Inhibitory effect of Chinese green tea on cigarette smoke-induced up-regulation of airway neutrophil elastase and matrix metalloproteinase-12 via antioxidant activity

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Abstract

Our recent study has indicated that Chinese green tea (Lung Chen), in which epigallocatechin-3-gallate (EGCG) accounts for 60% of catechins, protected cigarette smoke-induced lung injury. We now hypothesized that Lung Chen tea may also have potential effect on lung oxidative stress and proteases/antiproteases in a smoking rat model. Sprague-Dawley rats were exposed to either sham air (SA) or 4% cigarette smoke (CS) plus 2% Lung Chen tea or water by oral gavage. Serine proteases, matrix metalloproteinases (MMPs) and their respective endogenous inhibitors were determined in bronchoalveolar lavage (BAL) and lung tissues by gelatin/casein zymography and biochemical assays. Green tea consumption significantly decreased CS-induced elevation of lung lipid peroxidation marker, malondialdehyde (MDA), and CS-induced up-regulation of neutrophil elastase (NE) concentration and activity along with that of α_1-antitrypsin (α_1-AT) and secretory leukoproteinase inhibitor (SLPI) in BAL and lung. In parallel, significant elevation of MMP-12 activity was found in BAL and lung of the CS-exposed group, which returned to the levels of SA-exposed group after green tea consumption but not CS-induced reduction of tissue inhibitor of metalloproteinase (TIMP)-1 activity, which was not reversed by green tea consumption. Taken together, our data supported the presence of local oxidative stress and protease/anti-protease imbalance in the airways after CS exposure, which might be alleviated by green tea consumption through its biological antioxidant activity.

Introduction

Emphysema due to enzymatic destruction of the lung parenchyma by uninhibited proteolytic activity is one of the major pathology of chronic obstructive pulmonary disease (COPD), which is mainly caused by cigarette smoke (CS). Protease/anti-protease imbalance, as well as chronic oxidative stress and inflammation, induced by smoking and/or pollutants, are generally accepted as major mechanisms for development of emphysema [1]. Oxidative stress potentiates inflammation and protease/anti-protease imbalance by enhancing release of pro-inflammatory mediators and destructive enzymes from inflammatory cells such as neutrophils and macrophages in the alveoli, bronchioli and small airways [2]. Serine protease neutrophil elastase (NE) and matrix metalloproteinases (MMPs) are believed to play crucial roles in causing alveolar wall destruction leading to emphysema [3,4].

Using genetically modified animal model, NE has been shown to be directly involved in CS-induced development of emphysema due to its macrophage chemoattractant and inhibitory effect on tissue inhibitors of metalloproteinase (TIMP)-1 and secretory leukoproteinase inhibitor (SLPI) [5,6]. Increased NE level has been found in alveolar macrophages (AMs) of COPD patients compared to control subjects [7].

MMPs are a family of matrix degrading enzymes that are responsible for normal tissue remodeling. MMP-2, -9 and -12 are believed to be involved in COPD pathogenesis and progression [8]. MMP-2, -9 and -12 protein expressions in lungs and induced sputum of COPD patients were higher than that of healthy subjects [9,10]. Using a knock-out mice model, Hautamaki and colleagues [11] demonstrated that mice lacking MMP-12 did not develop emphysema following cigarette smoke exposure.

In the body system, inhibitors are present to counterbalance the elastolytic activities of NE and MMPs. SLPI and α_1-antitrypsin (α_1-AT) are endogenous inhibitors of NE while TIMPs regulate the proteinase activity of MMPs via formation of complexes. We recently reported that CS-induced lung injury was related to oxidative stress in the CS-exposed rat model [12]. We now hypothesize that CS may increase expressions and activities of proteases as a result of oxidative stress, which overwhelms the anti-proteolytic activities of endogenous inhibitors, leading to the observed alveolar destruction observed in the lung [12].

Chinese green tea is one of the common beverages consumed in Southeast Asia and Japan, and epidemiologic studies have shown the beneficial effects of drinking green tea in relation to cancer, obesity and cardiovascular diseases [13]. Epigallocatechin gallate (EGCG), one type of catechins, accounts for more than 80% of all active ingredients in Lung Chen tea that has been found to possess anti-proteolytic activity by suppressing MMP-2 and -9 expression and activities [14,15]. In this study, our aims were to address whether CS exposure would up-regulate proteases and whether Chinese green tea could modulate protease expression/activity in the lungs of rats exposed to CS, leading to protection of alveolar architecture seen in our previous study [12].

Materials and Methods

Green tea preparation

10% Lung Chen tea was freshly prepared everyday as previously described (12), in which 10 ml of Lung Chen tea or tap water (as control) was given daily by oral gavage for 56 days.

Cigarette smoke-exposed model

All animal procedures were approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR) of the University of Hong Kong (CULATR 1624-08). Male Sprague-Dawley rats (150~200g) purchased from Laboratory Animal Unit (LAU) of the University of Hong Kong, were randomly divided into four groups (n = 10 in each group) and exposed to either non-filtered cigarette smoke (CS) or sham air (SA) for 56 days as previously described [12]. In brief, the CS-exposed rats were placed in a 4% smoking chamber maintained by two peristaltic pumps and the SA-exposed rats in another chamber simultaneously. Rats were sacrificed by overdose of pentobarbitone 24h after last exposure.

Bronchoalveolar lavage (BAL) and tissue collection

The lungs were lavaged using a catheter via the trachea. A total of 3ml cold phosphate-buffered saline (PBS) was instilled twice before centrifugation at 100x g for 10 minutes. The cell-free supernatants (BAL fluid) were frozen in aliquots for further analysis.

Protein extraction

Lung homogenates were extracted using T-PER tissue protein extraction reagent (PIERCE, IL, USA) in the presence of protease inhibitors (Calbiochem, La Jolla, CA, USA). Protein concentration was measured by Bradford method with bovine serum albumin (BSA) as standards.

MDA level

MDA concentrations in lung homogenates were measured using TBARS assay kit (Cayman Chemical) according to manufacturer's instruction.

Total NE, α_1-AT and SLPI level

Total NE was measured using commercial available enzyme-linked immunosorbent assay (ELISA) kit (Bender MedSystems, Austria) according to manufacturer's instructions. α_1-AT concentration was measured by sandwich ELISA reported by Chan and colleagues [16]. SLPI was measured using sandwich ELISA kit (R&D Systems, MN, USA) following manufacturer's instructions.

NE activity assay

NE activity was assayed according to Chan's protocol [16]. In short, the activity was measured using 2mM MeO-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide (Calbiochem) as a substrate in 0.2M Tris –HCl and 0.5M NaCl (pH 8.0). The hydrolytic release of *p*-nitroaniline was measured at 410nm. The NE activity was then calculated with reference to a commercially available standard NE which had been standardized by active

site titration.

Myeloperoxidase (MPO) assay

MPO was measured spectrophotometically using Bradley's method [17]. Briefly, samples were mixed with 50mM potassium phosphate containing o-dianisidine dihydrochloride (Sigma Chemical, MO, USA) and 0.0005% H_2O_2 (Sigma Chemical). The change in absorbance at 460nm was measured. MPO activity was expressed as units per mg protein.

Gelatin and casein zymography

Gelatin and casein zymography was performed. Electrophoresis was carried out using 10% polyacrylamide gels containing either 1mg/ml gelatin or casein as substrate. 20 μg samples were diluted 1:1 with zymogram sample buffer (Bio-Rad, CA, USA). After electrophoresis, the gels were washed with wash buffer (0.5M Tris-HCl, pH 7.6, 0.5M $CaCl_2$, 2.5% Triton X-100) and incubated overnight with incubation buffer (0.5M Tris-HCl, pH 7.6, 0.5M $CaCl_2$, 1% NaN_3, pH 7.6, Triton X-100). Gels were then stained with Coomassie brilliant blue G-250 (30% methanol, 10% acetic acid) and destained accordingly. Clear bands indicate protease activity.

MMP-12 activity assay

MMP-12 activity in BAL was measured using the SensoLyte® 490 MMP-12 Assay Kit (AnaSpec, Fremont, CA, USA) according to manufacturer's instructions. End

point reading was performed using a fluorometer set at excitation 340 nm and emission 490 nm.

Reverse zymography

Reverse zymography was used according to Oliver's report (18). The resolving gels were prepared using 1mg/ml casein as substrate and 160ng/ml collagenase IV (Worthington Biochemical Corp., NJ, USA). The remaining steps are the same as gelatin zymography described above. Blue bands in a clear background are areas of inhibition. Semi-quantitative analysis was done using ImageJ.

Statistical analysis

The numbers of animals in each group for different measurements varied because some samples were not enough or under the detectable limits of the assays. Results are expressed as means ± SEM. One-way analysis of variance (ANOVA) with Bonferroni's post hoc test was used throughout the study. All statistical analyses were performed using computer software (Prizm 3.0, GraphPad, CA, USA). A value of $p < 0.05$ was considered as significant.

Results

MDA concentration

MDA level was significantly elevated after cigarette smoking in lung homogenates when compared to control group (13.8 ± 2.8 vs. 4.1 ± 1.1 μM/mg protein for CS and SA groups respectively, $p<0.01$) (Figure 1). This up-regulation was completely suppressed by pretreatment of Lung Chen tea (5.3 ± 1.5 μM/mg protein for Tea/CS groups respectively, $p<0.05$).

Serine protease concentration and activity

In order to observe the effect of cigarette smoking on serine protease, we measured NE concentration and activity in BAL and lung homogenates. The CS group showed significant up-regulation of NE concentrations in both BAL (1547.0 ± 65.2 ng/ml vs. 66.1 ± 4.3 ng/ml for CS and SA groups respectively, $p<0.01$) and lung (455.1 ± 71.0 vs. 25.9 ± 5.4 ng/mg protein for CS and SA groups respectively, $p<0.001$) (Figure 2a, 2b). NE activities were also increased in BAL (464.0 ± 19.6 mU/ml vs. 19.8 ± 1.3 mU/ml for CS and SA groups respectively, $p<0.01$) and lung homegentates (7.6 ± 3.1 vs. 0.6 ± 0.3 mU/mg protein for CS and SA groups respectively, $p<0.05$). Green tea consumption reduced CS-induced elevation of NE protein expression and activity in BAL and lung (Figure 2).

MPO activity

We also measured MPO, a major cytoplasmic component in neutrophils, which is released upon neutrophil activation and is widely used as a marker of neutrophil

activation. Figure 3a shows that MPO activity was increased in BAL (51 ± 3.0 mU/ml vs. 6.9 ± 0.7 mU/ml for CS and SA groups respectively, $p<0.01$) and lung homogenates (3179.0 ± 612.7 vs. 1106 ± 228.7 mU/mg protein for CS and SA groups respectively, $p<0.01$) from CS-exposed rats, which was prevented by Lung Chen tea (Figure 3b).

Anti-protease concentration in BAL

Interestingly, both α_1-AT (11770 ± 273 ng/ml vs. 293 ± 35 ng/ml for CS and SA groups respectively, $p<0.01$) and SLPI (86.0 ± 5.0 ng/ml vs. 3.8 ± 0.7 ng/ml for CS and SA groups respectively, $p<0.01$) concentrations were elevated after CS exposure. The up-regulation of α_1-AT and SLPI was reversed by Lung Chen tea (Figure 4).

Gelatin and casein zymography for activities of MMPs

Gelatin and casein zymography were performed to detect MMP-2/-9 and MMP-12 activity in BAL respectively. Pro- and active MMP-2 but not MMP-9 was detected with no significant difference between groups in BAL and lung homogenates using gelatin zymography (Figure 5a and b). On the other hand, we detected a significant elevation in active MMP-12 in BAL from CS group compared to SA group in casein zymography (Figure 5c), which was suppressed by Lung Chen tea. A weak band was observed in casein zymography using lung homogenates (Data not shown).

Quantification of MMP-12 activity

We found up-regulation of MMP-12 activity in BAL (4.5 ± 0.4 μM/ml vs. 2.9 ± 0.4 μM/ml for CS and SA groups respectively, $p<0.05$) and lung (1.1 ± 0.1 vs. 0.5 ± 0.1

μM/mg protein for CS and SA groups respectively, $p<0.01$) after CS exposure. The elevation was suppressed by Lung Chen tea (Figure 6), in line with casein zymogram result (Figure 5b).

Reverse zymography for activities of TIMPs

Reverse zymography with collagenase IV was done to detect the activity of TIMP-1 and TIMP-2 in lung homogenates. After CS exposure, TIMP-1 activity diminished compared to SA-exposed group (Figure 7a). No band for TIMP-2 was detected. Lung Chen tea had no effect on reduced TIMP-1 activity.

Discussion

The current study has demonstrated that local oxidative stress and protease/antiprotease imbalance in a CS-exposed rat model could be alleviated by green tea (Lung Chen) consumption. In line with previous findings, we confirmed CS-induced up-regulation of NE concentration and activity in BAL and lung, reflecting an increase in local protease activity [7,19]. We also found that cigarette smoking up-regulated α_1-AT and SLPI, suggesting a compensatory mechanism to protect the lungs from destruction. However, a loss of integrity of alveolar wall in this CS-exposed rat model was observed [12], which might be attributed to the incomplete inhibition of NE despite high levels of endogenous inhibitors, α_1-AT and SLPI [20].

Beside NE, MMPs have been demonstrated to be involved in the pathogenesis of COPD. MMP-9 has been found to cause emphysema when over-expressed in alveolar macrophages of mice [21]. Elevated MMP-9 level was found in induced sputum from COPD patients and smokers compared to healthy controls [22]. However, we could not detect any MMP-9 activity in BAL while the lung MMP-9 expression showed no significant change after smoking. Much attention has been paid to MMP-12 as Hautamaki *et al* [11] reported that mice lacking MMP-12 did not develop emphysema after smoking. MMP-12 activity and protein expression were up-regulated in induced sputum from COPD patients compared to healthy controls [9]. Valenca and colleagues [23] showed that smoking could induce MMP-12 protein expression in alveolar macrophages, in agreement with our findings.

Lung Chen tea contains abundant amount of green tea polyphenol, EGCG [24]. Our present findings that Lung Chen tea prevented CS-induced up-regulation of NE

protein level and activity as well as MMP-12 activity in BAL and lung, might be attributed to reduced neutrophil recruitment and reduction in reactive oxygen species (ROS) which have been reported with green tea [25]. To verify this, we measured MPO, a marker for neutrophil activation, and MDA, a marker for oxidative stress. We found increased levels of MDA in lung, and MPO activity in both BAL and lung from CS-exposed rats compared to SA-exposed rats, which returned to basal levels after Lung Chen tea consumption. The reduction of CS-induced elevated MDA level after Lung Chen tea might probably due to a direct ROS scavenging effect while the reduction in the CS-induced up-regulation of airway MPO activity by Lung Chen tea suggested the suppression of neutrophil activation. In a previous study, decreased MPO level was found in induced sputum of patients with COPD after treatment with oral glucocorticoids [26]. We have also observed an increase in the number of neutrophils after CS exposure in BAL by differential cell count, which was reduced in the presence of Lung Chen tea (data not shown). However, the mechanism of green tea-induced reduction of neutrophil influx remains unclear and will need further investigation.

MMP-12 was released from activated macrophages upon stimulation by elastin fragment, a degradation product during NE-mediated tissue injury [27]. It has been reported that EGCG could inhibit MMP-12 activity *in vitro* [28], in agreement with our data, suggesting that the reduction of MMP-12 activity by Lung Chen tea in CS-exposed rats is possibly through down-regulation of both neutrophil activation and NE activity.

Anti-protease, TIMP-1, is a small endogenous inhibitor for MMPs which binds and blocks the active site of the enzymes. Four TIMPs isoforms in human have been isolated: TIMP-1, -2, -3 and -4. The exact roles of these TIMPs are not clearly identified.

They probably play a very important role in regulating the activation and activity of MMPs in normal matrix turnover or tissue repair. It is believed that they have overlapping activities on different MMPs. TIMP-1 was reported to have a more specific binding activity to pro-MMP-9 while TIMP-2 binds more specifically to pro-MMP-2 [29]. Nonetheless, we only observed a band for TIMP-1 activity but not TIMP-2 activity in lungs from CS-exposed rats. The current findings of the elevation of NE and MMP-12 activities and the reduction of TIMP-1 activity reflect a CS-induced protease/anti-protease imbalance in rats' lung, which may lead to emphysema-like lung injury as an increase in mean linear intercept (L_m) seen in our previous report [12]. Alveolar macrophages from healthy subjects have been found to release more TIMP-1 than that from patients with COPD [30], in contrast to current findings. However, we found that treatment of Lung Chen tea could not prevent the down-regulation of TIMP-1 activity, in agreement with a previous study [31].

There are potential limitations in our study. First, we used brewed Lung Chen tea instead of pure EGCG because of lower cost and possibly synergic effects with other tea components on anti-oxidation and bioavailability [32]. This limits the clear delineation of the pathogenic mechanisms, as the mixed components of green tea might contain other substances with unknown effects. On the other hand, it is more relevant in reality as people drink green tea and not pure EGCG extracts as part of their lifestyle habit. Secondly, since Lung Chen tea was given on the same day immediately before and after CS exposure, therefore any demonstrated effects could only be considered as mainly preventive rather than interventional.

Clearly, our results demonstrate that cigarette smoking could induce protease/anti-protease imbalance by up-regulating NE and MMP-12 activity, and down-regulating TIMP-1 activity in a rat model. Lung Chen tea could suppress both CS-elevated NE and MMP-12 activity possibly by suppression of oxidative stress, and reduction in neutrophil recruitment and activation. Taken together, our findings suggest that green tea consumption may result in beneficial effects through alleviation of oxidative stress and protease/antiprotease imbalance during CS-induced lung injury in human, but more clinical research is warranted in future. In addition, inhibition of these proteases might be a more therapeutic strategy over the use of antioxidants.

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Declaration of interest

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Figure legends

Figure 1. MDA levels in lung homogenates of cigarette smoke (CS)- and sham air (SA)-exposed rats with or without Lung Chen tea consumption. $^{**}p < 0.01$ compares CS and SA group. $^{\#}p < 0.05$ compares the CS and Tea/CS group. Means ± SEM are shown.

Figure 2. Neutrophil elastase protein levels and activity in cigarette smoke (CS)- and sham air (SA)-exposed rats with or without Lung Chen tea consumption. a) Neutrophil elastase protein level in bronchoalveolar lavage (BAL). b) Neutrophil elastase protein level in lung homogenates. c) Neutrophil elastase activity in BAL. d) Neutrophil elastase activity in lung homogenates. $^{*}p < 0.05$ compares CS and SA group; $^{\#}p < 0.05$ compares CS and Tea/CS group.

Figure 3. Myeloperoxidase activity in cigarette smoke (CS)- and sham air (SA)-exposed rats with or without Lung Chen tea consumption. a) Myeloperoxidase activity in bronchoalveolar lavage. b) Myeloperoxidase activity in lung homogenates. $^{**}p < 0.01$ and $^{**}p < 0.01$ compares cigarette smoke and sham air group; $^{\#\#}p < 0.01$ and $^{\#\#}p < 0.01$ compares cigarette smoke and Tea/CS group.

Figure 4. Anti-protease levels in bronchoalveolar lavage (BAL) of cigarette smoke (CS)- and sham air (SA)-exposed rats with or without Lung Chen tea consumption. a) α_1-antitrypsin protein level in BAL. b) Secretory leukoproteinase inhibitor (SLPI) protein level in BAL. $^{**}p < 0.01$ compares CS and SA group; $^{\#\#}p < 0.01$ compares CS and Tea/CS group.

Figure 5. Gelatin and casein zymographic analysis of bronchoalveolar lavage (BAL) and lung homogenates from cigarette smoke (CS)- and sham air (SA)-exposed rats with or without Lung Chen tea consumption. a) Representative gelatin zymography for matrix metalloproteinase-2 in BAL. b) Representative gelatin zymography for matrix metalloproteinase-2 and matrix metalloproteinase-9 in lung homogenates. c) Representative casein zymography for matrix metalloproteinase-12 activity in BAL.

Figure 6. Fluorometric measurement of matrix metalloproteinase-12 activity in cigarette smoke (CS)- and sham air (SA)-exposed rats with or without Lung Chen tea consumption. a) Matrix metalloproteinase-12 activity in bronchoalveolar lavage (BAL). b) Matrix metalloproteinase-12 activity in lung homogenates. $^{*}p < 0.05$ compares CS and SA group; $^{\#}p < 0.05$ compares CS and Tea/CS group.

Figure 7. Reverse zymographic analysis of lung homogenates from cigarette smoke (CS)- and sham air (SA)-exposed rats with or without Lung Chen tea consumption. a) Representative reverse zymography for tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) activity in rat lung. b) Semi-quantification for TIMP-1 activity. $^{*}p < 0.05$ compares CS and SA group.

Figure 1

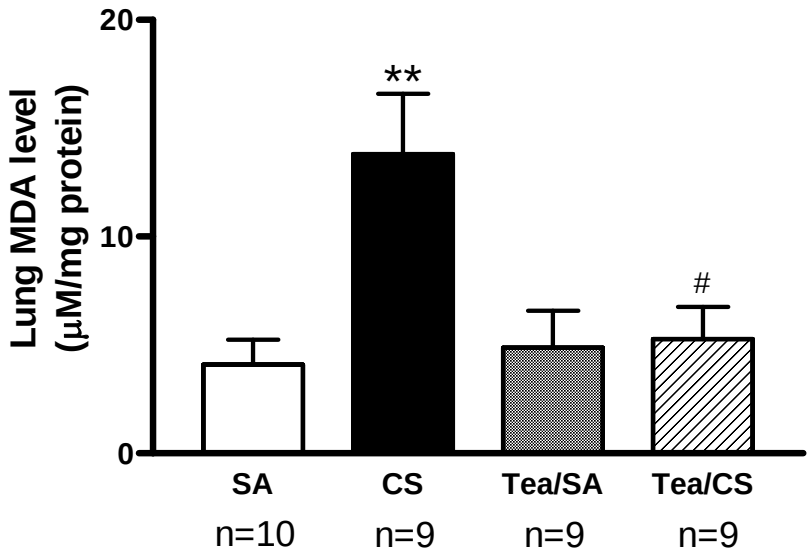
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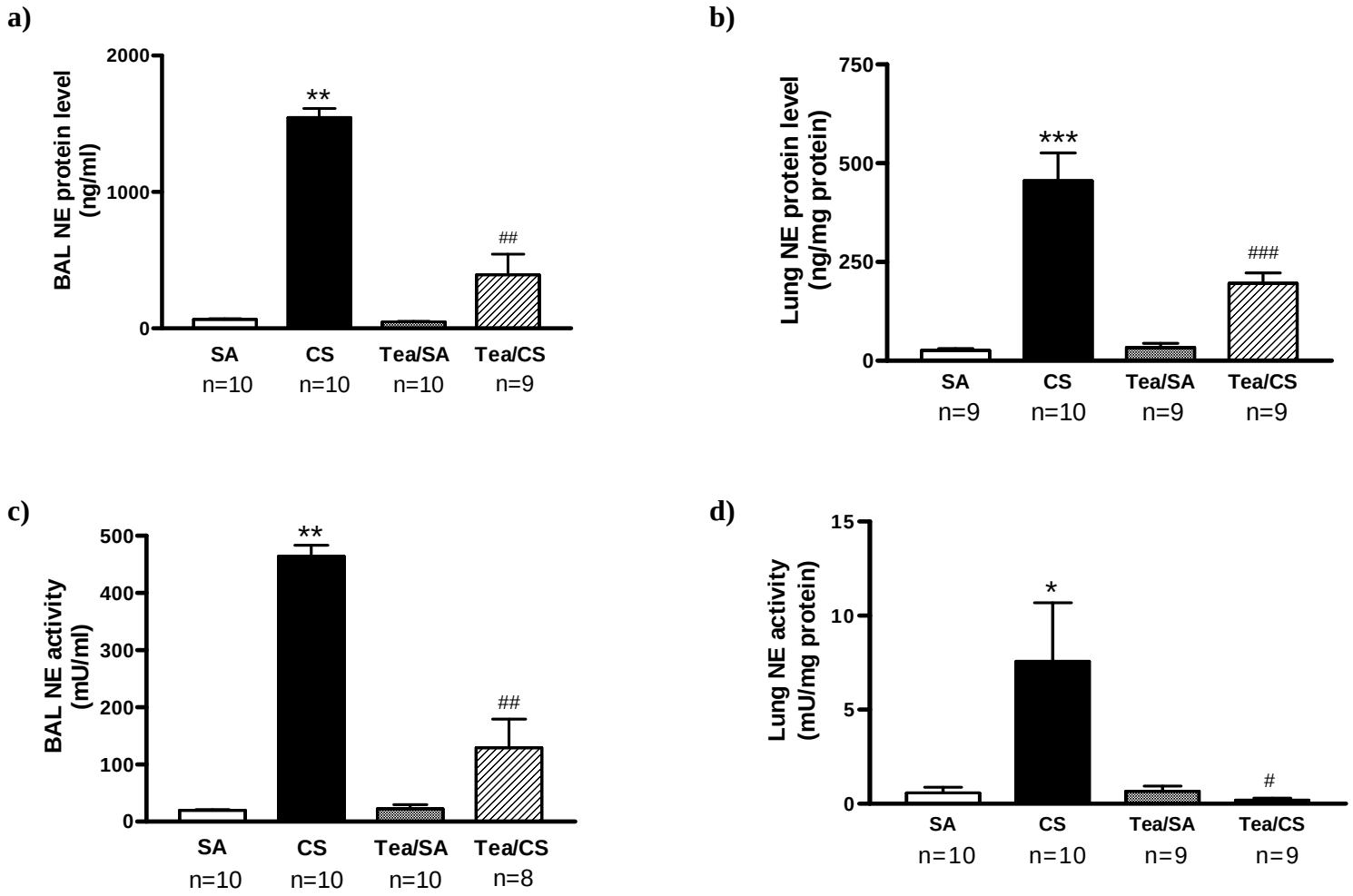
Figure 2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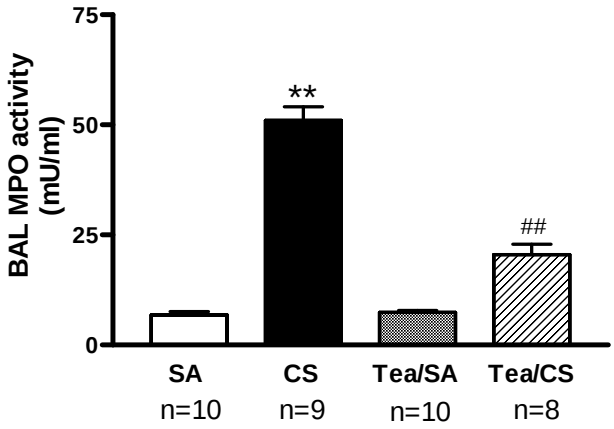
CS
Tea/SA
Tea/CS
n=10
n=10
n=9
n=9

Figure 3

a)


BAL MPO activity (mU/ml)
75
50
25
0
**
##
SA
CS
Tea/SA
Tea/CS
n=10
n=9
n=10
n=8

b)

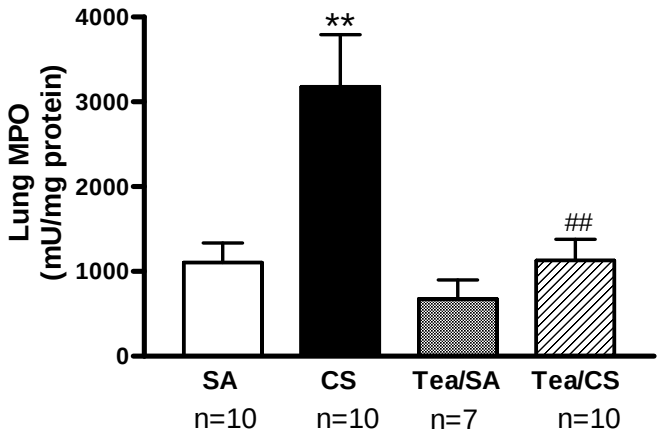
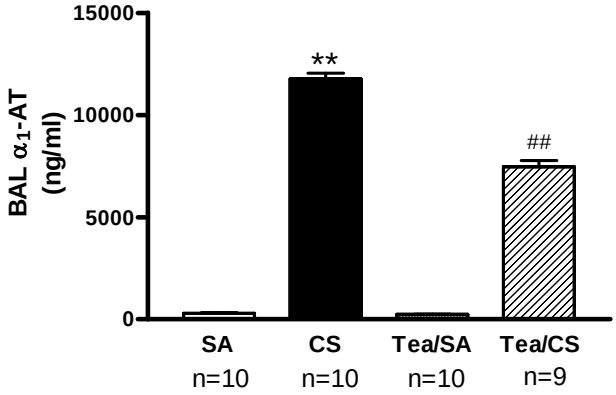

Lung MPO (mU/mg protein)
4000
3000
2000
1000
0
**
##
SA
CS
Tea/SA
Tea/CS
n=10
n=10
n=7
n=10

Figure 4

a)


15000
10000
5000
0
BAL α1-AT (ng/ml)
**
##
SA
n=10
CS
n=10
Tea/SA
n=10
Tea/CS
n=9

b)

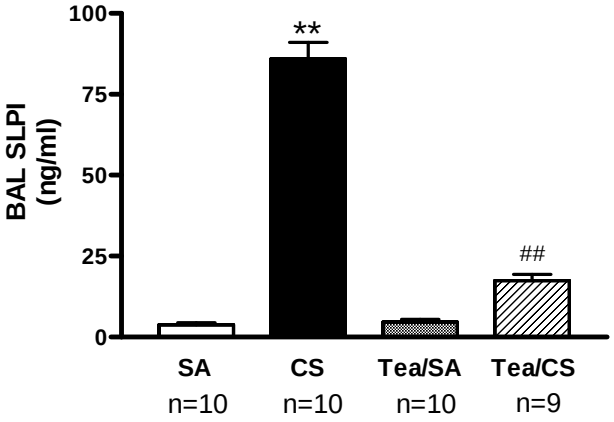
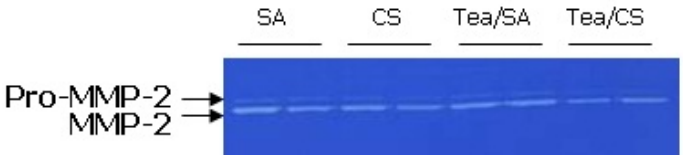
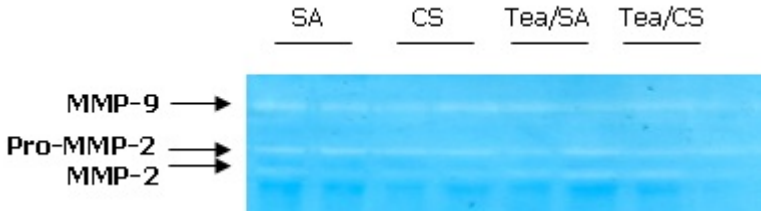

100
75
50
25
0
BAL SLPI (ng/ml)
**
##
SA
n=10
CS
n=10
Tea/SA
n=10
Tea/CS
n=9

Figure 5

a)


SA
CS
Tea/SA
Tea/CS
Pro-MMP-2
MMP-2

b)


SA
CS
Tea/SA
Tea/CS
MMP-9
Pro-MMP-2
MMP-2

c)

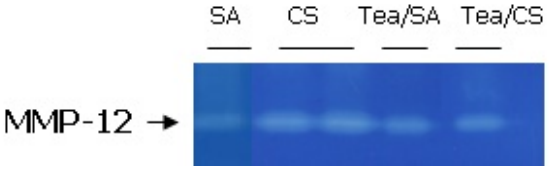
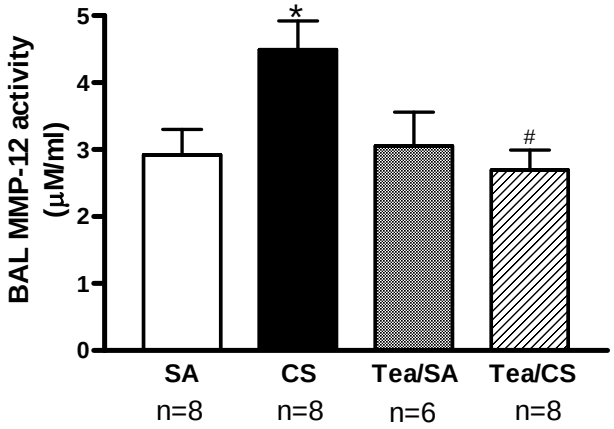

SA
CS
Tea/SA
Tea/CS
MMP-12

Figure 6

a)


BAL MMP-12 activity (µM/ml)
5
4
3
2
1
0
*
#
SA
CS
Tea/SA
Tea/CS
n=8
n=8
n=6
n=8

b)

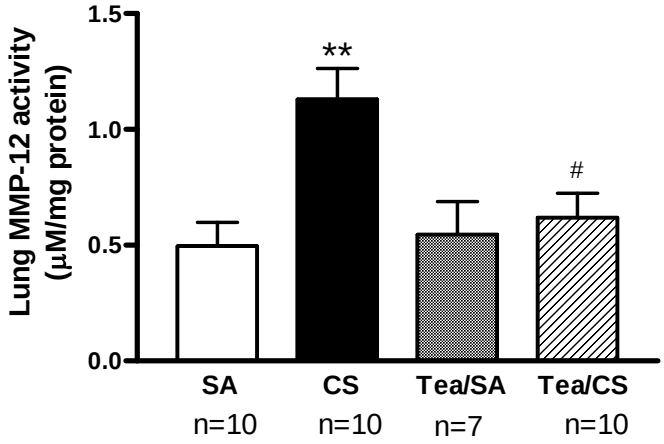
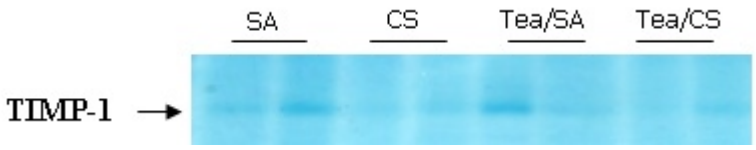
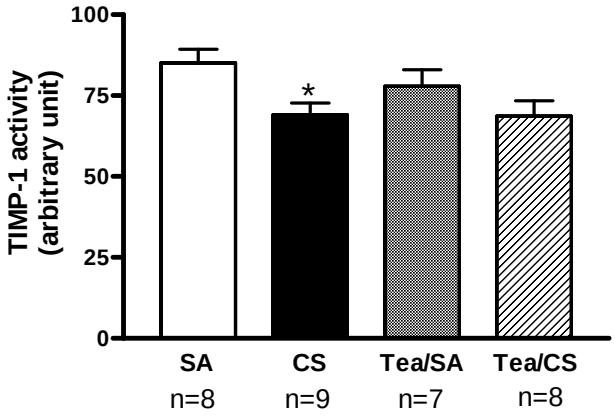

Lung MMP-12 activity (µM/mg protein)
1.5
1.0
0.5
0.0
**
#
SA
CS
Tea/SA
Tea/CS
n=10
n=10
n=7
n=10

Figure 7

a)


SA
CS
Tea/SA
Tea/CS
TIMP-1

b)


TIMP-1 activity (arbitrary unit)
100
75
50
25
0
*
SA
n=8
CS
n=9
Tea/SA
n=7
Tea/CS
n=8