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N-(2-mercaptopropionyl)-glycine but not Allopurinol prevented cigarette smoke-induced alveolar enlargement in mouse☆

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ABSTRACT

We investigated the possible protective effects of the Allopurinol (A), N-(2-mercaptopropionyl)-glycine (M) and N-acetylcysteine (N) against lung injury caused by long-term exposure to cigarette smoke (CS) in mouse. C57BL6 mice were exposed to 12 cigarettes a day for 60 days and concomitantly treated with either one of the antioxidant drugs diluted in saline (CS + A—50 mg/kg; CS + M—200 mg/kg/day; CS + N—200 mg/kg/day). Control groups were sham-smoked (AA). Long-term CS exposure results in extensive parenchyma destruction in CS group. Both CS + N and CS + M groups showed preserved alveolar structure and showed preserved lung function when compared to CS group. Macrophage and neutrophil counts were decreased in CS + M, and CS + N groups when compared to CS group ($p < 0.05$). Antioxidant enzyme activities were reduced in all treated groups. CS + A showed the highest reduction in catalase activity (−25%, $p < 0.01$). We conclude that M treatment reduced long-term CS-induced inflammatory lung parenchyma destruction and lung function, comparable to N treatment, however, antioxidant administration did not reverse CS-induced antioxidant enzyme activity reduction.

1. Background

The prevalence of chronic obstructive pulmonary disease (COPD) has increased worldwide owing to regular exposure of populations to risk factors such as tabagism, occupational silica and cadmium, and higher indices of pollution in both open and closed spaces. Indeed, the World Health Organization (WHO) now considers COPD an epidemic, and it is predicted to be the third leading cause of death by 2020 (Rabe et al., 2007).

Cigarette smoke (CS) is a complex mixture of over 4700 chemical compounds, including high concentrations of oxidants (10^{14} oxidant radicals/puff) (Church and Pryor, 1985). Oxidative stress develops when the balance between oxidants and antioxidants shifts in favor of the oxidants (Chow, 1993; Koyama and Geddes, 1998). CS-induced lung inflammation itself produces oxidative stress in the lungs in asthma and COPD (Biswas and Rahman, 2009; Boutten et al., 2010; MacNee, 2001; Rahman and Adcock, 2006; Ward, 2010). In order to minimize oxidant damage to biological molecules, the mammal lung is endowed with an integrated antioxidant system of enzymatic and expendable soluble antioxidants. Antioxidant enzymes include superoxide dismutase (SOD) family, catalase (CAT), glutathione peroxidase (GPx) (Chow, 1993; Halliwell and Gutteridge, 1990).

The injurious effects of long term-cigarette smoke exposure occur repeatedly during and immediately after cigarette smoking and may deplete lung antioxidant defenses (Chow, 1993; Oberley-Deegan et al., 2009; Tappia et al., 1995). In addition, intense phagocytic activity by recruited inflammatory cells increases generation of oxidants and other inflammatory markers (Pricop et al., 1999; Raley and Loegering, 1999). Previous reports from our group showed that antioxidants such as vitamin C and E, as well as mate tea supplementations have been shown to prevent acute lung inflammation (Lanzetti et al., 2008; Silva Bezerra et al., 2006) and improve mouse lung repair after CS-induced alveolar enlargement in mouse (Valenca et al., 2008a).

Therefore, we hypothesized that the administration of nonenzymatic antioxidant drugs may be an interesting approach aiming the treatment of cigarette smoke-induced alveolar enlargement in mice. We also investigated the effects of nonenzymatic antioxidant

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drugs on chronic lung inflammatory cell influx, lung function, and the activity of pulmonary antioxidant enzymes.

2. Methods

2.1. Chemicals

Adrenaline, Allopurinol, N-(2-mercaptopropionyl)-glycine, N-acetylcysteine and NADPH were purchased from Sigma Chemical (St. Louis, MO, USA). Pallflex filters were purchased from Imprint (São Paulo, Brazil). Diff-Quik was purchased from Baxter Dade AG (Dudingen, Switzerland). Bradford reagent was purchased from Bio-Rad (Hercules, CA, USA). Hydrogen peroxide was purchased from Vetec (Duque de Caxias, Brazil).

2.2. Experimental animals

C57BL6 male mice (8 weeks old, 20–24 g) were purchased from the Veterinary Institute, Fluminense Federal University (Niterói, Brazil). Mice were housed (5 per cage) in a controlled environment room with a 12-h light/12-h dark cycle (lights on at 6 pm) and ambient temperature of $25 \pm 2\,^{\circ}C$ (humidity ~ 80%). The animals had free access to water and food. Acclimatization was performed during the two weeks before the experimental procedures.

2.3. Cigarette smoke protocol

Cigarette smoke-induced lung injury has been extensively described as a useful research tool in order to study the mechanisms of either acute (Castro et al., 2004; Lanzetti et al., 2008; Silva Bezerra et al., 2006; Valenca et al., 2009, 2008b) or chronic lung inflammation, especially emphysema (Menegali et al., 2009; Rueff-Barroso et al., 2010; Valenca et al., 2004). Cigarette smoke-induced emphysema models vary not only on the number of cigarettes used, but also on the time of exposure (Bartalesi et al., 2005; Churg et al., 2009; Hodge-Bell et al., 2007). In the present study, we used a shorter however more intense cigarette-smoke exposure model, which has been described to induce emphysema as soon as 60 days from the beginning of the experiment (Menegali et al., 2009; Rueff-Barroso et al., 2010; Valenca et al., 2004).

C57BL6 male mice ($n = 40$) were exposed to 12 commercial full-flavor filtered Virginia cigarettes (10 mg of tar, 0.9 mg of nicotine and 10 mg of carbon monoxide) per day for 60 days by using a smoking chamber described previously (Menegali et al., 2009; Rueff-Barroso et al., 2010; Valenca et al., 2004; Valenca and Porto, 2008). Briefly, each group of mice was placed in the inhalation chamber (40 cm long, 30 cm wide and 25 cm high), inside an exhaustion chapel. A cigarette was coupled to a plastic 60 mL syringe so that puffs could be drawn in and subsequently expelled into the exposure chamber. We aspirated 1 L of smoke from one cigarette with this syringe (20 puffs of 50 mL) and immediately injected the puff into the chamber. The 10 animals of each group were maintained in this smoke-air condition for 6 min (~3%), and the inhalation chamber was opened, by removing its cover, and the smoke evacuated for 1 min by exhaustion of the chapel. This cigarette exposure was repeated four times (4×6 min) with intervals of 1 min (exhaustion). We repeated this procedure three times per day (morning, noon and afternoon) resulting in 72 min of CS exposure to twelve cigarettes. Each cigarette smoked produced 300 mg/m^3 of total particulate matter in the chamber (measured by weighing material collected on Pallflex filters). Carboxyhemoglobin (COHb) concentration was previously monitored in mice using the same experimental protocol (Beutler and West, 1984), and COHb was not toxic (Valenca et al., 2008b). Groups ($n = 10$ each) were defined as mice exposed to CS and treated with vehicle (CS), mice exposed to CS and treated with 50 mg/kg/day of Allopurinol (CS + A), mice exposed to CS and treated with 200 mg/kg/day of N-(2-mercaptopropionyl)-glycine (CS + M) and mice exposed to CS and treated with 200 mg/kg/day of N-acetylcysteine (CS + N). All treatments were performed by oral gavages once per day (simultaneously with CS exposure) and drugs were mixed with saline (vehicle). Mice exposed to ambient air were used as the control group (sham-smoked; $n = 10$) and were subjected to oral gavages with vehicle. The doses of Allopurinol (Faggioni et al., 1994), N-(2-mercaptopropionyl)-glycine and N-acetylcysteine (Heyman et al., 2003) were based on previous data from the literature with modification of the administration via.

A separate group of C57BL6 male mice ($n = 5$ for each group) were exposed to ambient air during sixty days by using the same protocol described above and simultaneously treated with vehicle (control group), 50 mg/kg/day of Allopurinol (AA + A), 200 mg/kg/day of N-(2-mercaptopropionyl)-glycine (AA + M) and 200 mg/kg/day of N-acetylcysteine (AA + N). All procedures were carried out in accordance with the Ethics Committee for Experimental Animal Use and Care (CEA) of Instituto de Biologia Roberto Alcantra Gomes.

2.4. Drugs

Allopurinol is a structural isomer of hypoxanthine and acts by inhibiting xanthine oxidase and thus lowering superoxide anion production. The sulfhydryl moiety in N-(2-mercaptopropionyl)-glycine, which contains a reduced sulfhydryl group, is able to scavenge both superoxide (O_2^-) and hydroxyl (OH^-), and therefore, prevent the initiation of lipid peroxidation (Date et al., 2002; Mitsos et al., 1986). N-acetylcysteine, with glutathione peroxidase-catalase-like activity (GPx-CAT), transforms hydrogen peroxide (H_2O_2) into water and oxygen (Rahman and Adcock, 2006; Rahman and Kilty, 2006; Valko et al., 2007). As N-acetylcysteine has been shown to be effective in reducing oxidative damage (Dekhuijzen and van Beurden, 2006; Dillioglugil et al., 2005; Mata et al., 2003; Pinho et al., 2005), we used N as an antioxidant control for A and M.

2.5. Tissue processing and stereological estimation

Twenty-four hours after the last CS exposure, mice were sacrificed and the right ventricle was perfused with saline to remove blood. The right lung was ligated and the left lung in all mice were inflated by instilling 4% formalin buffer at 25 cm H_2O pressure for 2 min in order to avoid leaks of formalin, then ligated, removed and weighed. Inflated lungs were fixed for 48 h before embedding in paraffin. Serial sagittal sections were obtained for histological and morphometrical analyses.

To obtain uniform and proportionate lung samples, 18 fields (six no overlapping fields in three different sections) were randomly analyzed using a video microscope (Zeiss-Axioplan—20× objective lens and JVC color video camera linked to a Sony Trinitron color video monitor; Carl Zeiss, Oberkochen, Germany), and a cycloid test-system superimposed on the monitor screen. The reference volume was estimated by point counting using the test points systems (PT). The points hitting the air spaces (PP) were counted to estimate the volume densities (Vv) of these structures ($V = PP/PT$). A total area of 1.94 mm^2 was analyzed to determine the volume density of airspaces (Vv air space) in sections stained with hematoxylin and eosin (H&E). Two investigators that performed the measurement counted on non-identified sections. Morphometrical method was adapted from Vlahovic et al. (1999).

2.6. BAL and cell counts

After each mouse was sacrificed and the right ventricle was perfused with saline to remove blood, the BAL fluid was performed in

all mice and obtained by injecting buffered saline (PBS) three consecutive times to a final volume of 1.5 mL in right lung. The fluid was withdrawn and stored on ice. Total cell number was determined in a Zi Coulter counter (Beckman Coulter, Fullerton, CA, USA). Differential cell counts were performed on cytospin preparations (Shandon, Waltham, MA, USA) stained with Diff-Quik (Baxter Dade, Dudingen, Switzerland). At least 200 cells per BAL fluid sample were counted using standard morphological criteria. At least 200 cells per BAL fluid sample were counted using standard morphological criteria (Castro et al., 2004). After BAL, right lungs were immediately removed and homogenized on ice with 10% (w/v) PBS (pH7.3) using an Ultra-Turrax® T8 homogenizer (Toronto, Canada) and then centrifuged at 600 × *g* for 5 min. Supernatants were kept at −20 °C for analysis of antioxidant enzyme activities (catalase, superoxide dismutase and glutathione peroxidase).

2.7. Biochemical assays

Catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities were determined in lung homogenates. CAT activity was measured by the rate of decrease in hydrogen peroxide concentration at 240 nm (Aebi, 1984). SOD activity was assayed by measuring inhibition of adrenaline auto-oxidation as absorbance at 480 nm (Bannister and Calabrese, 1987). GPx activity was measured by monitoring the oxidation of NADPH at 340 nm in the presence of H_2O_2 (Flohe and Gunzler, 1984). The total protein content in the samples from lung homogenates was determined by the method of Bradford (1976).

2.8. Mechanical ventilation

One hour after the last CS exposure protocol, animals were sedated with diazepam (1 mg i.p.), anesthetized with pentobarbital sodium (20 mg kg body weight^{-1} i.p.), tracheotomized, and a snugly fitting cannula (0.8 mm ID) was introduced into the trachea. The animals were then paralyzed with pancuronium bromide (0.1 mg/kg) and the anterior chest wall was surgically removed. A pneumotachograph (1.5 mm ID, length = 4.2 cm, distance between side ports = 2.1 cm) (Mortola and Matsuoka, 1993) was connected to the tracheal cannula for the measurements of airflow (V'). Lung volume (VT) was determined by digital integration of the flow signal. The pressure gradient across the pneumotachograph was determined by a Validyne MP45-2 differential pressure transducer (Engineering Corp., Northridge, CA, USA). The flow resistance of the equipment (Req), tracheal cannula included, was constant up to flow rates of 26 mL s^{-1} and amounted to 0.12 cm H_2O mL^{-1} s. Equipment resistive pressure (=Req.V') was subtracted from pulmonary resistive pressure so that the present results represent intrinsic values. Transpulmonary pressure was measured with a Validyne MP-45 differential pressure transducer (Engineering Corp., Northridge, CA, USA). All signals were conditioned and amplified in a Beckman type R Dynograph (Schiller Park, IL, USA). Flow and pressure signals were also passed through 8-pole Bessel filters (902LPF, Frequency Devices, Haverhill, MA, USA) with the corner frequency set at 100 Hz, sampled at 200 Hz with a 12-bit analog-to-digital converter (DT2801A, Data Translation, Marlboro, MA, USA), and stored on a microcomputer. All data were collected using LABDAT software (RHT-InfoData Inc., Montreal, QC, Canada).

Lung resistive (ΔP1) and viscoelastic/inhomogeneous (ΔP2) pressures, total pressure drop (ΔPtot = ΔP1 + ΔP2), static elastance (Est), and viscoelastic component of elastance (ΔE) were computed by the end-inflation occlusion method (Bates et al., 1988, 1985). Briefly, after end-inspiratory occlusion, there is an initial fast drop in transpulmonary pressure (ΔP1) from the pre-occlusion value down to an inflection point (Pi) followed by a slow pressure decay (ΔP2), until a plateau is reached. This plateau corresponds to the elastic recoil pressure of the lung (Pel). ΔP1 selectively reflects airway resistance in normal animals and humans and ΔP2 reflects stress relaxation, or viscoelastic properties of the lung, together with a small contribution of time constant of alveoli (Bates et al., 1988; Saldiva et al., 1992). Lung static elastance (Est) was calculated by dividing Pel by the tidal volume. ΔE was calculated as the difference between static and dynamic elastances, and reflects the viscoelastic component of elastance (Bates et al., 1988, 1985).

2.9. Statistical analysis

Data are expressed as means ± SEM. For comparison among groups, one-way ANOVA was performed followed by the Tukey post-test ($p < 0.05$). InStat Graphpad software was used to perform the statistical analyses (GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego, CA, USA).

3. Results

3.1. N-(2-mercaptopropionyl)-glycine and N-acetylcysteine but not Allopurinol prevented cigarette smoke-induced alveolar enlargement

After 60 consecutive days of cigarette exposure, lung from CS group showed significant histological alterations such as alveolar damage and increased alveolar spaces (Fig. 1). Both N and M groups displayed lung histological features similar to AA group, in which alveolar integrity and intact air spaces were observed. In contrast, Allopurinol treatment did not protect lung parenchyma from cigarette smoke-induced damage, therefore, showing histological features similar to CS group. Vv [air spaces] estimative corroborates with the above mentioned information, since both CS and CS + A groups showed increased Vv, but CS + N and CS + M values are similar to AA group (Fig. 2). No histological differences were observed among AA treated groups (data not shown).

3.2. Antioxidant treatments with N-(2-mercaptopropionyl)-glycine and N-acetylcysteine decreased neutrophil influx into the alveoli

Macrophage influx was increased by nearly 3 fold and there was a 52 fold increase in neutrophil influx into the BALF of CS group when compared to AA group. Although there was a trend towards the reduction of macrophages in CS + N and CS + M groups, the treatments with these antioxidant drugs significantly reduced neutrophil influx into the BALF. Allopurinol treatment surprisingly doubled both inflammatory cell influx into the BALF (Fig. 3). No differences were observed among AA treated groups (data not shown).

3.3. Cigarette-smoke induced redox imbalance is not prevented by antioxidant drug treatment

Long term CS protocol resulted in reduced SOD, CAT and GPx activities in mouse lung homogenates. Neither one of the antioxidant drugs administered to mice during the same period in which CS protocol was undertaken reversed the redox unbalance observed in Cs group. Besides, CAT activity was further reduced in both CS + A and CS + N groups when compared to CS group (Fig. 4).

Although no alteration was observed in SOD activity due to long term antioxidant drug treatments to non-smoking animals, Allopurinol resulted in decreased CAT activity in animals exposed to only air. Also, as expected, GPx activity was found to be increased in animals exposed to only air and treated with NAC (AA + N group) (Fig. 5).

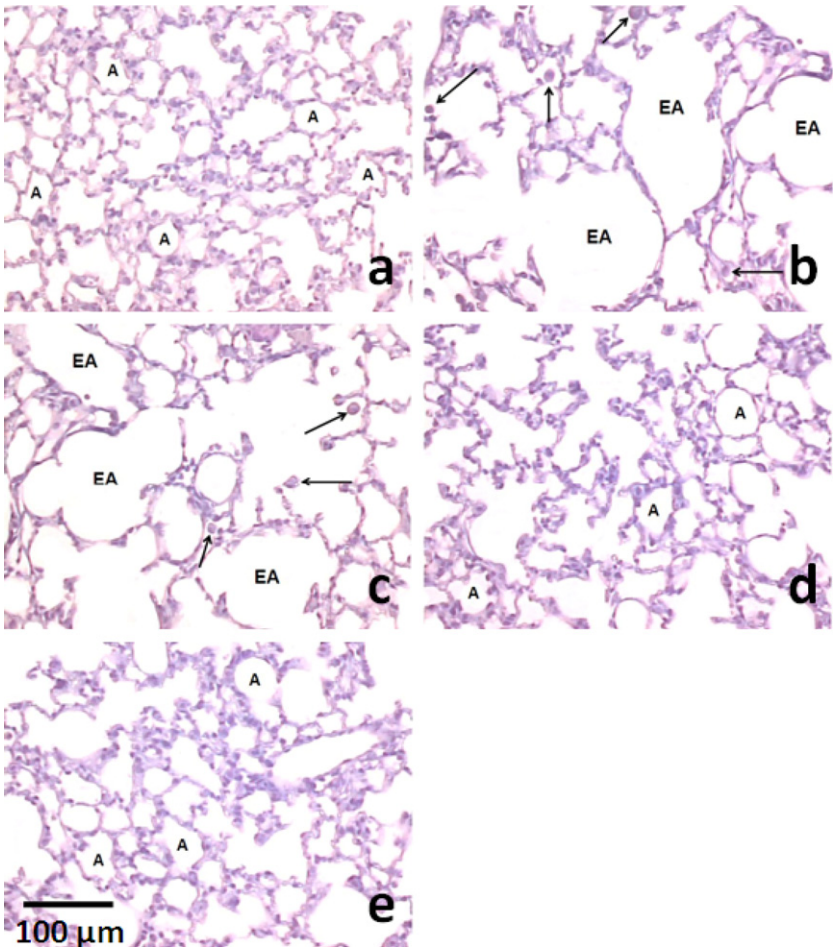

Fig. 1. Chronic cigarette-smoke exposure induced significant alveolar enlargement. N-(2-mercaptopropionyl)-glycine and N-acetylcysteine but not Allopurinol prevented smoke-induced alveolar enlargement. Representative examples of hematoxylin and eosin stained paraffin-embedded sections of mouse lungs. (a) Mice exposed to ambient air; (b) mice exposed to 12 commercial full-flavor filtered Virginia cigarettes per day for 60 days; (c) mice exposed to CS and treated with 50 mg/kg/day of Allopurinol; (d) mice exposed to CS and treated with 200 mg/kg/day of N-(2-mercaptopropionyl)-glycine; (e) mice exposed to CS and treated with 200 mg/kg/day of N-acetylcysteine.

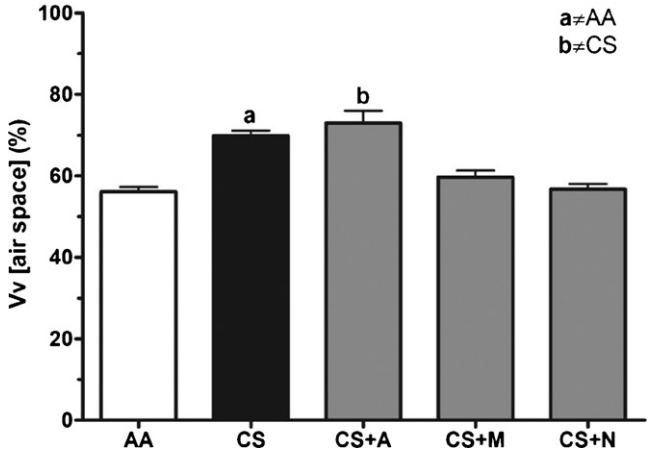

Fig. 2. Stereological estimation of volume density (Vv) of airspaces quantified cigarette smoke-induced lung parenchyma destruction. N-(2-mercaptopropionyl)-glycine and N-acetylcysteine treatments preserved lung alveolar structure. (AA) Mice exposed to ambient air; (CS) mice exposed to 12 commercial full-flavor filtered Virginia cigarettes per day for 60 days; (CS + A) mice exposed to CS and treated with 50 mg/kg/day of Allopurinol; (CS + M) mice exposed to CS and treated with 200 mg/kg/day of N-(2-mercaptopropionyl)-glycine; (CS + N) mice exposed to CS and treated with 200 mg/kg/day of N-acetylcysteine. (a) $p < 0.05$ AA group; (b) $p < 0.05$ versus CS group. $N = 10$ per group.

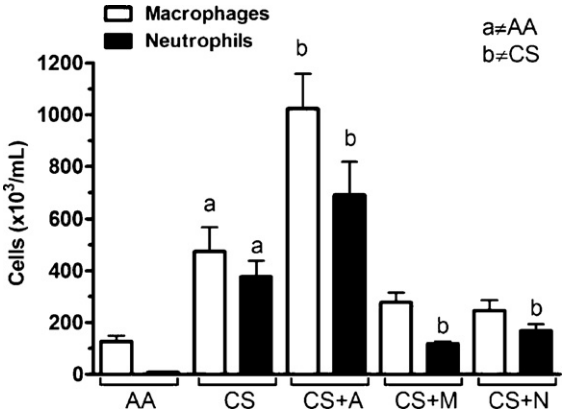

Fig. 3. Cigarette-smoke induced macrophage and neutrophil influxes into the brochoalveolar lavage (BAL). Allopurinol treatment exacerbated inflammatory cell recruitment to BAL. N-(2-mercaptopropionyl)-glycine and N-acetylcysteine treatments impaired cell influxes to the BAL. (AA) Mice exposed to ambient air; (CS) mice exposed to 12 commercial full-flavor filtered Virginia cigarettes per day for 60 days; (CS + A) mice exposed to CS and treated with 50 mg/kg/day of Allopurinol; (CS + M) mice exposed to CS and treated with 200 mg/kg/day of N-(2-mercaptopropionyl)-glycine; (CS + N) mice exposed to CS and treated with 200 mg/kg/day of N-acetylcysteine. (a) $p < 0.05$ AA group; (b) $p < 0.05$ versus CS group. $N = 10$ per group.

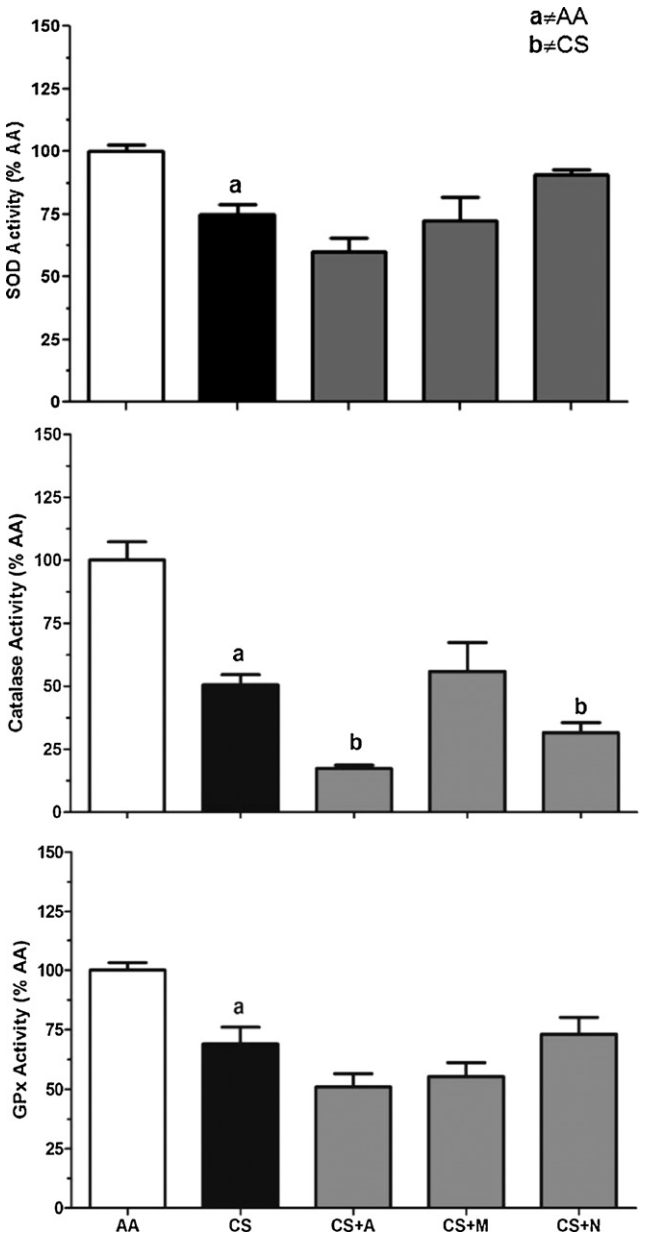

Fig. 4. Antioxidant enzyme activities were reduced due to chronic cigarette-smoke exposure and antioxidant drug treatment did not altered CS-induced redox imbalance. SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; (AA) mice exposed to ambient air; (CS) mice exposed to 12 commercial full-flavor filtered Virginia cigarettes per day for 60 days; (CS + A) mice exposed to CS and treated with 50 mg/kg/day of Allopurinol; (CS + M) mice exposed to CS and treated with 200 mg/kg/day of N-(2-mercaptopropionyl)-glycine; (CS + N) mice exposed to CS and treated with 200 mg/kg/day of N-acetylcysteine. (a) $p < 0.05$ AA group; (b) $p < 0.05$ versus CS group. $N = 10$ per group.

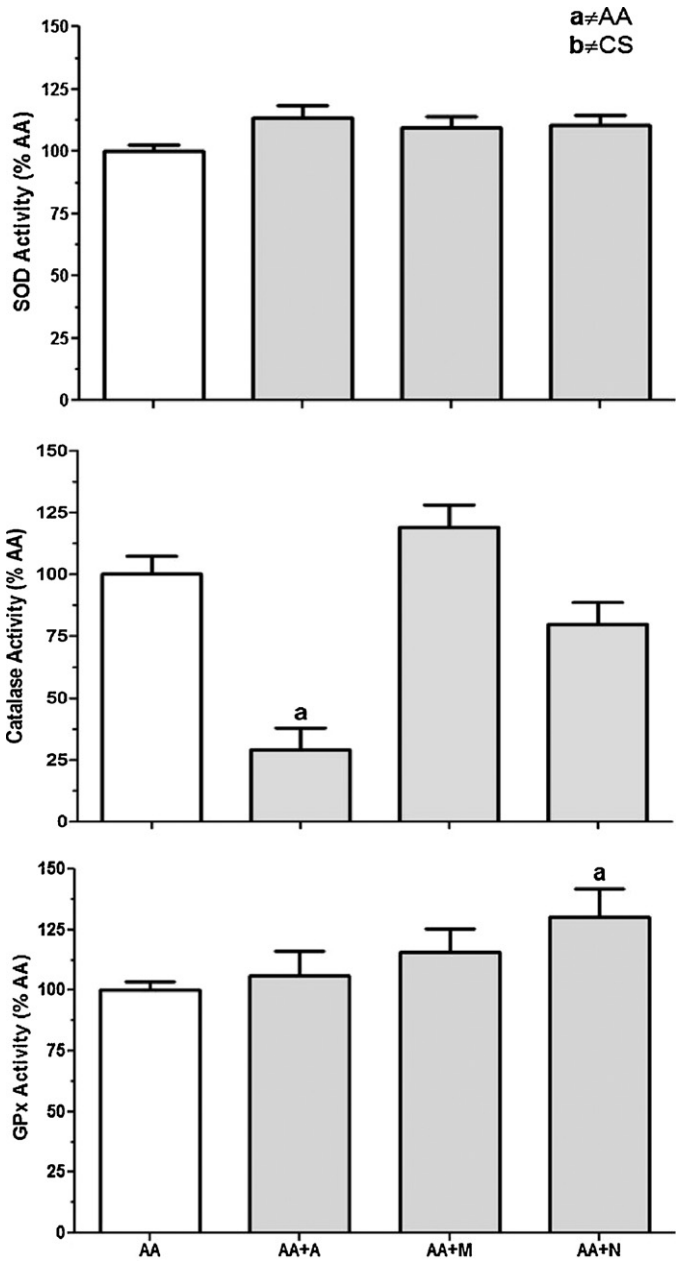

Fig. 5. Allopurinol treatment resulted in redox imbalance in groups exposed to ambient air. N-acetylcysteine chronic treatment increased glutathione peroxidase (GPx) activity in lung homogenates. SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; (AA) mice exposed to ambient air; (AA + A) mice exposed to ambient air and treated with 50 mg/kg/day of Allopurinol; (AA + M) mice exposed to ambient air and treated with 200 mg/kg/day of N-(2-mercaptopropionyl)-glycine; (AA + N) mice exposed to ambient air and treated with 200 mg/kg/day of N-acetylcysteine. (a) $p < 0.05$ AA group. $N = 10$ per group.

3.4. Long-term N-(2-mercaptopropionyl)-glycine and N-acetylcysteine treatments prevented lung functional impairment

Values of lung mechanics during spontaneous breathing obtained in each group are shown in Table 1. FRC was severely impaired due to long-term cigarette smoke exposure. CS group showed FRC values nearly two times higher than AA group. However, both N-(2-mercaptopropionyl)-glycine and N-acetylcysteine long-term treatments prevented lung function loss of mice in CS + M and CS + N when compared to CS group. Conversely, no improvement was observed in CS + A group, which showed FRC values similar to CS group. Similar results were observed when ΔPtot and ΔE were analyzed. However, no alteration was observed in Est when all groups were compared.

4. Discussion

There is considerable evidence from both animal and human studies that an increased oxidative burden occurs due to cigarette smoke (Montuschi et al., 2000; Rahman and Adcock, 2006; Rahman and MacNee, 1996; Rahman et al., 1996) and that oxidative stress plays an important role in many of the processes involved in the pathogenesis of lung alveolar enlargement (Rahman and MacNee,

Table 1
Lung functional data obtained during mechanical ventilation in each group. Results are presented as mean ± SEM. AA group: ambient air-exposed mice; CS: cigarette smoke-exposed mice; CS + A group: cigarette smoke-exposed mice treated with Allopurinol; CS + M group: cigarette smoke-exposed mice treated with N-(2-mercaptopropionyl)-glycine; CS + N group: cigarette smoke-exposed mice treated with N-acetylcysteine group. FRC: functional residual capacity; Est: respiratory system elastance; (ΔPtot): total resistance and resistive pressures; ΔE: dynamic elastance.

	Groups				
	AA	CS	CS + A	CS + M	CS + N
FRC	0.15 ± 0.01	0.29 ± 0.02[a]	0.24 ± 0.03	0.18 ± 0.02[b]	0.21 ± 0.01[b]
Est	31.09 ± 2.37	32.32 ± 3.17	31.68 ± 2.55	39.12 ± 1.25	34.93 ± 3.66
ΔPtot	1.70 ± 0.10	2.59 ± 0.17[a]	2.29 ± 0.15	1.69 ± 0.05[b]	1.79 ± 0.12[b]
ΔE	4.65 ± 0.22	7.27 ± 0.70[a]	7.51 ± 0.41	4.72 ± 0.32[b]	5.62 ± 0.46[b]

[a] $p < 0.05$ versus AA group.
[b] $p < 0.05$ versus CS group.

1996; Valenca et al., 2006, 2004). However, no effective prevention or/and treatment for cigarette-smoke induced alveolar enlargement has yet been described for humans. In this context, increasing lung antioxidant screen defenses through concomitant administration of antioxidant drugs in mice appears to be an interesting approach aiming the prevention cigarette smoke-induced lung damage.

In the present study, we evaluated the effects of N-(2-mercaptopropionyl)-glycine and Allopurinol on inflammatory cellular influx, redox imbalance and lung function in response to long-term exposure to cigarette smoke in C57BL6. N-acetylcysteine, an effective antioxidant recognized for ameliorates emphysema in mice was used as control. The cigarette-smoke induced alveolar enlargement protocol used in the present study differs from others on both dose and time matter. As we have previously reported, increasing the amount of smoke in which animals are exposed to results in early features of alveolar enlargement that could be considered as early emphysematous histopathological features which are noticed as soon as 60 days following cigarette-smoke protocol (Menegali et al., 2009; Rueff-Barroso et al., 2010; Valenca et al., 2004).

Among the antioxidant drugs used in the present study, treatment with N-acetylcysteine in humans is known to alter the pulmonary oxidant–antioxidant imbalance (Balansky et al., 2009; Linden et al., 1988; Rocksen et al., 2000) in favor of antioxidants and, therefore, was used here as an "control antioxidant drug". Previous studies from our group have described the CS-induced morphological alveolar alterations in mice. Long-term CS exposure leads to lung parenchyma massive destruction, increased pro-inflammatory makers, such as NFkappaB, TNF-alpha and IL-6 (Menegali et al., 2009; Teixeira et al., 2008; Valenca et al., 2006, 2004; Valenca and Porto, 2008).

Inhalation of cigarette smoke has been described to result in increased macrophage influx into the lung and BALF and also to increase neutrophil adhesion (Lehr et al., 1993; Valenca et al., 2006, 2004). Our present results corroborate with the above mentioned studies as long-term CS exposure also increased macrophages and neutrophil influxes to the BALF. Long-term treatment with N-acetylcysteine and N-(2-mercaptopropionyl)-glycine significantly reduced inflammatory cell influx into the BALF. Under endotoxemic condition, N-acetylcysteine has been shown to modulate macrophages chemotaxis and function (Víctor et al., 2003). Also, N-acetylcysteine is known to decrease macrophage activation and release of O_2^- (Chong et al., 2002). Although, N-acetylcysteine was used in the present study focusing prevention of CS-induced oxidative stress, several studies performed in patients have used this drug as treatment for COPD (Cai et al., 2009; De Benedetto et al., 2005; Dekhuijzen and van Beurden, 2006). The present data corroborate with the notion that N-acetylcysteine has beneficial effects reversing lung inflammatory cell influxes, but also showed that N-(2-mercaptopropionyl)-glycine was able to reduce CS-induced alveolar enlargement in mice. In contrast, Allopurinol administration did not result in any beneficial effects in mouse lungs, instead resulted in further increase of inflammatory cell influxes to the lungs. However, no similar effects were observed when Allopurinol was administered alone. The surprising effect of the further increase of inflammatory cell influx in CS + A group indicates a pro-inflammatory action of Allopurinol. Shenkar and Abraham (1999) reported that xanthine oxidase-blocking increased levels of NFkappaB in lung neutrophils and Kanellis et al. (2003) reported an additional increase of monocyte chemoattractant protein-1 (MCP-1) in congestive heart failure. There is no report regarding N-(2-mercaptopropionyl)-glycine action of inflammatory cells on CS-induced alveolar enlargement; however N-(2-mercaptopropionyl)-glycine has been described to reduce of TNF-alpha and IL-6 mRNA expression during posthemorrhage resuscitation (Tamion et al., 2000).

The present histological data suggest a relationship between cell influx into the BALF and lung parenchyma destruction once CS and CS + A groups showed elevated inflammatory cell counts and also increase volume density of airspaces. The destruction of the alveolar septa, in which mice showed enlarged alveoli that were irregular in size and shape and alveoli with multiple foci of septal discontinuities and isolated septal fragments (as observed in Fig. 2 and parenchyma destruction quantification is shown in Fig. 3), such as observed in CS group and also previously reported by our group (Valenca et al., 2006, 2004) and others (Churg et al., 2009; Dhami et al., 2000; Houghton et al., 2006; Shapiro et al., 2003), is thought to be a in part result from a proteolytic insult derived from the intravascular space.

Literature has described a close association between parenchyma destruction and inflammatory cell influx into pulmonary tissue (Brown et al., 1995; Valenca et al., 2004; Valenca and Porto, 2008). We believe that activation of macrophages and neutrophils sequestered in the pulmonary tissue could also induce the release of proteases, within the lung microenvironment with limited access for free radical scavengers and antiproteases. In addition, oxidant increase disturbs the balance of proteinase and antiproteinase activities, leading to the destruction of lung extracellular matrix via the action of metalloproteinases (Valko et al., 2007). Along with the positive association with alveoli apoptosis (Syrkina et al., 2008), oxidants are known to inactivate antiproteases, such as alpha-1-antitrypsin, creating a protease–antiprotease imbalance in the lungs, forming the basis of the protease/antiprotease theory of the pathogenesis of emphysema (Le Quement et al., 2008; Rahman and MacNee, 1996). In this context, only N-acetylcysteine has been described to reduce alveoli apoptosis by partly reversing the decrease in vascular endothelial growth factor (VEGF) secretion and VEGF receptor 2 protein expression in smoking-induced COPD in rats (Cai et al., 2009). However, no data have been published regard-

ing Allopurinol or N-(2-mercaptopropionyl)-glycine effects on protease/anti-protease balance.

The present lung function parameters showed that long-term CS exposure decreased lung viscoelastic properties which, in turn, increased lung compliance. The decrease in function showed by lung mechanics measurement corroborates with the conception that long-term CS exposure resulted in emphysema features in mice (Shapiro, 2002; Shifren et al., 2007). We believe that tissue destruction was associated with airway dysfunction in this mouse model of CS-induced alveolar enlargement. As expected, N-acetylcysteine administration prevented lung function impairment due to CS exposure (Cai et al., 2009; Dekhuijzen and van Beurden, 2006; Rubio et al., 2000). However, this is the first study showing N-(2-mercaptopropionyl)-glycine beneficial effects on lung function.

It is widely accepted that inhalation of volatile substances in cigarette smoke, as well as fine particulate matter, may increase oxidant levels in the lungs either directly (Liu et al., 2005) or through inflammatory cells response (MacNee, 2001; Morrison et al., 1999; Rahman and MacNee, 1996). Besides, intense phagocytic activity by recruited inflammatory cells increases generation of oxidants and other inflammatory markers (Pricop et al., 1999; Raley and Loegering, 1999). The constant release of oxidants due to long-term CS exposure may exhaust antioxidant defenses of the lungs (Chow, 1993). For example H_2O_2 could mediate enzyme inactivation in the context of peroxidase activity of either CuZn or extracellular SOD (Jewett et al., 1999). The present results corroborate with this statement as CS group showed a significant reduction of all antioxidant enzyme activities studied. CS-induced reduction of antioxidant enzyme activities undoubtedly potentiates extracellular matrix damage and tissue injury through increased formation of reactive oxygen and nitrogen species. This change in activity was associated with increased susceptibility to pulmonary oxidant stress and tissue damage (Folz et al., 1997).

Previous reports from our group and the present data on CS exposure have shown an imbalance of CAT, superoxide dismutase (SOD) and GPx in mouse lung as a biochemical signature of redox imbalance (Menegali et al., 2009; Valenca et al., 2006, 2008b). However, catalase activity seems to be responsive to xanthine oxidase inhibition as both AA and CS groups treated with Allopurinol displayed decreased CAT activity. The present data may support the notion that some antioxidants may become prooxidant in a certain milieu. Late in elevated range prooxidant with loss of supporting antioxidants in a milieu of oxidative—redox stress due to antioxidant enzyme depletion, as observed in CS + A group. In the present study, the further decrease of CAT activity in CS + A group suggests impairment of H_2O_2 detoxification. As uric acid and H_2O_2 interact, uric acid antioxidant properties are shifted towards a prooxidant feature (Hayden and Tyagi, 2004).

It is known that N-acetylcysteine increases plasma GSH levels dose dependently (Bridgeman et al., 1994). Our data are in accordance with this statement as AA + N group showed increased GPx activity in lung homogenates.

The present data suggest that CS-induced lung parenchyma remodeling appears dependent of antioxidant enzyme activity, as CS exposition results in decreased activity of all analyzed enzymes. However, preservation of lung histological features by either N-acetylcysteine or N-(2-mercaptopropionyl)-glycine treatments appears to be independent of antioxidant enzyme activity, either by acting as antioxidant themselves, or perhaps these drug fail in increasing antioxidant defense screen facing cigarette smoke.

It is now widely acknowledged the crosstalk between oxidants and inflammation. ROS are known to act as cell signaling molecules on several biological processes, for instance, they may activate key transcription factors such as NFkappaB which in turn induce expression of a variety of genes involved in inflammatory and immune responses (Birrell et al., 2008; Rahman, 2003; Rahman et al., 2005). As antioxidant enzymes analyzed in the present study did not seem to play a pivotal role in the determination of mouse CS-induced alveolar enlargement, and the main beneficial effects were observed to be related to the decrease in inflammatory cell influx to the lungs, we suggest that both N-(2-mercaptopropionyl)-glycine and N-acetylcysteine acted directly on both oxidants from CS and resultant of CS secondary effects.

Taken together, the present results suggest, for the first time, N-(2-mercaptopropionyl)-glycine beneficial action in prevented long-term CS-induced inflammatory cell influx, preserving lung parenchyma and thus lung function. In contrast, we also show that Allopuriniol administration was not able to reverse neither CS-induce alveolar enlargement and nor macrophage and neutrophil influxes into mouse lungs following CS-exposure. Therefore, N-(2-mercaptopropionyl)-glycine, but not Allopurinol, appears to be a potential therapeutic option aiming the prevention of cigarette-smoke induced alveolar enlargement.

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