Concomitant exposure to cigarette smoke and coal dust induces lung oxidative stress and decreases serum MUC5AC levels in male rats

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Abstract This study aimed to investigate whether concomitant exposure to cigarette smoke and coal dust could activate the epidermal growth factor receptor (EGFR) for MUC5AC expression. Thirty-two male Wistar rats were divided into the following groups (n = 8 each): control group (C); exposed to cigarette smoke plus coal dust at doses of 6.25 mg/m³ (CS + CD1); 12.5 mg/m³ (CS + CD2); and 25 mg/m³ (CS + CD3). The duration of exposure was 21 days, 1 hour/day. Lung malondialdehyde level was analyzed colorimetrically. Serum EGF and MUC5AC expression were measured by ELISA. Expression of lung EGFR and MUC5AC were measured by a confocal laser scanning microscopy. The level of lung malondialdehyde was higher significantly in all doses of exposure compared with control group (p < 0.05). The level of serum EGF was significantly increased in CS + CD2 group compared with control group or CS + CD1 group. The
expression of EGFR was not significantly different among all the treatment groups ($p > 0.05$). Serum MUC5AC levels were significantly lower in the two highest doses of coal dust compared with the control group. In conclusion, subchronic combined exposure to cigarette smoke and coal dust induces lung oxidative stress and inflammation and decreases serum MUC5AC level.

**Introduction**

Cigarette smoke contains toxic and/or carcinogenic gases and chemicals. Chemical composition of cigarette smoke depends on: (1) the type of cigarette; (2) cigarette design, such as the presence/absence of a filter; and (3) individual smoking patterns. A burning cigarette produces approximately 500 mg (92%) of gas and the remaining 8% is solid particulates. The gas phase consists mostly of CO$_2$, O$_2$, and N$_2$. Cigarette smoke also contains approximately $10^{15} - 10^{17}$ oxidants/free radicals and 4700 chemical compounds. Cigarette-smoke-derived toxic compounds, including aromatic hydrocarbons, have been shown to be lung carcinogens.$^1$

Lung cancer is a smoking-related disease and cause of death with an increasing incidence.$^1$ Numerous studies have demonstrated the role of cigarette smoke in an increased incidence of cancer in asbestos-exposed individuals.$^2$–$^4$ Cigarette-smoke exposure prior to naphthalene exposure can interfere with repair of bronchial epithelial cells in which squamous cells settle in the terminal bronchioles.$^5$ This indicates that combined exposure of cigarette smoke and other toxicants will induce carcinogenicity. To date, there is a lack of studies that analyze the mechanism of pathophysiology due to exposure to cigarette smoke and coal dust.

Smoking causes lung inflammation due to the influx of macrophages, neutrophils, dendritic cells, and CD8 T-lymphocytes as a source of inflammatory mediators. In addition, smoking also leads to oxidative stress.$^6$ Inflammation and oxidative stress underlie metaplasia, which are the earliest process prior to the occurrence of lung cancer. Epidermal growth factor receptor (EGFR) expression in the bronchial epithelium increases as a result of the activation of neutrophils and EGFR tyrosine kinase by its ligands. Subsequently, hypersecretion of mucus and goblet cell metaplasia will result. In vitro and in vivo studies showed that proinflammatory cytokines would up regulate the expression of EGFR (transforming growth factor-$

**Material and methods**

**Animals**

Thirty-two male Wistar albino rats, 16 weeks of age, weighing 175–200 g, were used for this study. They were divided into the following groups ($n = 8$ rats each): control group (C); exposed to cigarette smoke plus coal dust at doses of 6.25 mg/m$^3$ (CS + CD$_1$); exposed to cigarette smoke plus coal dust at doses of 12.5 mg/m$^3$ (CS + CD$_2$); and exposed to cigarette smoke plus coal dust at doses of 25 mg/m$^3$ (CS + CD$_3$). The duration of exposure was 21 days. Animals were housed in a clean wire cage and maintained under standard laboratory conditions with temperature of 25 $\pm$ 2°C and 12-hour dark/light cycle. Standard diet and water were provided ad libitum. Animals were acclimatized to laboratory conditions for 2 weeks prior to the experiment. Animal care and experimental procedures were approved by the institutional ethics committee of Faculty of Medicine, Brawijaya University, Malang, Indonesia.

**Coal dust preparation**

Coal dust preparation was performed as described in our previous studies.$^7$–$^{10}$ Two kilograms of sub-bituminous gross coals obtained from coal mining area in South Kalimantan, Indonesia, were pulverized by Ball Mill, Ring Mill, and Raymond Mill in the Carsurin Coal Laboratory of Banjarmasin. Coal dust particles were then filtered by Mesh MicroSieve (BioDesign, New York, NY, USA) to produce particles with the diameter $< 10$ $\mu$m (PM$_{10}$) that have been well characterized previously.$^7$–$^{10}$

**Cigarette smoke exposure**

Smoking exposure was done by smoking pump equipment that was designed and available in Pharmacology Laboratory, Medical Faculty, Brawijaya University of Malang. The rats in the control group were exposed to fresh air under similar conditions. Rats placed into whole-body exposure chambers ($26$ cm $\times$ $12$ cm $\times$ $12$ cm$^3$) made from fiberglass and were exposed to cigarette smoke for 7 min/cigarette, once a day in the morning prior to coal dust exposure, for 21 days. During exposure, the temperature...
was maintained at 22–25°C, and relative humidity was approximately 50%.

**Coal dust exposure**

The concentration of coal dust and produce for exposure were determined according previous studies. The exposure chamber was designed and available in the Laboratory of Pharmacology, Faculty of Medicine, Brawijaya University. The procedure work of the chamber is to supply an ambient resuspended PM10 coal dust that can be inhaled by rats. The chamber was 0.5 m³ and flowed by a 1.5—2 L/minute airstream that adopted the upper ground coal mine environmental airstream. To make a more comfortable minute airstream that adopted the upper ground coal mine environmental airstream. To make a more comfortable chamber, oxygen was also provided.7

**Tissue sampling**

After 21 days of exposure, the animals were subjected to euthanasia by ether inhalation and exsanguinated by cardiac puncture. The lungs were collected, weighed, and washed with physiological saline. The right lung was histologically processed with hematoxylin—eosin staining and confocal microscopy (EGFR and MUC5AC). The left lung was homogenized to measure the malondialdehyde (MDA) level colorimetrically. Serum EGF and MUC5AC were measured by enzyme-linked immunosorbent assay technique. All samples were labeled and stored at −80°C until analysis.

**MDA analysis**

The lung MDA levels were assayed by a previous method. Lungs were perfused using ice-cold PBS to obtain clean and free of blood puncture. Then, lungs were homogenized in KCl buffer (pH 7.6). The homogenate was mixed with 2.5 volumes of 10% (w/v) trichloroacetic acid to precipitate the protein. The precipitate was then centrifuged and the supernatant was reacted with 0.67% tetrabutylammonium in a boiling water-bath for 25 minutes. After cooling, the absorbance of the colored product was read at 532 nm using the spectrophotometer. The values obtained were compared with a series of MDA tetrabutylammonium salt (SigmaeAldrich, St Louis, MO, USA) as standard solutions.

**Analysis of EGF and MUC5AC**

The serum EGF and MUC5AC ELISA kit were purchased from USCNK, Life Science, Inc (Wuhan, Hubei, China). The analysis was done according to detail procedures in the kit.

**Labeling immunofluorescence staining of EGFR and MUC5AC**

Double-labeling immunofluorescence staining of EGFR and MUC5AC was done according to previous studies.12

**Statistical analysis**

Data are presented as mean ± standard deviation and the differences between groups were analyzed using one-way analysis of variance (ANOVA) with SPSS 15.0 statistical package for Windows (SPSS, Chicago, IL, USA). Only probability values of p < 0.05 were considered statistically significant and later subjected to Tukey’s posthoc test.

**Results**

**Characteristics of cigarette smoke**

The present study used *Trubus Alami* brand clove cigarettes, which were produced in Tulungagung. These cigarettes contained 2.90 mg of tar and 44.30 mg of nicotine. Carbon monoxide level of cigarette smoke was 102.33 ppm.13

**Lung morphology**

Lung morphology after exposure to cigarette smoke and coal dust is shown in Fig. 1. Subsequent to 21 days of exposure to cigarette smoke and coal dust, there were morphological changes with features of intense and massive inflammation, excess mucus that covered the lumen, and fibrotic process. It was seen that the widened lumen was surrounded by inflammatory cells, the alveoli were more intensely inflamed with widening of alveolar lumen, and the alveolar epithelial bridge was lost, resulting in local emphysema (Fig. 1B–1D). Fibrogenesis occurred outside the lumen so that the lumen was surrounded by increasingly dominant fibrous connective tissues in accordance with exposure dosing (Fig. 1B–D).

**Levels of MDA**

Levels of MDA for different coal dust exposures are presented in Table 1. The level of lung MDA was higher significantly in all doses of exposure compared with the control group (p < 0.05). MDA level was significantly elevated in CS + CD2 or CS + CD3 compared with CS + CD1 group (p < 0.05). The level was also increased significantly in CS + CD2 over that of the CS + CD2 group (p < 0.05).

**EGF level**

Mean levels of serum EGF for different groups is presented in Table 1. The level of serum EGF was significantly increased in the CS + CD2 group compared with the control group and with CS + CD1 (p < 0.05).

**EGFR expression**

Confocal laser scanning microscopic analysis of EGFR expression for 21-day exposure to cigarette smoke and coal dust is presented in Table 1 and Fig. 2. The expression of EGFR was not significant differences among all the treatment groups (p > 0.05).

**MUC5AC expression**

Lung MUC5AC expression levels for all groups can be seen in Table 1 and Fig. 2. The expression of lung MUC5AC was not significantly different between groups (p > 0.05). Out of the 6.25 mg/m³, 12.5 mg/m³, and 25 mg/m³ doses of coal
dust exposure, only the two highest doses significantly decreased the serum MUC5AC expression compared with the control group \( (p < 0.05) \), as seen in Table 1.

**Discussion**

A burning cigarette produces approximately 500 mg (92%) of gas and the remaining 8% is solid particulates. Additionally, cigarette smoke contains acetaldehyde, hydroquinone, formaldehyde, benzo(a)pyrene, cresol, nicotine, catechol, acrolein, coumarin, anthracene, nitrogen oxides, and heavy metals. In the present study, cigarette smoke contained 2.90 mg of tar and 44.30 mg of nicotine. Contents of tar and nicotine used in this study were higher than those (13 mg and 1.4 mg of tar and nicotine, respectively) used in the prior study. Tar contains high concentrations of stable radical, which continually accumulate in the smoker’s lung. Subsequent to 21 days of concomitant exposure to cigarette smoke and coal dust, there were bronchoalveolar morphological changes with features of intense and massive

**Table 1** Levels of lung and serum biomarkers after 21-day’s exposure to cigarette smoke and coal dust.

<table>
<thead>
<tr>
<th>Level</th>
<th>Doses of coal dust exposure</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0 mg/m³</td>
</tr>
<tr>
<td>Lung Malondialdehyde (ng/mL)</td>
<td>0.0211 ± 0.0098</td>
</tr>
<tr>
<td>Serum EGF (pg/mL)</td>
<td>103.66 ± 7.75</td>
</tr>
<tr>
<td>Lung EGFR (AU)</td>
<td>826.36 ± 57.25</td>
</tr>
<tr>
<td>Lung MUC5AC (AU)</td>
<td>582.06 ± 361.65</td>
</tr>
<tr>
<td>Serum MUC5AC (pg/ml)</td>
<td>0.80 ± 0.28</td>
</tr>
</tbody>
</table>

Values are presented as mean ± standard deviations: \(^a\)\(p < 0.05\) compared with control group, \(^b\)\(p < 0.05\) compared with the group exposed to cigarette smoke plus coal dust at doses of 6.25 mg/m³, \(^c\)\(p < 0.05\) compared with the group exposed to cigarette smoke plus coal dust at doses of 12.5 mg/m³.

AU = arbitrary unit; EGF = epidermal growth factor; EGRF = epidermal growth factor receptor.
inflammation, mucous secretion that filling the lumen, and fibrotic process. It was apparent that lumen widening was surrounded by inflammatory cells. Increasingly intense inflammation occurred in alveolar areas with a widened alveolar lumen, and broken interalveolar septa were observed, resulting in local emphysema. Fibrogenesis occurred outside the lumen so that the lumen was surrounded by increasingly dominant fibrous connective tissues in accordance with exposure dosing. Previous studies conducted cigarette smoke exposure for 1 month, 2 months, and 4 months. Goblet cell hyperplasia, mucus secretion in airway epithelium, and damaged interalveolar septa were observed at 1 month of exposure. Meanwhile, alveolar interstitium filled with collagen fibers was observed at 4 months of exposure. Our study observed morphological features similar to those observed at 1 month of the above study. Accelerated pulmonary fibrosis seen in our study was caused by the interaction of cigarette smoke and coal dust and the high contents of active components in cigarette smoke.

Lung inflammation is defined as small clusters of inflammatory cells in the alveoli consisting of alveolar macrophages and lymphocytes. Inflammation has specific purposes. Acute inflammation protects organs from damage, but chronic inflammation is associated with disease progression. In the present study, exposure to cigarette smoke and coal dust (12.5 mg/m³) caused a significant increase in EGF expression compared with control. We hypothesized that the interaction of cigarette smoke and coal dust at a dose of 12.5 mg/m³ led to an increase in the release of the extracellular domain of pro-EGF into mature EGF mediated by metalloproteinases of the ADAM family, particularly ADAM 10. Numerous studies have shown an increased activity of matrix metalloproteinases (MMPs) as a result of exposure to cigarette smoke. Cigarette smoke activates MMPs to release EGF, which subsequently induces EGFR phosphorylation to activate mitogen-activated protein kinases. In the present study, EGFR expression was not significantly different in different treatment groups so that an increase in EGF was not followed by upregulation of EGFR.

The present study showed that exposure to cigarette smoke and coal dust increased pulmonary oxidative stress (p < 0.01). This finding indicates that components of cigarette smoke and coal dust interact to induce oxidative stress. Cigarette smoke contains 10¹⁷ oxidant molecules per puff of both mainstream and sidestream smoke. Gas phase and particulate phase of cigarette smoke contain nitric oxide, superoxide radicals, and organic peroxy radicals. Gas-phase radicals are highly reactive and have a short half-life. Particulate-phase radicals are relatively stable and consist of hydroquinone—semiquinone—quinone complex. These complexes represent an active redox system capable of reducing molecular oxygen to form superoxide radicals. In addition, cigarette smoke also contains long-lived metals, such as nickel and cadmium.

Free radicals of cigarette smoke will interact with antioxidants in the epithelial lining fluid of the airway epithelium and cellular membrane directly to induce damage. Previous studies found an increase in oxidative damage to lung tissue that included lipid peroxidation (MDA). Inhalation of coal dust will form reactive oxygen compounds via direct and indirect mechanisms. Direct
mechanism involves bioactive components of coal dust and indirect mechanism involves the oxidative burst during activation of macrophages and polymorphonuclear leukocytes. With the direct mechanism, oxidative capacity of coal dust bioactive component is caused primarily by transition-metal contents, including Fe, Cr, Mn, Co, Ni, Cu, Zn, and silica. Some of these metals are capable of catalyzing the Fenton reaction to produce reactive oxygen compounds. Comparison of oxidative stress in healthy individuals with that of coal miners showed a highly significant difference in plasma MDA levels. With indirect mechanism, superoxide radicals form during phagocytosis of inhaled particles, which will dismutase to form hydrogen peroxide. In the presence of transition metal ions, such as Fe or Cu ions, hydrogen peroxide is converted to hydroxyl radicals (Fenton or Haber–Weiss reaction). The formation of excess reactive oxygen compounds will exceed antioxidant capacity, resulting in pulmonary oxidative stress. Furthermore, production of proteolytic and elastase enzymes, in conjunction with reactive oxygen compounds, will denature proteins and damage carbohydrates and lipid peroxidation and, consequently, lead to changes, such as fibrosis. The present study found that fibrosis may be associated with oxidative stress.

Compared with nonsmokers, the risk of lung cancer incidence in smokers is 22-fold higher in men and 12-fold higher in women. It has been known that the remodeling of the upper and lower respiratory tracts into squamous metaplasia, resulting in the development of diseases, was associated with exposure to cigarette smoke. There is no study of both the development and incidence of lung cancer caused by combining exposure to cigarette smoke and coal dust. Various ingredients of cigarette smoke bring about an increase in MUC5AC. Exposure to acrolein would increase MUC5AC mRNA expression. The benz(a)pyrene caused up regulation of MUC5AC. Previous studies found increased expression of MUC5AC as a result of exposure to cigarette smoke. MUC5AC hypersecretion is consistent with activation of EGFR-AP-1/nuclear factor (NF)-κB signaling pathways. In our study, exposure to 25 mg/m³ of cigarette smoke and coal dust led to a significant decrease in MUC5AC expression compared with control. Our finding indicated that the active components of cigarette smoke or coal dust may inhibit the signaling for serum MUC5AC production. Previous studies showed that lipopolysaccharide exposure is able to induce goblet cell metaplasia, and that increased MUC5AC expression could be inhibited by administration of an MMP inhibitor. In addition, compound act as inhibitor for NF-κB also potential to inhibits MUC5AC expression. Thus, we hypothesized that combined cigarette smoke and coal dust in the present study may produce effects analogous to those of an MMP or NF-κB inhibitors.

Conclusion

Our data suggest that subchronic combined exposure to cigarette smoke and coal dust induces lung oxidative stress and inflammation and also decreases the serum MUC5AC level.

Conflicts of interest

The authors declare that there are no conflicts of interest.

References


