expressed as means (SD).

* $P \leq 0.001$, between patients with COPD and healthy control subjects.

† $P \leq 0.001$, between patients with COPD and healthy smokers.

‡ $P \leq 0.01$, between patients with COPD and healthy control subjects.

§ $P \leq 0.05$, between patients with COPD and healthy smokers.

‖ $P \leq 0.05$, between healthy smokers and healthy control subjects.

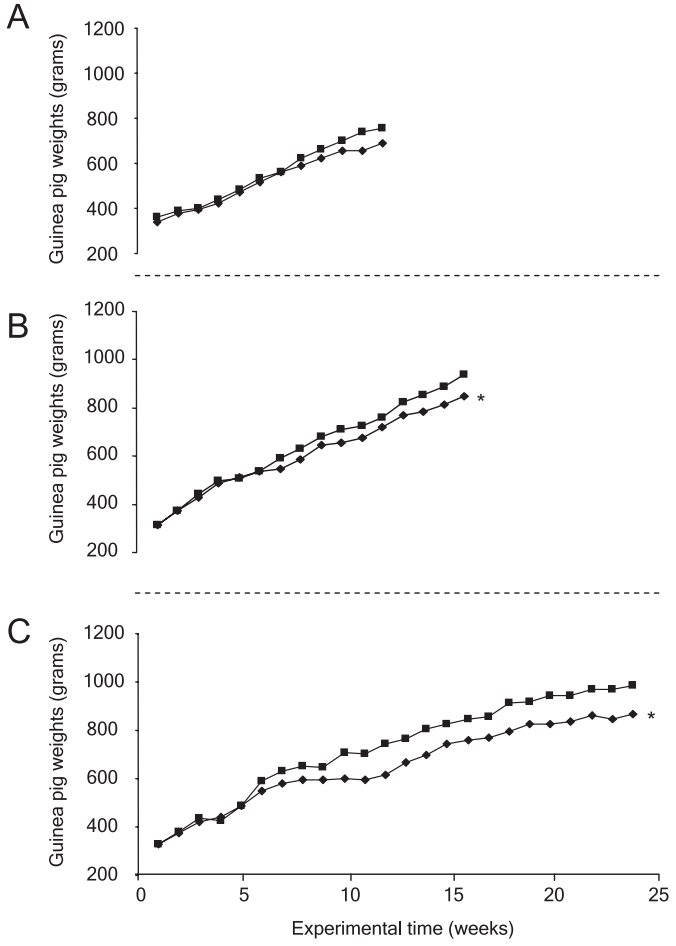

Figure 1. (*A*) Rate of body weight gain of cigarette smoke (CS)–exposed guinea pigs (*solid diamonds*) and corresponding control animals (*solid squares*) over 3 months. No significant differences were observed in the rate of body weight gain between CS-exposed and control guinea pigs at 3 months. (*B*) Rate of body weight gain of CS-exposed guinea pigs (*solid diamonds*) and corresponding control animals (*solid squares*) over 4 months. The rate of body weight gain was significantly reduced (*$P < 0.05$) in the CS-exposed guinea pigs compared with the control subjects at 4 months. (*C*) Rate of body weight gain of CS-exposed guinea pigs (*solid diamonds*) and corresponding control animals (*solid squares*) over 6 months. The rate of body weight gain was significantly decreased (*$P < 0.05$) in the CS-exposed guinea pigs compared with control animals at 6 months.

Animal studies. Guinea pigs exposed to CS for 3 months did not experience any significant reduction in their body weight gain compared with corresponding control subjects (Figure 1A). Importantly, guinea pigs exposed to CS for 4 and 6 months exhibited a significant decrease in their body weight gain compared with the corresponding control animals (Figures 1B and 1C, respectively).

Molecular Markers of Muscle Redox Balance and Oxidized Proteins

Human studies. Total protein carbonylation levels were significantly greater in the vastus lateralis of both smokers and patients with COPD than in the control subjects (Figure 2A). Muscle protein carbonylation levels were, in turn, significantly higher in the limb muscles of patients with COPD than in those of healthy smokers (Figure 2A). Several glycolytic enzymes, creatine kinase, ATP synthase, carbonic anhydrase-3, and actin were identified to be consistently carbonylated in the quadriceps of smokers, patients with severe COPD, and healthy control subjects (Figure 2B and Table 2; and *see* Table E1 in the online supplement). Interestingly, the enzymes enolase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), creatine kinase, ATP synthase, and carbonic anhydrase-3 exhibited significantly greater levels of carbonylation in the vastus lateralis of smokers and patients with severe COPD than in healthy control subjects (Figure 2C). Importantly, among both smokers and patients with severe COPD, a significant inverse relationship was found between muscle protein carbonylation levels and QMVC (Figure 2D). Levels of malondialdehyde (MDA)–protein adducts, were significantly increased in the quadriceps of both smokers and patients with COPD compared with control subjects (Figure 2E).

Total protein tyrosine nitration levels were significantly greater only in the limb muscles of patients with COPD, but not in smokers, than in control subjects (Table 3). Protein content of the mitochondrial enzyme manganese-superoxide dismutase (Mn-SOD) was significantly increased in the vastus lateralis of both patients with COPD and smokers compared with control subjects (Table 3), whereas muscle catalase levels did not differ among the study groups (Table 3). Compared with healthy control subjects, creatine kinase activity was significantly reduced only in the vastus lateralis of the patients with severe COPD, but not in the smokers (Table 3).

Animal studies. Chronic CS exposure induced a significant increase in reactive carbonyls in both diaphragm and gastrocnemius muscles of guinea pigs after 3, 4, and 6 months of exposure compared with corresponding control animals (Figures 3A and 3B, respectively). In respiratory and limb muscles of the guinea pigs, enzymes involved in glycolysis, creatine kinase, ATP synthase, actin, and tropomyosin were shown to be carbonylated in both CS-exposed and control animals (Figure 3C, Table 2, and Table E2). Carbonylation levels of the enzyme creatine kinase were significantly higher in the diaphragm of CS-exposed guinea pigs at 3 and 6 months of exposure than in the corresponding muscles of control subjects (Figure 3D). Moreover, proteins such as enolase, aldolase, GAPDH, creatine kinase, actin, and tropomyosin displayed greater carbonylation levels in the gastrocnemius of guinea pigs exposed to CS for 3 and 6 months compared with corresponding control muscles (Figure 3E). Interestingly, chronic exposure to CS also induced a significant rise in MDA–protein adducts in the diaphragm and gastrocnemius of guinea pigs exposed to CS for 3, 4, and 6 months compared with control rodents (Figures 3F and 3G, respectively).

In CS-exposed animals, muscle protein tyrosine nitration levels were greater for all time cohorts, except for the diaphragm, in which increased protein nitration levels did not reach statistical significance after 3 months of exposure (Figures 4A–4C). Enzymes involved in glycolysis, creatine kinase, and actin were also shown to be tyrosine nitrated in both CS-exposed and control animals (Table 2 and Table E3). Importantly, enolase, aldolase, and creatine kinase exhibited significantly greater levels of tyrosine nitration in the diaphragm of CS-exposed guinea pigs at 3 and 6 months than in control subjects (Figure 4D). Furthermore, GAPDH and creatine kinase also showed significantly higher levels of tyrosine nitration in the gastrocnemius of CS-exposed rodents at 3 and 6 months than in corresponding control muscles (Figure 4E).

Mn-SOD protein content did not differ significantly between CS-exposed guinea pigs and control animals in any of the muscles (Figures 5A and 5B). Interestingly, only the gastrocnemius from rodents exposed to CS for 4 and 6 months exhibited a significant increase in catalase compared with control animals (Figures 5C

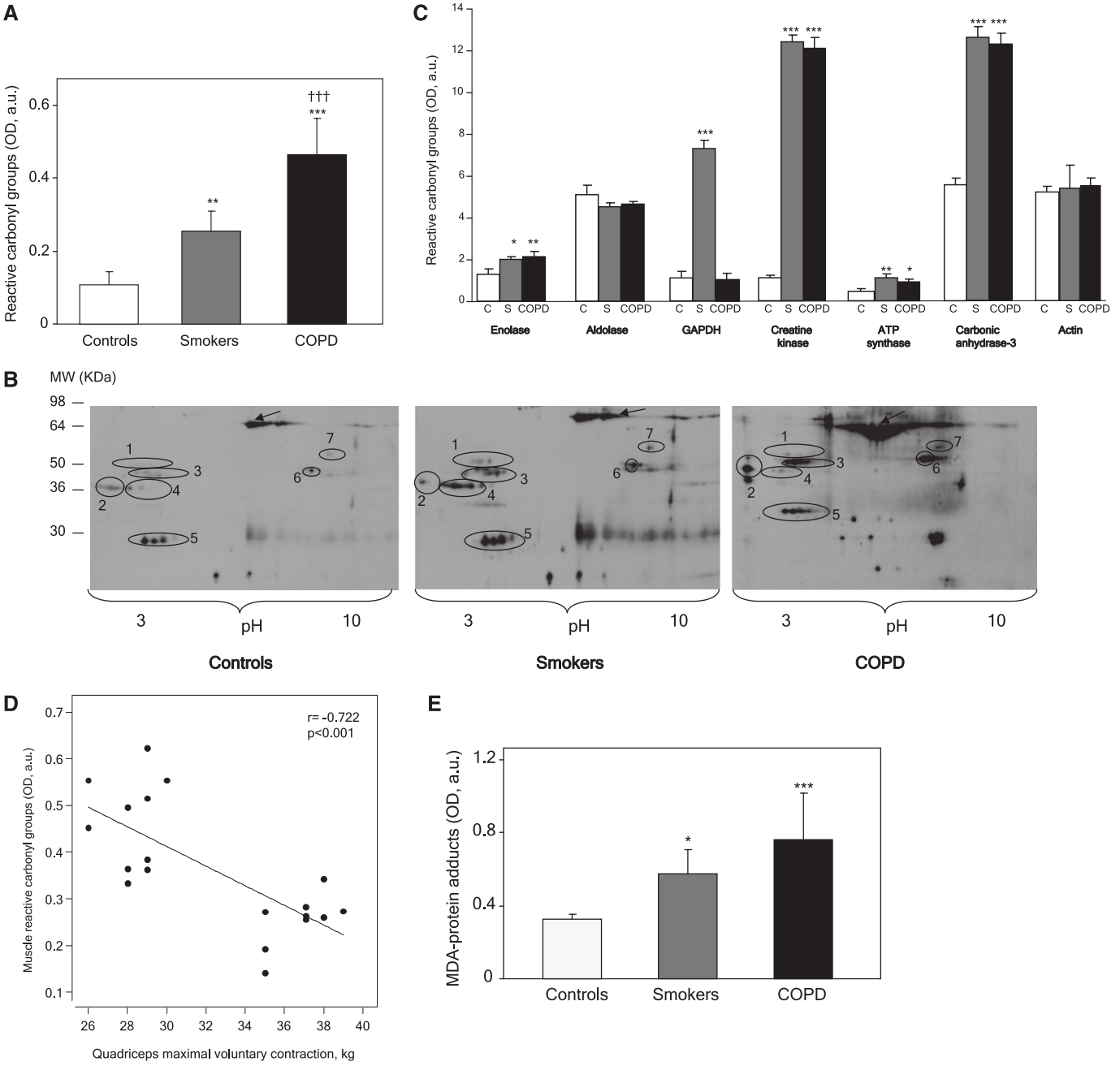

Figure 2. (*A*) Mean values and standard deviation (optical density [OD] values expressed as arbitrary units [a.u.]) of total reactive carbonyl groups were significantly higher in the quadriceps of both patients with chronic obstructive pulmonary disease (COPD) (***$P < 0.001$) and healthy smokers (**$P < 0.01$) than in control subjects. Moreover, levels of reactive carbonyls were significantly increased in the vastus lateralis of patients with COPD than in smokers (†††$P < 0.001$). (*B*) Representative two-dimensional immunoblots corresponding to the detection of carbonylated proteins in crude muscle homogenates of vastus lateralis of a healthy control subject (*left*), a smoker (*middle*), and a patient with severe COPD (*right*). β-Enolase (*1*), fructose biphosphate aldolase A (*2*), creatine kinase (*3*), glyceraldehyde-3-phosphate dehydrogenase (*4*), carbonic anhydrase-3 (*5*), actin (*6*), and ATP synthase (*7*) were consistently oxidized in the vastus lateralis of the three study groups. Albumin was also carbonylated in the muscles of both control and cachectic rats (*arrow* in each panel). (*C*) Mean values and standard deviation of total reactive carbonyls (OD values expressed as arbitrary units) of each identified protein in limb muscles of smokers, patients with COPD, and healthy control subjects. Note that levels of reactive carbonyls of several muscle proteins (enolase, glyceraldehyde-3-phosphate dehydrogenase [GAPDH], creatine kinase, ATP synthase, and carbonic anhydrase-3) were significantly greater in the vastus lateralis of the smokers (S) and patients with severe COPD (COPD) than in control subjects (C). Statistical significance is expressed as follows: smokers (S) versus control individuals (C): *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. (*D*) Among all the smokers and patients with COPD, muscle protein carbonylation levels, expressed as OD values expressed as arbitrary units, inversely correlated with quadriceps maximal voluntary contraction. (*E*) Mean values and standard deviation (OD values expressed as arbitrary units) of total malondialdehyde (MDA)–protein adducts were significantly greater in the quadriceps of both patients with COPD (***$P < 0.001$) and healthy smokers (*$P < 0.05$) than in control subjects.

TABLE 2. IDENTIFIED OXIDIZED AND NITRATED PROTEINS IN SKELETAL MUSCLES OF HUMANS AND GUINEA PIGS

	β-Enolase	Aldolase	Triose-phosphate Isomerase-1	GAPDH	Creatine Kinase	ATP Synthase	Carbonic Anhydrase-3	Actin	Tropomyosin
Identified Carbonylated roteins									
Humans									
Quadriceps muscle									
Nonsmokers	+	+		+	+	+	+	+	
Smokers	+	+		+	+	+	+	+	
Patients with severe COPD	+	+		+	+	+	+	+	
Guinea pigs									
Diaphragm									
Control subjects	+	+			+	+		+	
CS exposed	+	+			+	+		+	
Gastrocnemius									
Control subjects	+	+	+	+	+	+		+	+
CS exposed	+	+	+	+	+	+		+	+
Identified Nitrated Proteins									
Guinea pigs									
Diaphragm									
Control subjects	+	+	+		+			+	
CS exposed	+	+	+		+			+	
Gastrocnemius									
Control subjects	+	+	+	+	+			+	
CS exposed	+	+	+	+	+			+	

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; CS = cigarette smoke; GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

and 5D). Interestingly, creatine kinase activity levels were significantly decreased in both diaphragm and gastrocnemius muscles of CS-exposed guinea pigs at 4 and 6 months compared with their respective control subjects (Figures 6A and 6B).

Muscle Inflammatory Cells and Cytokines

Human studies. Muscle levels of the cytokines IL-6 and TNF-α were not significantly modified in any of the three study groups (Table 4). Levels of inflammatory cells, although low in all muscles, were significantly greater in the vastus lateralis of patients with severe COPD compared with either smokers or healthy control subjects (Table 4).

Animal studies. Chronic exposure to CS did not have any significant effects on muscle levels of the cytokines IL-6 and TNF-α and those of inflammatory cells (leukocytes and macrophages) in guinea pigs at any time (Table 5).

Muscle Fiber Structure

Human studies. Proportions of type I fibers were significantly reduced, whereas those of type II fibers were significantly increased in the vastus lateralis muscles of patients with COPD compared with either smokers or healthy control subjects (Table 3). The proportions of quadriceps muscle fibers did not significantly differ between smokers and control subjects (Table 3). The size of quadriceps type I or type II fibers did not significantly differ among the three study groups (Table 3).

Animal studies. Compared with control rodents, the diaphragm of guinea pigs exposed to CS for 6 months exhibited a significant decrease in the proportions of type I fibers, whereas those of type II fibers exhibited a significant rise in the same animals (Table 6). No significant differences were observed in muscle fiber size between exposed and nonexposed animals in any time cohort (Table 6).

TABLE 3. MUSCLE OXIDATIVE STRESS, CREATINE KINASE ACTIVITY, AND FIBER PHENOTYPE IN HUMAN STUDY SUBJECTS

	Control Subjects (*n* = 10)	Smokers (*n* = 9)	COPD (*n* = 10)
Redox markers			
Protein nitration, a.u.	0.61 (0.09)	0.78 (0.22)	0.99 (0.32)*
Mn-SOD, a.u.	0.14 (0.06)	0.23 (0.07)*	0.22 (0.07)*
Catalase, a.u.	0.15 (0.04)	0.16 (0.03)	0.18 (0.08)
Enzyme activity			
Creatine kinase activity, U/L	662.1 (148.3)	664.8 (108.06)	454.0 (67.4)†‡
Muscle fiber type, %			
Type I fibers	42 (7)	38 (8)	20 (5)§‖
Type II fibers	58 (7)	62 (8)	80 (5)§‖
Muscle fiber size (CSA), μm^2			
Cross-sectional area, type I fibers	1,907 (463)	2,046 (480)	2,149 (221)
Cross-sectional area, type II fibers	2,014 (654)	1,868 (365)	2,119 (389)

Definition of abbreviations: a.u. = arbitrary units; CSA = cross-sectional area; Mn-SOD, manganese superoxide dismutase. Values are expressed as means (SD).

* $P \leq 0.05$, between either healthy smokers or patients with COPD and healthy control subjects.

† $P \leq 0.01$, between patients with COPD and healthy control subjects.

‡ $P \leq 0.01$, between patients with COPD and healthy smokers.

§ $P \leq 0.001$, between patients with COPD and healthy control subjects.

‖ $P \leq 0.001$, between patients with COPD and healthy smokers.

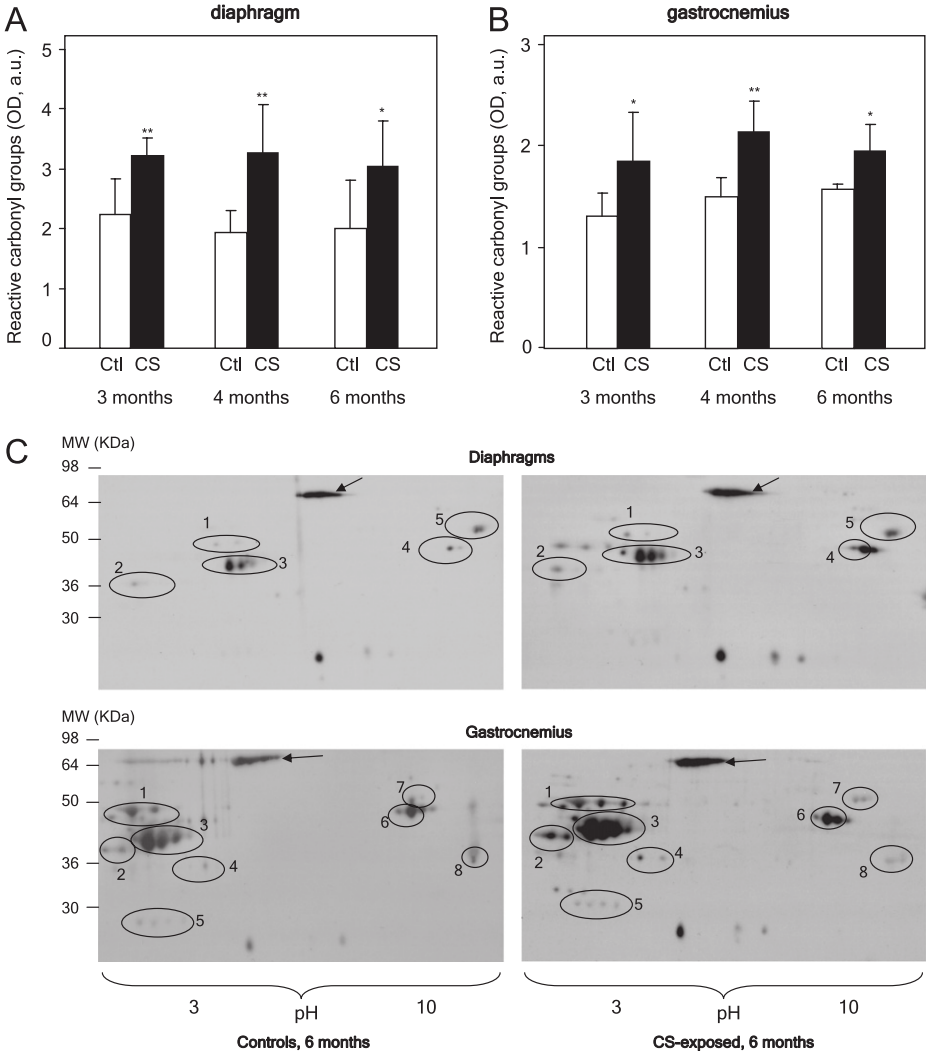

Figure 3. (*A*) Mean values and standard deviation (optical density [OD] values expressed as arbitrary units [a.u.]) of total reactive carbonyl groups were significantly higher in the diaphragm of guinea pigs exposed to cigarette smoke (CS) for 3, 4, and 6 months compared with control muscles (Ctl). Statistical significance is expressed as follows: CS-exposed animals (CS) versus control guinea pigs (Ctl): $*P < 0.05$ and $**P < 0.01$. (*B*) Mean values and standard deviation (OD values expressed as arbitrary units) of total reactive carbonyl groups were significantly greater in the gastrocnemius of guinea pigs exposed to CS for 3, 4, and 6 months compared with control muscles (Ctl). Statistical significance is expressed as follows: CS-exposed animals (CS) versus control guinea pigs (Ctl): $*P < 0.05$ and $**P < 0.01$. (*C*) Representative two-dimensional immunoblots corresponding to the detection of carbonylated proteins in crude muscle homogenates of diaphragm (*top*) and gastrocnemius (*bottom*) of control and CS-exposed guinea pigs at 6 months (*left* and *right*, respectively). β-Enolase (*1*), fructose biphosphate aldolase A (*2*), creatine kinase (*3*), actin (*4*), and ATP synthase (*5*) were consistently oxidized in the diaphragm of both CS-exposed and control guinea pigs. β-Enolase (*1*), fructose biphosphate aldolase A (*2*), creatine kinase (*3*), glyceraldehyde-3-phosphate dehydrogenase (*4*), triosephosphate isomerase (*5*), actin (*6*), ATP synthase (*7*), and tropomyosin (*8*) were consistently oxidized in the gastrocnemius of both CS-exposed and control guinea pigs. Albumin was also carbonylated in the muscles of both control and CS-exposed rodents (*arrow* in each panel). (*D*) Mean values and standard deviation of total reactive carbonyls (OD values expressed as arbitrary units) of each identified protein in the diaphragm of CS-exposed guinea pigs (S) and control animals (C) at 3 and 6 months (*left* and *right*, respectively). Note that levels of reactive carbonyls in creatine kinase protein were significantly greater in the diaphragm of CS-exposed guinea pigs than in control animals at 3 and 6 months of exposure. Statistical significance is expressed as follows: CS-exposed (S) versus control animals (C): $**P < 0.01$ and $***P < 0.001$. (*E*) Mean values and standard deviation of total reactive carbonyls (OD values expressed as arbitrary units) of each identified protein in the gastrocnemius of cigarette smoke (CS)-exposed guinea pigs (S) and control animals (C) at 3 and 6 months (*left* and *right*, respectively). Note that levels of reactive carbonyls of several muscle proteins (enolase, aldolase, glyceraldehyde-3-phosphate dehydrogenase [GAPDH], creatine kinase, actin, and tropomyosin) were significantly greater in the gastrocnemius of CS-exposed guinea pigs than in control animals. Statistical significance is expressed as follows: CS-exposed (S) versus control animals (C): $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. (*F*) Mean values and standard deviation (OD values expressed as arbitrary units) of total malondialdehyde (MDA)–protein adducts were significantly greater in the diaphragm of guinea pigs exposed to CS for 3, 4, and 6 months compared with control muscles (Ctl). Statistical significance is expressed as follows: CS-exposed animals (CS) versus control guinea pigs (Ctl): $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. (*G*) Mean values and standard deviation (OD values expressed as arbitrary units) of total MDA–protein adducts were significantly greater in the gastrocnemius of guinea pigs exposed to CS for 3, 4, and 6 months compared with control muscles (Ctl). Statistical significance is expressed as follows: CS-exposed animals (CS) versus control guinea pigs (Ctl): $**P < 0.01$ and $***P < 0.001$.

Changes in Lung Structure of Guinea Pigs

In the bronchioles of guinea pigs exposed to CS for 4 and 6 months, there was prominent goblet cell metaplasia with a four- to sevenfold increase in the number of goblet cells compared with nonexposed animals (Table 6). The alveolar space size, as measured by the nonlinear intercept, slightly increased with aging, but did not differ between CS-exposed and control animals (Table 6), indicating that CS-exposed rodents did not develop emphysema over the study period.

DISCUSSION

In skeletal muscles of both humans and guinea pigs chronically exposed to CS and of patients with COPD compared with control muscles, the following modifications were observed: (*1*) a mild but significant reduction in quadriceps muscle force in the healthy smokers, (*2*) an inverse relationship between muscle protein carbonylation levels and quadriceps force among smokers and patients with COPD, (*3*) increased protein oxidation in the quadriceps of smokers and patients with COPD as well as in diaphragm and gastrocnemius of CS-exposed animals, (*4*) a significant rise in protein nitration in both respiratory and limb muscles of CS-exposed rodents but not in human smokers, (*5*) a significant increase in oxidative modifications of proteins involved in glycolysis, energy production and distribution, carbon dioxide hydration, and muscle contraction in both humans and guinea pigs, (*6*) a CS exposure–induced, significant

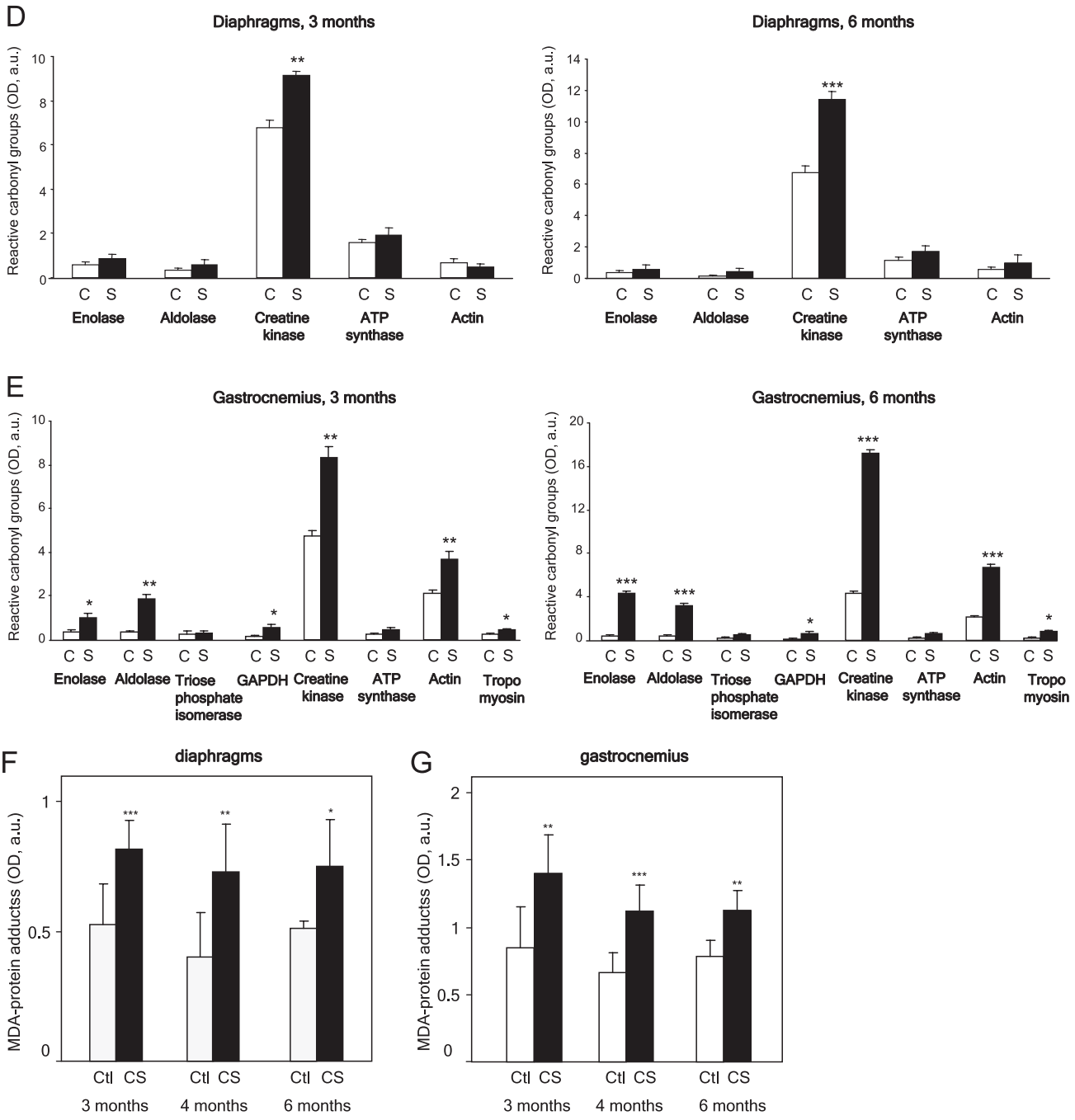

Figure 3. (*Continued*)

reduction in creatine kinase activity in both respiratory and limb muscles of guinea pigs, and (*7*) a lack of any significant effect on muscle inflammatory cell or cytokine levels subsequent to chronic exposure to CS.

Interestingly, in the present investigation, quadriceps muscle force was significantly reduced, although mildly, in healthy smokers compared with control subjects. This finding is in line with a previous study, in which healthy smokers exhibited greater peripheral muscle fatigue (11). Also, as previously reported (9, 26, 30), patients with severe COPD, independently of their muscle mass, exhibited a significant reduction in quadriceps muscle function (27%) compared with healthy control subjects and smokers. In addition, among the population of smokers and patients with severe COPD, muscle protein carbonylation levels were inversely correlated with quadriceps muscle force. This is in agreement with former studies from our group (26, 30), in which muscle protein oxidation was also shown to negatively correlate with quadriceps muscle function in patients with COPD.

The present investigation is the first to provide evidence of the posttranslational oxidative modifications induced by both ROS and RNS on muscle proteins in human smokers and in animals chronically exposed to CS. Interestingly, in agreement with our initial hypothesis, protein oxidation, as measured by either reactive carbonyls or MDA–protein adducts, was significantly increased in the muscles of both smokers and exposed guinea pigs. Increased levels of protein tyrosine nitration, a biological marker of excessive RNS production, reached

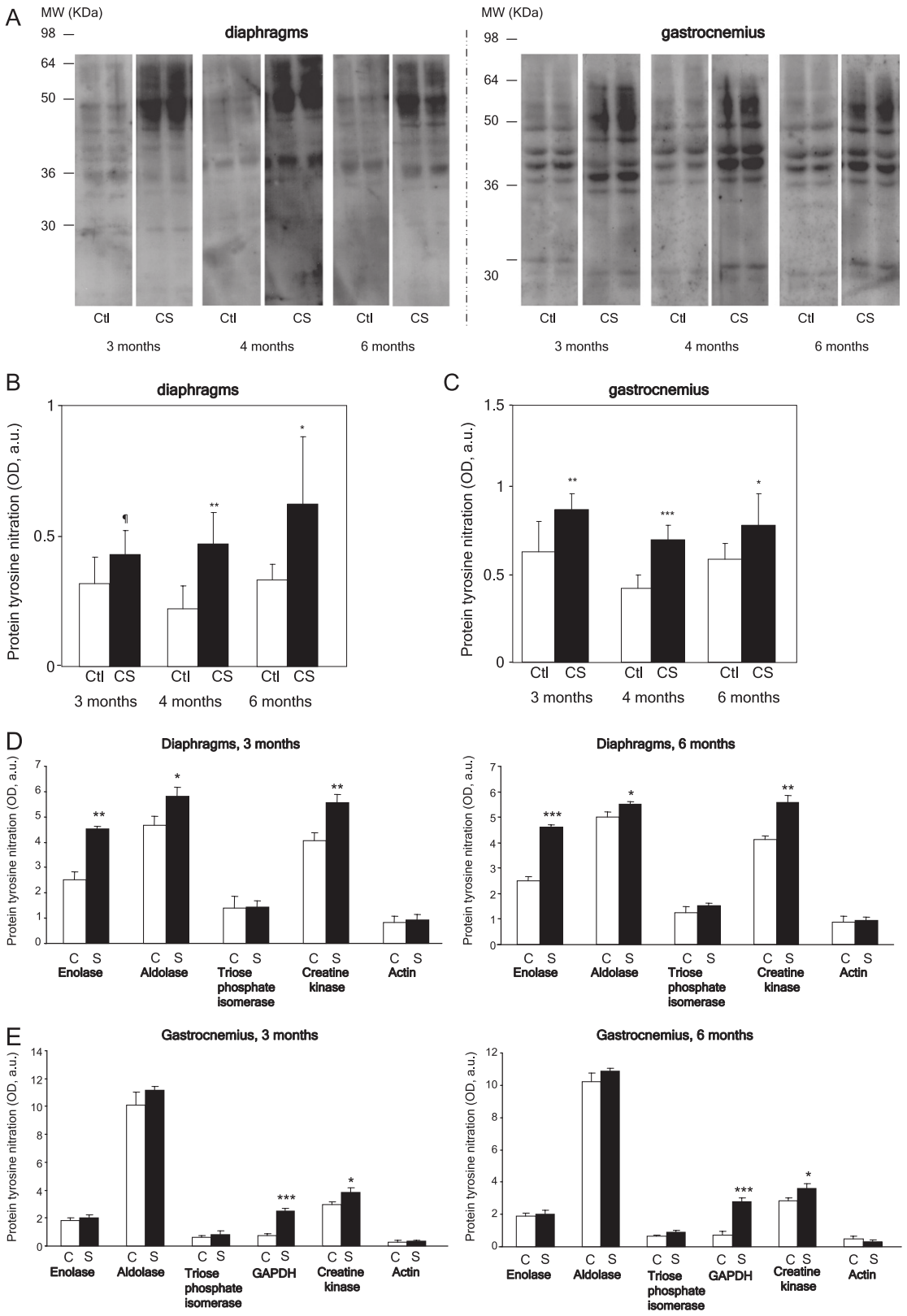

Figure 4. (*A*) Representative examples of protein tyrosine nitration immunoblots in the diaphragm and gastrocnemius muscles of cigarette smoke (CS)–exposed and control (Ctl) guinea pigs at 3, 4, and 6 months. Several tyrosine-nitrated proteins were detected. (*B*) Mean values and standard deviation (optical density [OD] values expressed as arbitrary units [a.u.]) of total protein nitration were significantly higher in the diaphragm of guinea pigs exposed to CS for 3, 4, and 6 months compared with control muscles (Ctl). Statistical significance is expressed as follows: CS-exposed animals (CS) versus control guinea pigs (Ctl): $P = 0.070$, $^{*}P < 0.05$, and $^{**}P < 0.01$. (*C*) Mean values and standard deviation (OD values expressed as arbitrary units) of total protein nitration were significantly greater in the gastrocnemius of guinea pigs exposed to CS for 3, 4, and 6 months compared with control muscles (Ctl). Statistical significance is expressed as follows: CS-exposed animals (CS) versus control guinea pigs (Ctl): $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. (*D*) Mean values and standard deviation of total protein tyrosine nitration (OD values expressed as arbitrary units) of each identified protein in the diaphragm of CS-exposed guinea pigs (S) and control animals (C) at 3 and 6 months (*left* and *right*, respectively). Note that levels of nitrotyrosine formation of the proteins enolase, aldolase, and creatine kinase were significantly greater in the diaphragm of CS-exposed guinea pigs than in control animals. Statistical significance is expressed as follows: CS-exposed (S) versus control animals (C): $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. (*E*) Mean values and standard deviation of total protein tyrosine nitration (OD values expressed as arbitrary units) of each identified protein in the gastrocnemius of CS-exposed guinea pigs (S) and control animals (C) at 3 and 6 months (*left* and *right*, respectively). Note that levels of nitrotyrosine formation of the proteins glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and creatine kinase were significantly greater in the gastrocnemius of CS-exposed guinea pigs than in control animals. Statistical significance is expressed as follows: CS-exposed (S) versus control animals (C): $^{*}P < 0.05$ and $^{***}P < 0.001$.

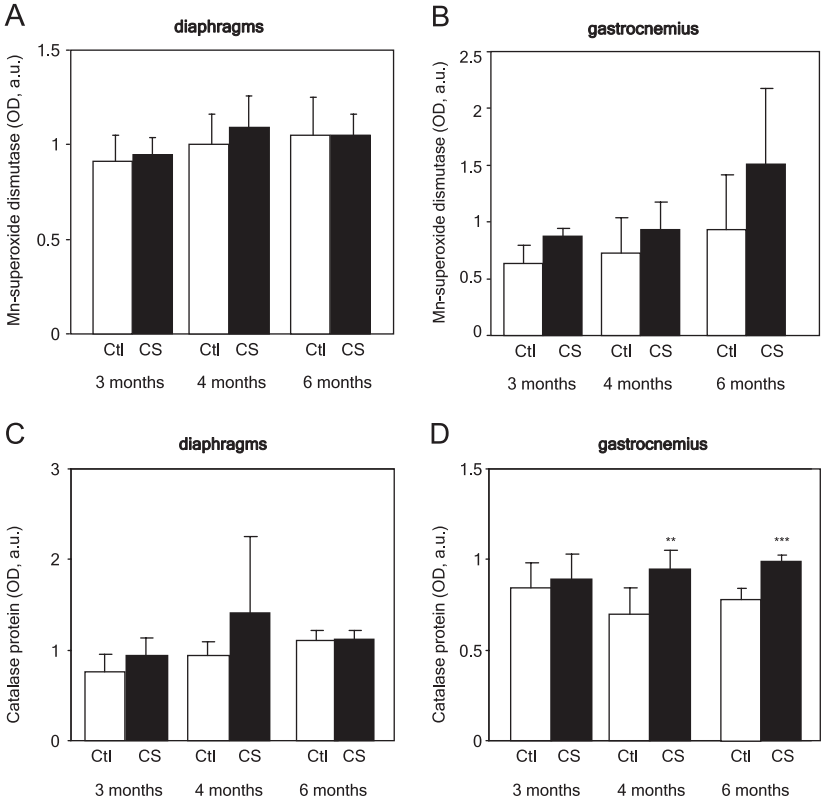

Figure 5. (*A*) Mean values and standard deviation (optical density [OD] values expressed as arbitrary units [a.u.]) of manganese (Mn)-superoxide dismutase protein did not significantly differ in the diaphragm of guinea pigs exposed to cigarette smoke (CS) for 3, 4, and 6 months compared with control muscles (Ctl). (*B*) Mean values and standard deviation (OD values expressed as arbitrary units) of Mn-superoxide dismutase protein did not significantly differ in the gastrocnemius of guinea pigs exposed to CS for 3, 4, and 6 months compared with control muscles (Ctl). (*C*) Mean values and standard deviation (OD values expressed as arbitrary units) of catalase protein did not significantly differ in the diaphragm of guinea pigs exposed to CS for 3, 4, and 6 months compared with control muscles (Ctl). (*D*) Mean values and standard deviation (OD values expressed as arbitrary units) of catalase protein were significantly greater in the gastrocnemius of guinea pigs exposed to CS for 4 and 6 months compared with control muscles (Ctl). Statistical significance is expressed as follows: CS-exposed animals (CS) versus control guinea pigs (Ctl): $^{**}P < 0.01$, and $^{***}P < 0.001$.

statistical significance only in the diaphragm and gastrocnemius of CS-exposed animals and in the quadriceps of patients with severe COPD, but not in the vastus lateralis of human smokers. This is in keeping with a previous investigation in which increased protein tyrosine nitration was demonstrated in the vastus lateralis of patients with COPD and no differences were detected in the levels of several nitric oxide end-products, including nitrotyrosine, in the quadriceps of human smokers compared with control subjects (14).

Peroxynitrite, which is formed from the near-diffusion-limited reaction between nitric oxide and superoxide anions, accounts for most protein tyrosine nitration in skeletal muscles (28). In the current study, Mn-SOD protein content, but not catalase, was significantly increased in the quadriceps of both human smokers and patients with severe COPD compared with control subjects. In CS-exposed guinea pigs, however, muscle Mn-SOD levels did not differ from those in control animals, and chronic CS exposure induced a significant rise in total protein tyrosine nitration in the respiratory and limb muscles of guinea pigs. In view of these findings, it could be concluded that in patients with severe COPD and in the muscles of guinea pigs, greater production of RNS than in healthy smokers may have outcompeted with Mn-SOD for superoxide anion, eventually leading to the formation of significantly increased levels of protein tyrosine nitration within the vastus lateralis muscle.

Importantly, in the guinea pig model, the effects of oxidants on muscle proteins were observed in both respiratory and limb muscles, suggesting that chronic CS exposure probably exerted direct deleterious effects on all muscles of the exposed animals. Likewise, the significant increase in muscle protein oxidation observed in human smokers, free of lung or cardiovascular disease, is likely to be attributed to a direct action of ROS and RNS (aldehydes, peroxides, nitrogen oxides, and peroxyl radicals, among others) contained in CS. Moreover, the effects of oxidants on muscles occurred at an earlier stage than the effects observed in the respiratory system. These findings reinforce the concept that CS per se is likely to be involved in direct tissue toxicity in the skeletal muscles of CS-exposed guinea pigs, regardless of lung and bronchial alterations. In fact, our findings are in total agreement with previous investigations, in which a rise in various oxidative stress markers was demonstrated in the blood, lungs, and other organs of human smokers and

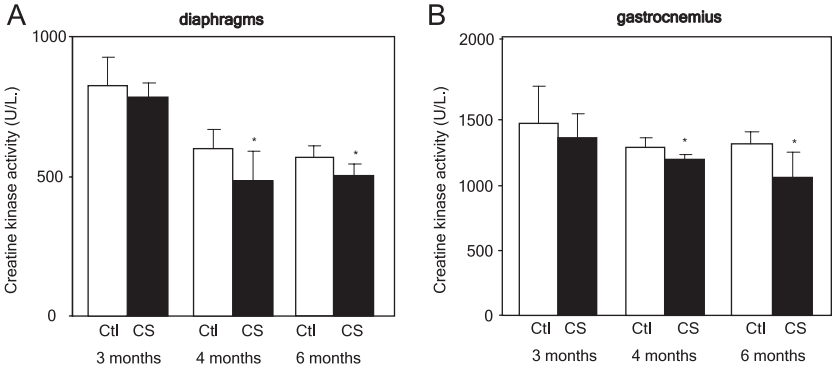

Figure 6. (*A*) Mean values and standard deviation (activity units [U]/L) of creatine kinase activity were significantly lower in the diaphragm of guinea pigs exposed to cigarette smoke (CS) for 4 and 6 months compared with control muscles (Ctl). Statistical significance is expressed as follows: CS-exposed animals (CS) versus control guinea pigs (Ctl): $^{*}P < 0.05$. (*B*) Mean values and standard deviation (activity units [U]/L) of creatine kinase activity were significantly lower in the gastrocnemius of guinea pigs exposed to CS for 4 and 6 months compared with control muscles (Ctl). Statistical significance is expressed as follows: CS-exposed animals (CS) versus control guinea pigs (Ctl): $^{*}P < 0.05$.

TABLE 4. MUSCLE INFLAMMATION IN HUMAN STUDY SUBJECTS

	Control Subjects ($n = 10$)	Smokers ($n = 9$)	COPD ($n = 10$)
IL-6, pg/ml	0.29 (0.19)	0.23 (0.12)	0.32 (0.30)
TNF-α, pg/ml	1.64 (0.37)	1.72 (0.33)	1.64 (0.24)
Total inflammatory cells, cells/mm^2	0.99 (0.60)	0.88 (0.51)	2.57 (1.70)*†

Definition of abbreviations: TNF = tumor necrosis factor.
Values are expressed as means (SD).
* $P \leq 0.05$ between patients with COPD and healthy control subjects.
† $P \leq 0.05$ between patients with COPD and healthy smokers.

animals chronically exposed to CS (1–7). In line with this, in a previous study (7), guinea pigs acutely exposed to CS also exhibited a significant increase in plasma lipid peroxidation together with a reduction in muscle glutathione levels immediately after the exposure.

It should also be mentioned that inflammatory events, that is, muscle proinflammatory cytokines and inflammatory cell infiltration, are not likely to contribute to muscle protein oxidation or nitration in any of the models of chronic exposure to CS analyzed in this investigation. In fact, only the vastus lateralis of patients with severe COPD exhibited a significant increase in inflammatory cell infiltration compared with either smokers or healthy control subjects. Nevertheless, this significant increase is likely to be of little biological relevance, because absolute levels were extremely low in all muscle specimens from both humans and rodents.

It is worth mentioning that in the current experimental setting, exposure to CS for 6 months was insufficient to induce pulmonary emphysema. In fact, bronchiole goblet cell metaplasia, but not lung morphometric modifications, was the only histological alteration found in the respiratory tract of CS-exposed guinea pigs. These findings are in line with a previous investigation from our group (7), but are in contrast with previously published studies in which guinea pigs were also chronically exposed to CS (15, 35, 36). Indeed, the length of CS exposure required to cause emphysema varies across animal species and is dependent on the methods of exposure and cigarette dose (15, 17). In keeping with this, in the present study, guinea pigs were exposed to a relatively moderate content of nicotine and other compounds in the cigarette smoke, as established by Diamond and colleagues (37). On this basis, differences in the dose of nicotine and other chemicals contained in CS, relatively high in some investigations (35–37) and moderate in others (7), might account for the discrepancies among studies regarding the development of emphysema in guinea pigs chronically exposed to CS. It should also be discussed that although chronic CS exposure did not induce lung destruction in the current study, it may have been sufficient to promote elastase-induced emphysema, as previously demonstrated (37).

In the present investigation, to understand the pathophysiological consequences of posttranslational oxidative modifications of the muscle proteins, the nature of the oxidatively modified proteins was identified. Importantly, this study is the first to show that highly abundant proteins involved in glycolysis, energy production and distribution, carbon dioxide hydration, and muscle contraction were significantly more oxidized in the quadriceps of human smokers and patients with severe COPD, and in the diaphragm and gastrocnemius of guinea pigs chronically exposed to CS. Interestingly, the line has been put forward that these specific proteins are prone to suffer oxidative modifications under certain experimental conditions. For instance, the diaphragm of endotoxemic rats exhibited increased oxidative modifications of glycolytic enzymes, creatine kinase, carbonic anhydrase-3, and contractile actin (32), as well as the diaphragm and vastus lateralis of patients with severe COPD (33). In the current investigation, creatine kinase and carbonic anhydrase-3 displayed the greatest oxidative modifications in the vastus lateralis of healthy smokers and patients with severe COPD. These findings are in agreement with previous studies from our group (25, 33), in which creatine kinase was also shown to be highly modified by oxidants and its activity significantly reduced in the muscles of patients with severe COPD. In the current investigation, creatine kinase activity was significantly reduced only in the vastus lateralis of patients with severe COPD, but not in smokers. It is likely that the amount of oxidants in muscles of the latter was still not sufficient to induce a significant decrease in the activity of this enzyme. On the other hand, chronic exposure to CS induced a significant decrease in creatine kinase activity in both the diaphragm and gastrocnemius muscles of guinea pigs at 4 and 6 months but not at 3 months. Modifications of the activity of creatine kinase may have relevant implications in muscle performance in response to chronic exposure to CS and in severe COPD. Clearly, future studies will shed light on the specific mechanisms whereby posttranslational oxidative modifications may lead to muscle protein loss and dysfunction in active smokers and patients with severe COPD.

Despite potential controversies with previous studies (7, 35, 36), in the present investigation CS was shown to influence body weight as demonstrated by the observed reduction in body weight gain in animals chronically exposed to CS for as little as 4 months. Although not specifically quantified, food intake between CS-exposed and control rodents was similar, even after 4 months. It should be noted that a reduction in body

TABLE 5. MUSCLE INFLAMMATION IN GUINEA PIGS AT VARIOUS PERIODS OF CIGARETTE SMOKE EXPOSURE

	3 mo		4 mo		6 mo	
	Control ($n = 7$)	CS Exposed ($n = 7$)	Control ($n = 7$)	CS Exposed ($n = 7$)	Control ($n = 7$)	CS Exposed ($n = 7$)
IL-6, pg/ml						
Diaphragm	7.80 (1.57)	9.42 (1.60)	5.47 (1.76)	7.79 (3.18)	5.38 (0.98)	5.22 (0.74)
Gastrocnemius	5.48 (1.21)	6.40 (1.70)	4.48 (0.76)	4.20 (1.05)	4.25 (0.34)	5.41 (1.96)
TNF-α, pg/ml						
Diaphragm	0.39 (0.17)	0.51 (0.23)	0.25 (0.05)	0.38 (0.29)	0.27 (0.10)	0.23 (0.05)
Gastrocnemius	0.38 (0.29)	0.38 (0.54)	0.30 (0.19)	0.25 (0.25)	0.21 (0.06)	0.24 (0.17)
Total inflammatory cells, cells/mm^2						
Diaphragm	0.99 (0.91)	1.45 (1.00)	0.45 (0.28)	0.75 (0.39)	0.31 (0.31)	0.46 (0.30)
Gastrocnemius	0.41 (0.24)	0.57 (0.18)	0.44 (0.22)	0.66 (0.51)	0.43 (0.58)	0.76 (0.02)

Definition of abbreviations: CS = cigarette smoke; TNF = tumor necrosis factor.
Values are expressed as means (SD).

TABLE 6. MUSCLE FIBER PHENOTYPE AND LUNG STRUCTURE IN GUINEA PIGS AT VARIOUS PERIODS OF CIGARETTE SMOKE EXPOSURE

	3 mo		4 mo		6 mo	
	Control ($n = 7$)	CS Exposed ($n = 7$)	Control ($n = 7$)	CS Exposed ($n = 7$)	Control ($n = 7$)	CS Exposed ($n = 7$)
Muscle fiber type, %						
Diaphragm, type I	37 (5)	34 (2)	29 (3)	30 (4)	35 (5)	30 (2)*
Gastrocnemius, type I	10 (3)	10 (4)	11 (5)	8 (3)	9 (3)	13 (6)
Diaphragm, type II	63 (5)	66 (2)	71 (3)	70 (4)	65 (5)	70 (2)*
Gastrocnemius, type II	90 (3)	90 (4)	89 (5)	92 (3)	91 (3)	87 (6)
Muscle fiber size (CSA), μm^2						
Diaphragm, type I	734 (143)	666 (304)	593 (146)	647 (195)	697 (192)	757 (134)
Gastrocnemius, type I	894 (256)	779 (198)	797 (200)	787 (212)	1,010 (399)	1,296 (582)
Diaphragm, type II	850 (135)	743 (127)	706 (204)	685 (148)	1,013 (130)	908 (203)
Gastrocnemius, type II	1,154 (325)	1,129 (247)	1,148 (228)	1,125 (246)	1,545 (253)	1,328 (248)
Lung structure						
Goblet cell metaplasia, cells/mm	0.4 (1.9)	0.4 (1.4)	1.0 (3.3)	4.3 (8.0)*	0.5 (1.6)	3.6 (6.3)*
Mean linear intercept, μm	60 (18)	61 (18)	74 (24)	72 (20)	75 (29)	72 (37)

Definition of abbreviations: CS = cigarette smoke; CSA = cross-sectional area.
Values are expressed as means (SD).
* $P \leq 0.05$, between CS-exposed and control guinea pigs.

weight gain, rather than the characteristic body weight and muscle mass loss of patients with COPD, was the outcome variable in the current study. On this basis, exploring whether the pathophysiological mechanisms leading to decreased body weight gain in CS-exposed animals share similarities with those involved in muscle mass loss and dysfunction in smokers and patients with COPD, which might even precede the pulmonary disease, warrants further attention in future studies.

In the current investigation, only the diaphragm, but not the limb muscles, of guinea pigs exposed to CS for 6 months exhibited a switch to a more glycolytic phenotype. Previous studies have yielded discrepant results regarding muscle phenotype changes in response to chronic CS exposure. In this regard, our findings are in agreement with those reported in earlier studies (38, 39), in which proportions of type I fibers were shown to be reduced in the quadriceps of smokers. More recently, Gosker and colleagues (40) have demonstrated that the soleus muscle of mice chronically exposed to CS also exhibited a reduction in the proportions of type IIa oxidative fibers compared with control muscles. Nonetheless, in another study (14), the size, but not the proportions, of type I and type IIa fibers was shown to be reduced in the quadriceps of smokers compared with control subjects. Interestingly, in an animal model of hypertensive rats exposed to CS, the soleus also exhibited a reduction in the percentage of type I fibers along with a decrease in the areas of all muscle fibers (12), whereas the extensor digitorum longus exhibited only a reduction in the size, but not in the proportions, of both oxidative and glycolytic fibers (13). In view of these results, it could be concluded that the predominance of an oxidative or glycolytic phenotype in a given muscle may account for the differences among studies related to CS-induced effects on muscle structure.

Study Limitations

See the online supplement for additional information.

A first limitation in the current investigation has to do with the relatively small number of healthy smokers, patients with COPD, and healthy control subjects studied. However, it should also be considered that muscle biopsies were obtained from current smokers free of lung or cardiovascular disease, from healthy control subjects, and from patients with severe and very severe COPD. In addition, the experimental model used, that is, guinea pigs chronically exposed to CS, also helped elucidate the oxidative phenomena directly induced by CS on skeletal muscle proteins in two different compartments, the respiratory and limb muscles.

A second limitation has to do with the lack of functional data concerning either the respiratory or limb muscles of guinea pigs. An initial step in this field of investigation was to explore the specificity of the oxidative phenomena of skeletal muscle proteins as well as their differential regulation in response to chronic exposure to CS in two different models: human and animal studies. On the other hand, it should also be taken into account that peripheral muscle function was, indeed, evaluated in smokers, patients with severe COPD, and healthy control subjects in the present investigation.

A third limitation is related to the nature of the identified proteins by means of two-dimensional electrophoresis and proteomics analyses in the muscle homogenates from both humans and guinea pigs. It is likely that less abundant muscle proteins or proteins of larger sizes may not have been detected in this system. Future investigations will be designed to explore whether proteins of specific muscle compartments and/or higher molecular weights could also be modified by ROS and RNS in response to chronic CS exposure.

Conclusion

In the present study, it is demonstrated for the first time that CS exerts a mild but significant reduction in quadriceps muscle force together with direct oxidative modifications of specific muscle proteins, without inducing any significant rise in muscle inflammation. The posttranslational oxidative alterations of the muscle proteins may negatively influence their function, for example, creatine kinase activity, eventually rendering the modified proteins more susceptible to increased protein breakdown, which in turn would lead to muscle loss and dysfunction in smokers and patients with COPD. In the animal model, CS-induced oxidative stress occurred in the muscles as early as 3 months after exposure. Importantly, this event preceded the characteristic bronchiolar and parenchymal changes induced by CS in the lungs, suggesting a direct toxic effect of CS on skeletal muscle proteins.

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