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Systemic effects of cigarette smoke exposure in the guinea pig

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Summary Chronic obstructive pulmonary disease is associated with systemic effects including reduced body weight, oxidative stress and altered circulating TNFα levels. The present study was aimed to investigate whether chronic exposure to cigarette smoke induces these systemic changes in a guinea pig model.

Seven animals/group were exposed to the smoke of seven cigarettes/day, 5 days/week, during 2, 4 and 6 months (chronic exposure). Three animals/group were sacrificed immediately, 3h or 24h after exposure to seven cigarettes (acute exposure). Chronically smoke-exposed animals exhibited lower body weight gain, starting at 5th week, and goblet cell metaplasia in small bronchioles. At 6 months there was a trend for increased plasma and lung tissue TNFα levels. No changes, neither in skeletal muscle glutathione (GSH) nor in plasma lipid peroxidation, were observed at any time point after chronic exposure. However, skeletal muscle GSH decreased and plasma lipid peroxidation increased immediately after acute smoke exposure, equaling control levels thereafter.

We conclude that cigarette smoke exposure in the guinea pig induces a transient and repeated oxidative effect, which might result in impaired systemic metabolism and consequent failure of smoke-exposed animals to gain weight. The effects of cigarette smoke on body weight antecede and appear to be independent from the alterations produced in small airways.
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Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by an inappropriate/excessive inflammatory response of the lung to respiratory pollutants, mainly cigarette smoking.[1] In addition to the typical pulmonary pathologic changes of COPD, several effects occurring outside the lung have been described, the so-called systemic effects of the disease.[2] There is increasing awareness that these systemic effects are clinically relevant and may contribute to a better understanding and management of the disease.

Recent studies have clearly shown that COPD is associated not only with an abnormal inflammatory response in the lung parenchyma, but also with evidence of systemic inflammation, namely systemic oxidative stress, activation of circulating inflammatory cells and increased levels of proinflammatory cytokines.[3]

The level of lipid peroxidation in plasma, as an index of oxidative stress, is significantly increased by both cigarette smoking and COPD, the latter being particularly significant during exacerbation episodes.[4] Numerous studies have reported increased levels of circulating cytokines and acute reactants in the peripheral circulation of patients with COPD, particularly, increased concentrations of TNFα, its receptors (TNFR-55 and TNFR-75), IL-6, IL-8, C-reactive protein, lipopolysaccharide-binding protein and FAS and Fas ligand.[5–8] It is interesting to note that chronic elevation of TNFα is associated with muscle wasting that can be ameliorated with antibodies against TNF.[9,10]

Cigarette smoking alone can cause, in the absence of COPD, significant extrapulmonary diseases (e.g. coronary artery disease). Young smokers and even passive smokers may show endothelial dysfunction of the systemic vessels[11,12] and systemic oxidative stress.[13] Oxidative stress causes muscle fatigue[14] and facilitates proteolysis.[15,16] This may be particularly relevant in COPD since the regulation of glutathione (GSH), the most important intracellular antioxidant,[17] is abnormal in the skeletal muscle of COPD patients.[18] Indeed, an optimal GSH content is necessary to ensure the appropriate redox environment needed for myogenesis.[19]

Cigarette smoking has the potential to contribute to systemic effects of COPD. Nevertheless, although it is accepted that cigarette smoking is the main risk factor for COPD,[1] much less attention has been paid to its potential effects on skeletal muscle structure and function. There is a wide variety of animal models of cigarette smoke-induced lung disease that have been used to investigate different aspects of COPD.[20] However, little is known about the potential systemic effects of cigarette smoking in these animal models.

Guinea pigs chronically exposed to cigarette smoke develop pulmonary histological changes (emphysema, goblet cell metaplasia in small bronchioles, vascular remodeling) and functional abnormalities (increased lung volumes, gas trapping, pulmonary hypertension)[21] that are similar to those seen in humans with COPD. Accordingly, we used the guinea pig model to investigate whether chronic or acute exposure to cigarette smoke induces systemic changes, such as increased plasma levels of TNFα and oxidative stress, both in plasma and skeletal muscle.

Materials and methods

Materials and reagents

GSH and GSSG reductase were purchased from Sigma (Madrid, Spain). 2′-7′-Dichlorofluorescein was purchased from Molecular Probes (Eugene, OR, USA).

Experimental protocol

Chronic exposure

Groups of seven male Hartley guinea pigs, initially weighting 300 g, were exposed to the smoke of seven commercial cigarettes (11 mg tar, 0.8 mg nicotine per cigarette) each day, 5 days per week, for periods of 2, 4 and 6 months using a nose-only inhalation system.[21] Control animals (Ctrl) followed the same procedures except cigarette smoke exposure. Diet was given ad libitum and water was supplemented with Vit C (1 g/L) (Roche Farma S.A., Madrid, Spain).

At the end of each period the animals were anesthetized 24 h after the last session of smoke exposure. Whole blood was obtained from carotid artery and collected in tubes with EDTA. After centrifugation at 1200g 10 min, plasma samples were immediately stored at −70 °C until analyzed.

Skeletal muscle samples from the *gastrocnemius* muscle and lung samples were obtained. Fresh muscle (2 g) was used to isolate mitochondria. The remaining fresh muscle tissue and lung samples were immediately placed in liquid N_2 and stored at −70 °C until analyzed.

Acute exposure

Three male Hartley guinea pigs per group were exposed to the smoke of seven commercial

cigarettes and sacrificed for study immediately, 3 h or 24 h after exposure. Control animals (Ctrl) followed the same procedures except cigarette smoke exposure.

Skeletal muscle and lung tissue samples were processed and analyzed as described above. Whole blood was drawn from the carotid artery. Carboxyhemoglobin concentration was determined using a gas analyzer (CIBA-Corning 860, CIBA-Corning Diagnostics Corporation, Medfield, MA, USA).

Mitochondria isolation and GSH determination
Mitochondria skeletal muscle was obtained by a modification of the Rustin method.[22] Briefly, approximately 2 g of muscle was homogenized in medium containing 20 mM Tris pH = 7.2, 0.25 M sucrose, 40 mM KCl, 2 mM EGTA and 1 mg/ml BSA (medium A) and centrifuged twice at 500g for 8 min. The supernatants of both centrifugations were further centrifuged at 12000g for 8 min. The supernatant was discarded and the pellet was centrifuged again in medium A plus 5% Percoll. The resulting pellet containing the mitochondrial fraction was resuspended in 0.5 ml homogenization medium. Mitochondria purity was confirmed by specific activity of succinate dehydrogenase in the final mitochondrial fraction with respect to that of the homogenate. Homogenate and mitochondria samples were treated with 5% trichloroacetic acid, the mixture was centrifuged to discard precipitated protein, and the content of GSH and GSSG was analyzed by HPLC.[23]

Reactive oxygen species determination
Hydrogen and other organic peroxides were monitored spectrofluorimetrically in isolated mitochondria samples, using chlorometyl-2′-7′-dichlorodihydrofluorescein diacetate (2 μmol/L) determining the fluorescence of 2′-7′-dichlorofluorescein (DCF) as described previously in detail.[23] Relative fluorescence units were normalized per mg of cellular protein.

Lipid peroxidation
Lipid peroxidation, as malondialdehyde (MDA) and 4-hydroxinonenal (4-HNE), was analyzed in frozen plasma samples spectrophotometrically using a commercial kit (Byoxytech. LPO-586, Oxys Research, Portland, OR, USA).

TNFα levels determination
TNFα levels were determined in plasma and lung homogenates samples by ELISA (BD Biosciences, San Jose, CA, USA).

Morphometric studies in lung tissue
Formalin fixed paraffin-embedded lung tissue sections were cut and stained with H&E and alcian blue to evaluate the number of secretory cells in small airways as an index of bronchial damage induced by cigarette smoke.[24] The number of secretory cells in the airway epithelium of small noncartilagenous bronchioles was counted and expressed as the number of cells per mm of epithelial length.

Nutritional status
Plasma nutritional profile was assessed to check for potential malnourishment in smoke-exposed animals. Cholesterol was determined with a Bayer reagent (B01-4124-01), triglycerides with an Olympus System reagent (66003), and total proteins by a modified Biuret method (OSR6232), and albumin with a Bayer bromocresol green (B01-4121-01). All parameters were determined spectrophotometrically by an autoanalyzer ADVIA 1650 (Bayer Tarrytown, New York, USA).

Statistics
All data were expressed as mean ± SD. Comparisons between groups were performed using an analysis of variance (ANOVA) or the Kruskal–Wallis test, when appropriate. Comparisons were carried out using the Student's test. Correlations between variables were analyzed with Pearson's coefficient. Probability values lower than 0.05 were considered as significant.

Results

To validate the method of smoke exposure used in this study, blood carboxyhemoglobin (HbCO) concentration was measured in guinea pigs acutely exposed to cigarette smoke. The concentration of HbCO in arterial blood, measured immediately after exposing the animals to the smoke of seven cigarettes, was 13 ± 1%.

Effects of long-term exposure to cigarette smoke

Skeletal muscle and plasma oxidative stress
GSH concentration in skeletal muscle was measured to evaluate the potential systemic impact of cigarette smoke. The levels of total GSH (GSH plus GSSG) in skeletal muscle homogenate and mitochondria after 2, 4 and 6 months of cigarette smoke exposure, are shown in Fig. 1(A and B). Although no significant differences in GSH levels were observed between the groups at the different exposure

periods, there was a trend for lower GSH levels in mitochondria (Fig. 1B) after 2 months of cigarette smoke exposure compared with the control group ($p = 0.07$).

Because cigarette smoke is associated with an overproduction of reactive oxygen species (ROS), we determined ROS generation by the skeletal muscle mitochondria, as well as levels of MDA plus 4-HNE in plasma as end products of oxidative stress. No evidence for increased ROS was detected at 2 and 4 months of exposure (data not shown). At 6 months there was a trend to increase ROS generation in mitochondria of smoke-exposed animals (1.5 fold increase in smokers vs. controls, $p = 0.08$). MDA+4-HNE plasma levels were not significantly different in control and smoke-exposed animals at any time of exposure (Fig. 1C). In agreement with the results of plasma lipid peroxidation and skeletal muscle GSH levels, there was no evidence of an increased systemic oxidative stress.

Body weight

The evolution of body weight of control and smoke-exposed animals over time is shown in Fig. 2. The rate of body weight gain in guinea pigs exposed to cigarette smoke was lower than in control animals. Difference in weight gain started at week 5 and persisted thereafter. Smoke-exposed animals weighed approximately 100 g less than controls at

every time point of the study. To discard malnourishment as a potential cause of reduced body weight gain we measured parameters of nutritional profile in plasma (Table 1). No differences in the
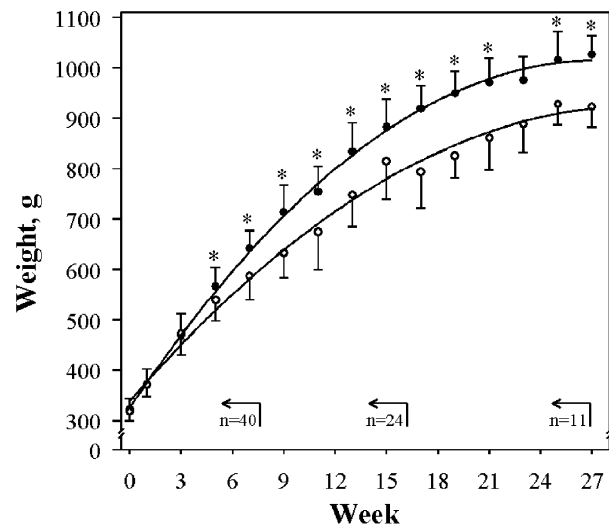
Figure 2 Rate of body weight gain over time. Smoke-exposed guinea pigs (open symbols), initially weighting 300 g, showed a reduced rate of weight gain, beginning at the 5th week of exposure until the final of the study, as compared with control guinea pigs (closed symbols). Symbols show mean ± SD. $n =$ denotes the total number of animals studied until that time point. *$p < 0.05$ compared with smoke-exposed group.
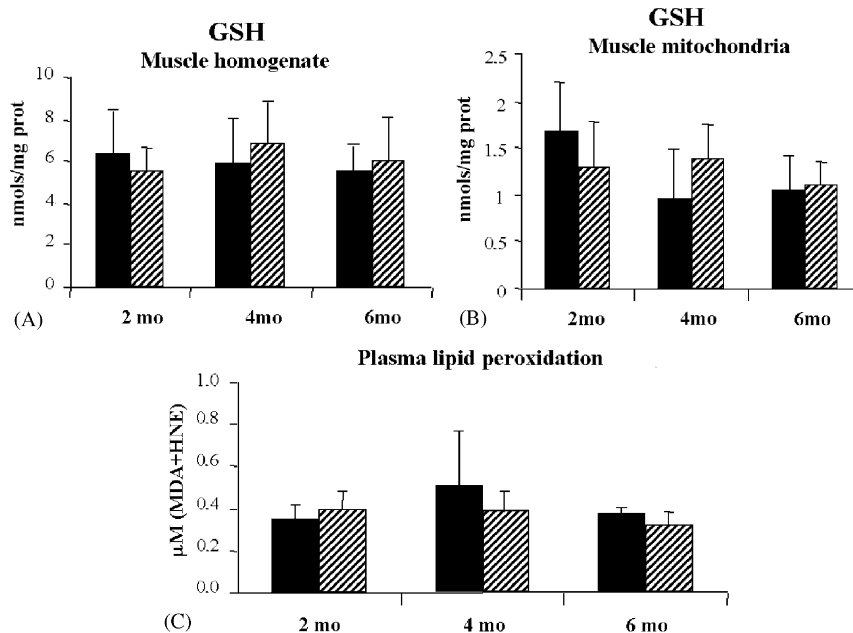
Figure 1 Oxidative stress in skeletal muscle and plasma. Total GSH content (GSH+GSSG) in skeletal muscle and plasma lipid peroxidation were determined as markers of systemic oxidative stress, after 2, 4, and 6 months in cigarette smoke-exposed (dashed bars) and control (filled bars) guinea pigs. No differences were shown in GSH levels in total skeletal muscle homogenate (A) and isolated mitochondria (B) and plasma levels of MDA+4-HNE (C). Results are expressed as mean ± SD.

Table 1 Nutritional profile.

	2 months		4 months		6 months	
	Ctrl	Smkr	Ctrl	Smkr	Ctrl	Smkr
Cholesterol (mg/dl)	38±14	42±10	37±12	38±3	49±16	45±10
Triglicerides (mg/dl)	69±44	62±27	74±38	80±30	169±106	183±96
Total proteins (g/l)	40±4	44±2	43±12	44±2	50±3	48±4
Albumin (g/l)	27±2	29±1	28±7	29±1	31±3	31±3

Definition of abbreviations: Ctrl: control; Smkr: cigarette smoke-exposed.

levels of cholesterol, total protein content and albumin were observed in smoke-exposed animals as compared to control ones, at any time point. Triglycerides increased after 6 months of exposure at the same level both in control and smoke-exposed animals.

### Histological changes in the airways

To evaluate the deleterious effect of chronic cigarette smoke exposure on the airways we evaluated the extent of goblet cell metaplasia in small noncartilaginous bronchioles (Fig. 3). There was a significant increase in the number of goblet cells at 4 and 6 months in smoke-exposed animals, confirming airway epithelial damage induced by cigarette smoke (Fig. 3A). Interestingly, airway epithelial damage was apparent much later than the reduction of body weight gain (Figs. 2 and 3).

Inflammatory response

Cigarette smoke may induce an inflammatory response in the lung with enhanced release of TNFα. To evaluate this response in our experimental model, we determined TNFα production both in plasma and whole-lung tissue. At 2 and 4 months of exposure no TNFα release was detected (detection limit with the ELISA kit is 1–2 pg/ml). After 6 months of exposure there were detectable TNFα levels (Fig. 4). Whole-lung TNFα content in smoke-exposed and control animals was similar (Fig. 4A). Interestingly there was a trend to increase TNFα levels in plasma of smoke-exposed animals compared with the control group ($p = 0.06$), likely suggesting a systemic inflammatory process (Fig. 4B).

Effects of acute exposure to cigarette smoke

Skeletal muscle, lung and plasma oxidative stress

Having shown that cigarette smoke did not induce significant chronic oxidative stress neither in the
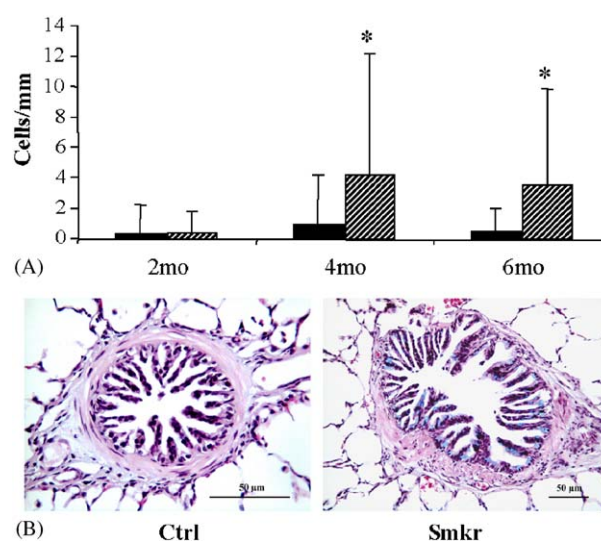
Figure 3 Goblet cell metaplasia in small airways. (A) Number of secretory cells in the airway epithelium after 2, 4 and 6 months in smoke-exposed (dashed bars) and control (filled bars) guinea pigs. (B) Transverse sections of small bronchioles stained with alcian blue from control (left panel) and smoked-exposed (right panel) guinea pigs. Note the increased number of goblet cells (light blue stained cells) in the epithelial surface of smoke-exposed animals. Internal scale bar $= 50\,\mu m$. *$p < 0.05$ compared with control animals.

lung nor in the plasma of guinea pigs, we tested the hypothesis that cigarette smoke might induce oxidative stress acutely.

Compared with control animals, exposure of guinea pigs to seven cigarettes caused a significant reduction of GSH levels in skeletal muscle, in both total homogenate and isolated mitochondria (Fig. 5A and B, respectively), which was apparent immediately after finishing the cigarette smoke exposure. However, levels measured 3 and 24 h after finishing the smoke exposure were similar to those measured in controls. Lung homogenate GSH

levels did not differ at any time in smoke-exposed animals, as compared to controls (Fig. 5C).

In agreement with the reduction of GSH content in skeletal muscle immediately after cigarette smoke exposure, lipid peroxidation in plasma increased significantly. By contrast, at 3 h and 24 h post-exposure MDA plus 4-HNE levels were comparable to paired controls (Fig. 5D).

Inflammatory process

Release of TNFα after acute cigarette smoke exposure was measured both in plasma and whole-lung tissue. No TNFα release could be detected in plasma at any time point. In lung tissue, TNFα could be detected, although there was no difference between controls and cigarette smoke-exposed animals. TNFα levels at all time points averaged 21.9 ± 3 pg/mg prot in controls and 20.8 ± 1.5 pg/mg prot in smoke-exposed guinea pigs.

Discussion

There is consistent evidence indicating that COPD has an important systemic component.[2] Our study examined the systemic manifestations of cigarette smoking after long-term and acute exposure in a guinea pig model, in terms of systemic oxidative stress measured both in plasma and skeletal muscle. Although the amount of smoke that the guinea pigs inhaled in this study differs from that of smokers, the current model has been widely used to study the effects of cigarette smoke on lung and
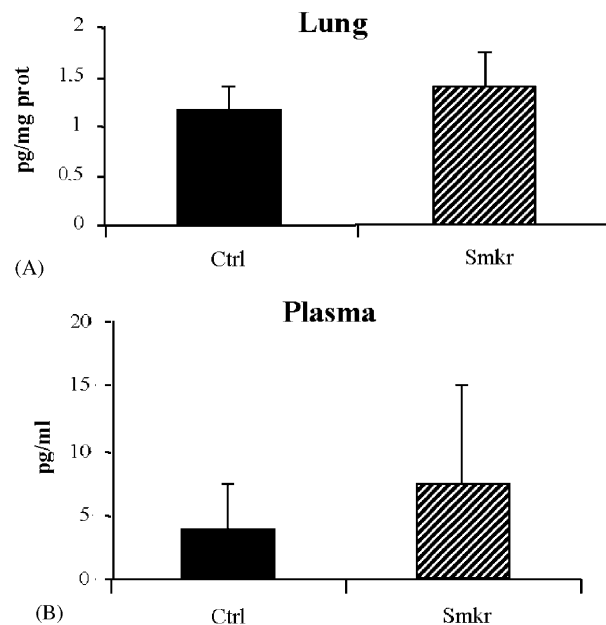
Figure 4 TNFα levels after 6 months of exposure. TNFα content in whole-lung homogenate (A) and plasma (B) of control (Ctrl) and smoke-exposed guinea pigs (Smkr). There was a trend for increased TNFα levels in plasma of smoke-exposed guinea pigs ($p = 0.06$).
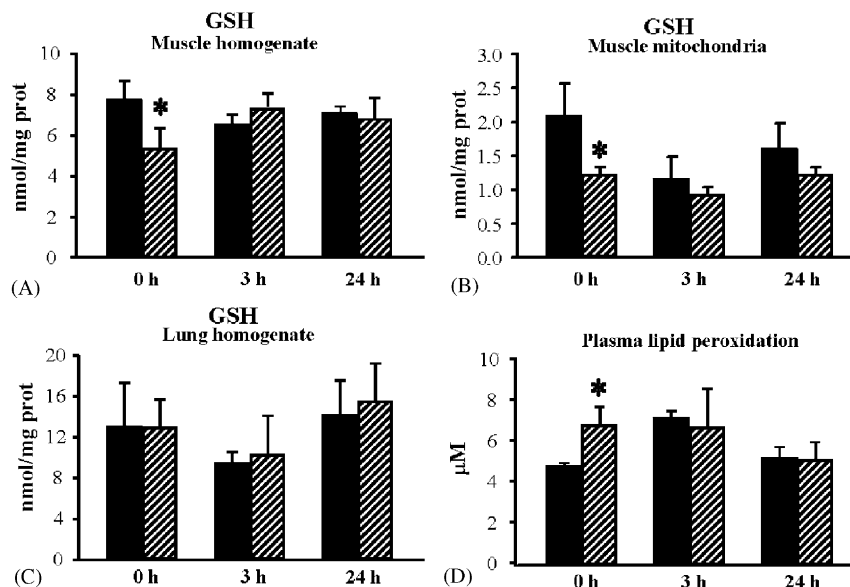
Figure 5 Oxidative stress after acute cigarette smoke-exposure in control (filled bars) and smoke-exposed (dashed bars) guinea pigs. Skeletal muscle GSH content in total homogenate (A) and isoalted mitochondria (B) decreased immediately after cigarette smoke exposure (*$p < 0.05$). No differences were shown in measurements performed 3 and 24 h after smoke exposure. No differences were shown in GSH levels of whole-lung homogenate at any time after smoke exposure (C). Plasma lipid peroxidation, measured as MDA levels plus 4-HNE, increased immediately after acute cigarette smoke exposure (*$p < 0.05$) returning to control values at 3 and 24 h post-exposure (D).

extrapulmonary tissues.[20] To our knowledge, this is the first study that has evaluated the level of oxidative stress in skeletal muscle in rodents exposed to cigarette smoke, since other studies have focused their investigation in local pulmonary, hepatic, renal cardiac or bladder muscle,[25,26] but not in skeletal muscle.

Our data reveal no significant evidence of systemic oxidative stress after long-term exposure to cigarette smoke, since neither the levels of MDA plus 4-HNE, as signs of plasma lipid peroxidation, nor the levels of total GSH, both in homogenate and mitochondria of skeletal muscle, underwent significant changes as compared to control values after 2, 4 and 6 months of exposure (Fig. 1).

Cigarette smoke is a complex mixture of over 4700 identified constituents[4] that includes numerous reactive substances such as a large quantity of reactive aldehydes[26] and free radical species, not only oxygen free radicals but also nitrogen species.[27] All of these compounds are capable to react with protein thiols and with the main nonprotein thiol such as GSH. Indeed, recent observations reported that cigarette smoke depletes GSH in the lining fluid of the respiratory tract due to conjugation of GSH with acrolein and crotonaldehyde.[28] In contrast to these findings in respiratory tissue our data show that chronic cigarette smoke exposure did not result in significant oxidative stress in plasma or skeletal muscle. However, cigarette smoke caused oxidative stress acutely in extrapulmonary tissues, as evidenced by the increase in lipid peroxidation in plasma and the reduction of GSH content in skeletal muscle. The different outcome between chronic and acute exposure to cigarette smoke shown here in skeletal muscle may be indicative of an adaptive response. For instance, acute smoking has been shown to deplete GSH in the lung, whereas chronic smoking increased GSH levels in epithelial lining fluid.[29]

In our study pulmonary GSH was not affected by smoke exposure at any time point, indicating that there was a significant store of GSH in this organ that did not interact with cigarette smoke. This result could be explained by differences in the efficiencies of antioxidant systems and repair enzymes among tissues. Indeed, there is a larger amount of GSH in the lung (16 nmols/mg prot) respect to skeletal muscle (7 nmols/mg prot), so that a reduced tissue response would be expected. Our findings are in agreement with those of Bilimora et al.[25] that revealed that exposure to cigarette smoke at a dose about 4 times greater than that used in our study, resulted in only 22% depletion of pulmonary GSH immediately after smoke exposure, being completely replaced 3 h

after treatment. Studies in rats have revealed that GSH lung levels decreased immediately[30,31] and 1 h after smoke exposure returning to normal levels after 2–6 h of exposure.[32] This indicates that there are differences between species and cigarette doses, but even at exceptionally high levels of exposure, there is a significant store of GSH in lung tissue that does not interact with cigarette smoke.

Interestingly, the acute study shows a systemic impact of cigarette smoke by a mechanism that questions the idea that cigarette smoke intermediates should elicit a local action on pulmonary tissue prior to exert any systemic action.[25] Importantly, acute smoke exposure did result in tissue damage, as suggested by increased products of lipid peroxidation and decreased skeletal muscle GSH levels, both in homogenate and mitochondria, immediately after smoke exposure. Membrane lipid peroxidation is a consequence of oxidative stress. The aldehyde 4-hydroxy-2-nonenal (4-HNE) is a stable end product of lipid peroxidation, which is highly reactive and diffusible and can cause oxidative damage in DNA, lipids and proteins in tissues far from the original site. The delicate balance that exists between the toxicity of oxidants and the protective effects of intra- and extracellular antioxidant defense systems is critical for the maintenance of normal skeletal muscle function. Inherent to its function, skeletal muscle is continuously exposed to fluctuations in its redox environment, as during exercise when the mitochondrial respiratory chain increases ROS production.[33] In addition, proteins of the contractile apparatus itself may be oxidized by ROS, which may impair force development and muscle contractility, and also compromise muscle function indirectly by inducing muscle atrophy.[34] Furthermore, oxidative stress may also result in apoptosis of skeletal myocytes[35] thereby contributing to muscle atrophy.

In agreement with the acute results, a reduction in body weight gain was observed in smoke-exposed animals starting at the 5th week after initiating smoke exposure, which was maintained after 6 months of cigarette smoking. This observation is consistent with a general alteration of body composition induced by cigarette smoke products. It is interesting to note that reduction of body weight gain was apparent much earlier than the histological changes in the airways, likely suggesting that changes in skeletal muscle may be independent from changes occurring in the lung. Since cigarette smoke might alter food intake, we investigated the nutritional profile to evaluate malnourishment as a potential cause of reduced body weight gain. The lack of changes in total

protein content, triglycerides, cholesterol and albumin indicates that the loss of approximately 100 g of body weight in smoke-exposed animals was not due to inanition. In this respect, it should be noted that studies on the nutritional abnormalities associated with COPD suggest that these patients may suffer from cachexia rather than malnourishment. Indeed, in COPD the caloric intake is normal or even greater than normal, not lower as in malnourishment, and the response to nutritional support is often poor.[36] Such observations are consistent with our findings in the guinea pig model and suggest that reduced weight gain might result from a toxic effect of cigarette smoke exposure rather than a consequence of reduced food intake.

Cigarette smoke, due to its high concentration of free radicals,[37] may cause transient and repeated oxidative imbalances that might result in impaired muscle metabolism and promote muscle atrophy, which may become apparent in terms of body weight after several weeks of smoke exposure.

Cigarette smoking can also elicit complex physiological and pathological responses including inflammatory-immune system activation. Therefore, oxidative damage of cell components can be derived not only from a direct reaction with reactive substances contained in cigarette smoke, but also from smoke-induced secondary events such as activation and infiltration of phagocytes into the lung. Inflammatory lung cells release inflammatory cytokines, such as TNFα, IL-6, IL-1b, that may reach the systemic circulation and contribute to the activation of inflammatory cells.[38] In our study, we did not find TNFα release in lung tissue, which is consistent with a lack of lung damage. Only, after 6 months of cigarette exposure we observed a trend to increase TNFα levels, both in plasma and lung tissue, likely suggesting lung inflammation, which is consistent with the presence of goblet cell metaplasia in small airways.

In conclusion, cigarette smoke exposure in the guinea pig induces a transient and repeated oxidative effect, which might result in impaired systemic metabolism and consequent failure of smoke-exposed animals to gain weight. This effect antecedes and appears to be independent from the characteristic changes occurring in the lung.

Acknowledgements

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