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ORGAN TOXICITY AND MECHANISMS

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Increased expression of iNOS and c-fos via regulation of protein tyrosine phosphorylation and MEK1/ERK2 proteins in terminal bronchiole lesions in the lungs of rats exposed to cigarette smoke

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Abstract Epidemiological evidence suggests that smoking is a major cause of human lung cancer. However, the mechanism by which cigarette smoke induces the cancer remains unestablished. To evaluate the effects of cigarette smoke on the expression of inducible nitric oxide synthase (iNOS), nuclear protooncogenes and related mitogen-activated protein kinases (MAPKs) in rat lung tissue, a histopathological study of the effects of gas-phase cigarette smoke on rat lung tissue were carried out. The terminal bronchioles were found to be infiltrated predominantly by lymphocytes in the peribronchiolar region and a mild to moderate degree of emphysema was noted in the alveolar spaces. The terminal bronchioles also showed marked lipid peroxidation, dilatation, and peribronchiolar fibrosis. Immunohistochemical evaluation showed that the expression of iNOS, NF-κB, MAPKs (MEK1, ERK2), phosphotyrosine protein and c-fos was increased in the terminal bronchioles but protein kinase C (PKC), MEKK-1, c-jun, p38 and c-myc showed no change. These results provide evidence to suggest that exposure to cigarette smoke results in oxidant stress which leads to the stimulation of iNOS and c-fos together with the induction of protein tyrosine phosphorylation and MEK1/ERK2 which in turn may promote lung pathogenesis.

Key words Cigarette smoke · c-Fos · NF-κB · Mitogen-activated protein kinases · Inducible nitric oxide synthase · Protein tyrosine phosphorylation

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Introduction

Epidemiological studies have shown that smoking is a major cause of human cancer at a variety of sites (Chow et al. 1993; Heineman et al. 1994; McLaughlin et al. 1990; McLaughlin et al. 1995). However, the mechanism by which cigarette smoke induces cancer remains unestablished. Among the 3800 compounds identified in cigarette smoke, a large number show mutagenic and carcinogenic activity (Vineis and Caporaso 1995). High concentrations of reactive oxygen species (ROS) and nitrogen oxides (NO_x) produced from cigarette smoke (Church and Pryor 1985; Nakayama and Kodama 1984; Pryor and Prier 1983) may also be involved in its carcinogenicity (Zhu et al. 1998). However, a direct molecular role for cigarette smoke in the development of lung cancer has not yet been defined. Previous studies have shown that exposure of 3T3 cells to mainstream cigarette smoke results in the expression of c-fos (Muller 1995). However, the mechanism behind the cigarette smoke-induced expression of c-fos and other signal proteins still remains to be elucidated.

Protein phosphorylation at tyrosine residues is a key component in the regulation of eukaryotic cell growth, differentiation and other responses (Segar and Krebs 1995). The phosphotyrosine proteins have been linked to many cellular events, involving stimulation of mitogen-activated protein kinases (MAPKs). MAPKs are members of a serine/threonine kinase family (Cobb and

Goldsmith 1995). A well-defined MAPK subfamily consists of extracellular signal-regulated protein kinases (ERKs), which are mainly activated by growth factors but can also be activated by some cell stress signals (Guyton et al. 1996; Marshall 1994). One of the events required for stress-induced proliferation, inflammation and carcinogenesis is the transcriptional elevation of the expression of several genes (Bernstein and Colburn 1989; Cerutti et al. 1992; Dong et al. 1994; Stevenson et al. 1994; Tseng et al. 1994) including c-jun, c-fos, c-myc and inducible nitric oxide synthase (iNOS) which are known to be activated by stress signals through stimulation of tyrosine kinases which in turn modulate downstream events including activation of MAPKs (Repp 1991) and expression of nuclear protooncogenes.

Inappropriate regulation of NF-κB-mediated transcription has also been associated with pathological conditions including acute inflammation, viral replication and cancer (Baldwin 1996; Grilli et al. 1993; O'Neill and Kaltschmidt 1997). To elucidate the mechanism of c-fos expression and the role of signaling proteins in cigarette smoke-induced lung injury, we designed an animal exposure system. We investigated the expression of protein kinase C (PKC), tyrosine phosphoproteins, MAPKs, NF-κB, iNOS and nuclear protooncogenes in the terminal bronchiole areas of the lung tissue of rats exposed to cigarette smoke.

Materials and methods

Animal treatment

Male Wistar rats (120 ± 10 g body weight) were purchased from the National Science Council, Taiwan. The animals were housed three per cage in an environmentally controlled animal house. All animals were handled according to the guidelines of the Taiwan Society for Laboratory Animal Sciences for the care and use of laboratory animals. The rats were divided into four exposure groups, with six rats in each group. For the exposure tests, rats were placed in whole-body exposure chambers (Fig. 1) and exposed to the smoke from 5, 10 or 15 cigarettes (New Paradise, Taiwan) or filtered room air. The filtered breathing air was introduced into the chamber at a flow rate of 200 l/min. Each group was exposed for 1 h per day for 5 days per week over 2 months. During exposure, the temperature was maintained at 22–25°C and the relative humidity at approximately 40%. In order to control the exposure conditions in each experiment, the concentration of NO_x in the exposure chambers was monitored continuously. The rats were killed 2 months after cessation of exposure by decapitation and the blood was collected for assay of nitric oxide. The lungs were excised for the immunohistochemical determination of PKC, NF-κB, iNOS, nuclear protooncogenes, tyrosine phosphoproteins and MAPKs.

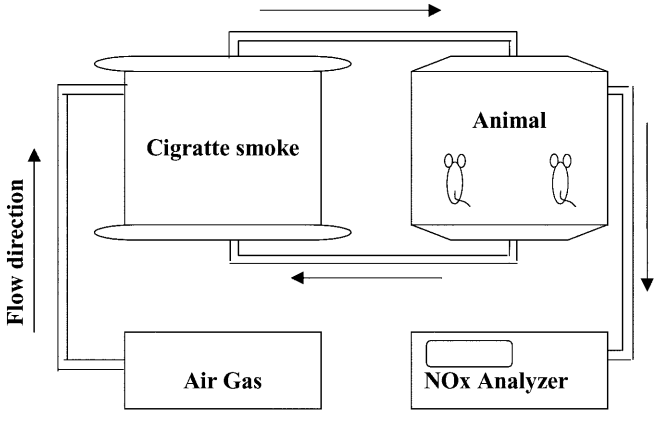


Fig. 1 Block diagram of the chamber used for cigarette smoke exposure

Measurement of nitric oxide production

The induction of iNOS after exposure to cigarette smoke was estimated in terms of the formation of nitric oxide which was quantified as serum nitrite using a procedure based on the Griess reaction with sodium nitrite as a standard (Green et al. 1982).

Histochemical detection of lipid peroxidation

The rats were killed and the lungs were quickly removed and rinsed in ice-cold saline. Tissue samples (approximately 5×10 mm in size) were cut, immediately frozen under a CO_2 stream, and processed for histochemistry. Lung sections (15 μm thick) from smoke-exposed rats were stained for 2.5 h in the dark at room temperature with Schiff's reagent. After the reaction, the sections were rinsed three times with sulfite water (5 ml 10% $K_2S_2O_5$, 5 ml 1 *N* HCl, water to 100 ml), dehydrated in alcohol, cleared in xylene, and mounted on balsam (Pompella et al. 1987).

Histopathological examination

Immediately after death, a complete necropsy was performed and the lungs were examined for lung injury. The lungs were washed with physiological saline and inspected for gross lesions. Tissues were fixed in 10% buffered formalin, processed for histological examination according to conventional methods. Step sections (five sections per block of tissue) were prepared from the lung tissue and stained with hematoxylin and eosin.

Immunohistochemical assessment of protein expression

After excision, the lungs were fixed in 10% buffered-formalin solution for 18–24 h, dehydrated, embedded in paraffin and cut into sections of 5 μm thickness. For immunohistochemical examination, the sections were deparaffinized in xylene, rehydrated in 0.05 *M* Tris buffer, pH 7.6, for 10 min and boiled in 0.01 *M* citrate buffer, pH 6.0, for 5 min. The sections were then removed and allowed to cool at room temperature for 20 min and rinsed twice with Tris buffer for a total of 30 min. Endogenous peroxidase activity was blocked by a 15-min incubation in 3% hydrogen peroxide. In order to increase antigenic exposure, tissue sections were incubated in 0.1% Triton X-100 for 45 min at room temperature. Following this, the samples were incubated with diluted primary antibodies, anti-c-fos, c-jun, iNOS, NF-κB, MEK1, ERK1/ERK2, PKC-α, PY-20, MEKK-1, JNK and p38 (Transduction Laboratories), and c-myc (Santa Cruz Biotechnology), for 45 min at room temperature. After rinsing twice with Tris buffer for a total of 20 min, the bound primary antibodies were detected by sequential incubation with biotinylated secondary antibody (biotinylated anti-rabbit or anti-mouse immunoglobulins; LSAB kit, DAKO) for 30 min, streptavidin peroxidase (LSAB kit, DAKO) for 15 min and diaminobenzidine for 5–10 min at room temperature with two rinses with Tris buffer in between. The sections were then washed with distilled water and counterstained with Mayer's hematoxylin. After dehydration and mounting, the expression of proteins in the lung tissue was assessed by microscopic examination of the immunoperoxidase staining. The native control for each experiment was a DAKO

antibody diluent with background-reducing compounds, which is intended for use as a diluent in the preparation of primary antibodies and negative control reagents. Positively stained areas (yellow-brown in color) were observed and photographed by microscopy.

Results

Levels of exposure to cigarette smoke

An important free radical in cigarette smoke is nitric oxide (Pryor and Prier 1983) which is present at up to 500 ppm and probably represents one of the major exogenous sources of nitric oxide. To control the exposure conditions, the concentrations of NO_x in the exposure chamber were displayed and monitored by a continuous analyzer. The concentrations of NO_x in the chamber rose after the initiation of cigarette smoke emission. The concentrations of NO_x increased to peak levels of 7.1, 14.5 and 25.0 ppm with 5, 10, and 15 cigarettes, respectively, 10 min after initiation of smoke emission then declined to basal levels after 1 h (data not shown).

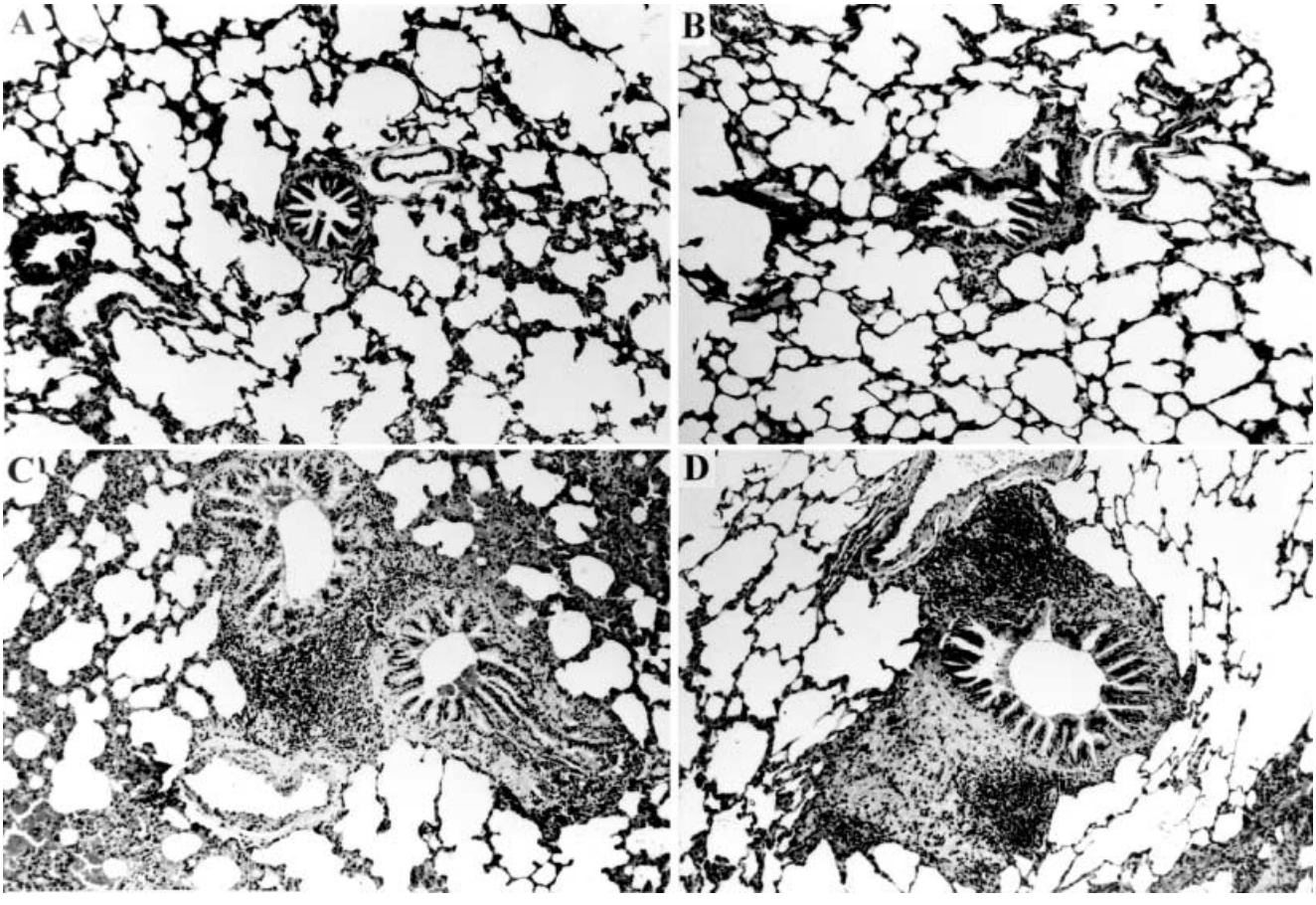
Lung injury caused by cigarette smoke

Histopathological analysis showed that the lungs of cigarette smoke-exposed rats showed marked changes in the terminal bronchioles and the alveolar ducts. The terminal bronchioles were infiltrated by lymphocytes in the peribronchial region and emphysema was noted in the alveolar ducts (Fig. 2). Dilatation of the terminal bronchioles and peribronchiolar fibrosis also occurred at higher doses of cigarette smoke (Fig. 2C,D). No marked changes were seen in the same areas of the lungs from unexposed rats. The lymphocyte infiltration, emphysema, dilatation and peribronchiolar fibrosis caused by cigarette smoke were directly related to the number of cigarettes.

Lipid peroxidation in rat lung tissues

As expected, lung sections obtained from control animals were absolutely unstained after incubation with Schiff's reagent (Fig. 3A), while the sections obtained from smoke-exposed lungs exhibited a purple-red stain of various intensities surrounding the terminal bronchiole

Fig. 2A–D Histopathological evaluation of the effect of cigarette smoke on the rat lung. **A** Lung section from a control rat shows no noticeable change. **B**,**C**,**D** Lung sections obtained from rats exposed to the smoke from 5 (**B**), 10 (**C**), and 15 (**D**) cigarettes. The terminal bronchioles and the alveolar ducts show light (**B**), mild (**C**) and severe (**D**) lymphocytic infiltration. Peribronchiolar fibrosis and dilatation of terminal bronchiole are also apparent (**C**,**D**) (H and E, ×100)



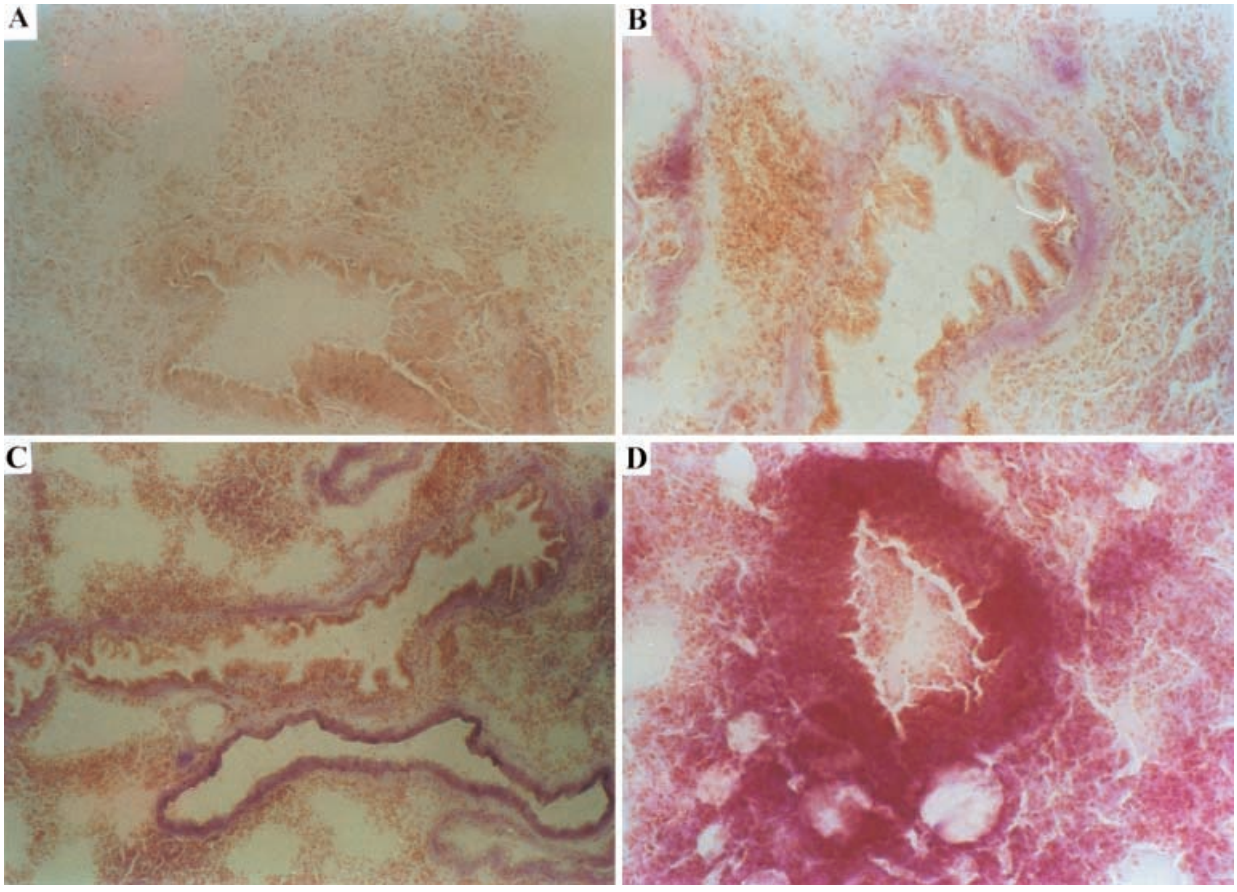


Fig. 3A–D Lipid peroxidation caused by gas-phase cigarette smoke in rat lung. The purple color indicates positive staining which represents the distribution of peroxidation. **A**–**D** Frozen sections of lung obtained from control rats (**A**), and rats exposed to the smoke from 5 (**B**), 10 (**C**), and 15 (**D**) cigarettes. The terminal bronchioles show weak (**B**), moderate (**C**) and prominent (**D**) peroxidation (×200)

areas (Fig. 3B–D). Smoke exposure led to leukocyte infiltration and emphysema mainly in terminal bronchioles and alveolar duct areas of the lungs (Fig. 2). This also occurred in the same regions of the lungs from most of the rats used in relation to lipid peroxidation positive staining. This may explain why lung injury caused by cigarette smoking is associated with oxidant stress.

Expression of MAPKs, phosphotyrosine proteins and PKC

Immunohistochemical evaluation of MAPKs and phosphotyrosine proteins in rat lungs exposed to the smoke from 15 cigarettes confirmed that smoke exposure increased the expression of phosphotyrosine proteins (Fig. 4B), MEK1 (Fig. 4D) and ERK2 (Fig. 4F) but not MEKK-1, JNK, p38 kinase and PKC (data not shown) in terminal bronchioles and alveolar duct area. A positive correlation between the protein expression levels and the number cigarettes was found (data not shown).

Expression of nuclear protooncogenes

In order to evaluate the expression of c-jun, c-fos and c-myc, tissue sections from the lungs of rats exposed to cigarette smoke were examined immunohistochemically using the biotin-avidin peroxidase detection system. As shown in Fig. 5B, the expression of c-fos, represented as yellow-brown staining in the terminal bronchioles, was increased markedly following exposure to 15 cigarettes. The expression of c-fos increased in relation to the number of cigarettes (data not shown). In contrast, the expression of both c-jun and c-myc was not changed by smoke exposure (data not shown). These results indicate that injury to the lungs of the rats may have been caused by the induction of c-fos through MEK/ERK via activation of tyrosine kinases.

Expression of NF-κB and iNOS

In order to elucidate the correlation between nitric oxide and cigarette smoke-induced lung injury, we also studied the effects of gas-phase cigarette smoke on the expression of iNOS and NF-κB. Exposure of rats to the smoke from 15 cigarettes caused a marked increase in iNOS (Fig. 5D) and NF-κB (Fig. 5F) as detected immunohistochemically. Yellow-brown positive staining indicating expression of iNOS and NF-κB was also located in the terminal bronchioles and alveolar ducts. The

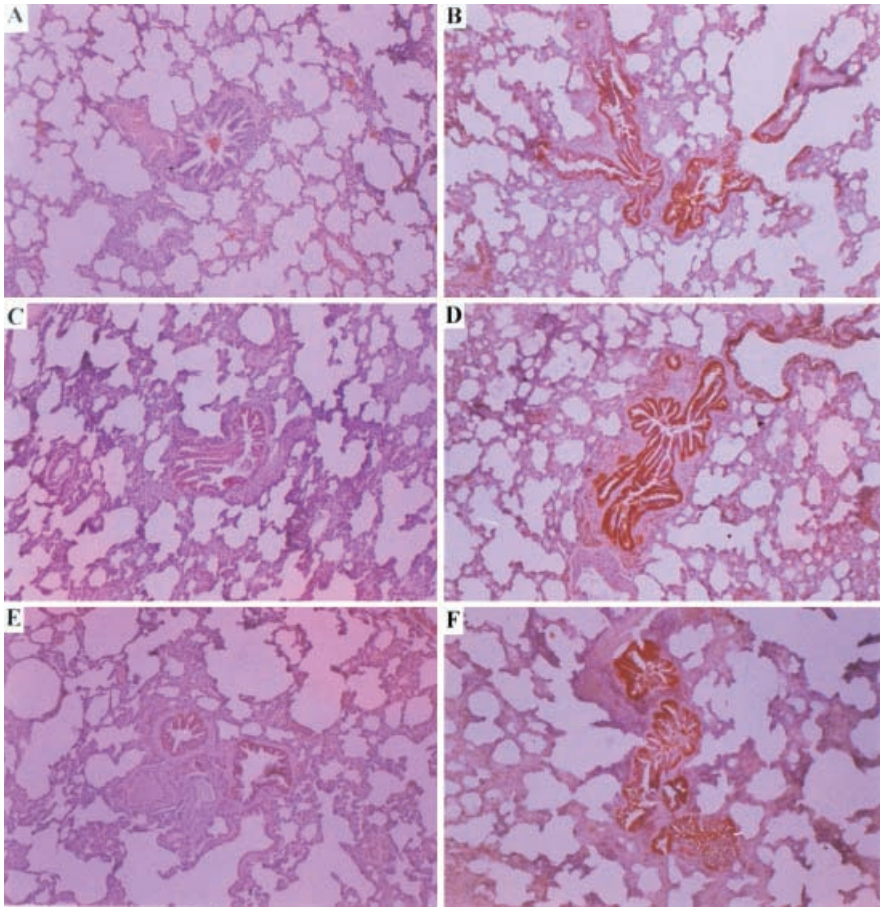


Fig. 4A–F Immunohistochemical evaluation of the effect of gas-phase cigarette smoke on the expression of phosphotyrosine proteins (**A**,**B**), MEK1 (**C**,**D**) and ERK2 (**E**,**F**) in rat lung indicated as yellow-brown staining. Positive staining is apparent in the terminal bronchioles and alveolar ducts of lung from unexposed rats (**A**,**C**,**E**), and from rats exposed to the smoke from 15 cigarettes (**B**,**D**,**F**) (×100)

expression of iNOS and NF-κB was significantly increased in a dose-dependent manner (data not shown), and might have contributed to the lung inflammation and emphysema in cigarette smoke-exposed rats.

Serum nitrite concentration

The concentrations of nitrite in the serum of rats exposed to cigarette smoke for 1 h per day for 5 days per week over 2 months were 3.0, 4.8 and 6.2 μM following exposure to the smoke from 5, 10 and 15 cigarettes, respectively (Fig. 6). Thus rats exposed to cigarette smoke produced nitric oxide and the concentration was dependent on the number of cigarettes.

Discussion

To study the role of signaling molecules in lung pathogenesis induced by cigarette smoke, we exposed rats to various concentrations of cigarette smoke. The rats were exposed to gas-phase cigarette smoke in a chamber for 1 h per day for 2 months. No similar animal model for studying gas-phase cigarette smoke exposure has been reported previously. The experimental conditions were designed to mimic passive smoke exposure of humans in a confined room. Exposure to cigarette smoke resulted in increases in the levels of NO_x and produced lipid peroxidation in rat lungs. In addition, 2 months after cessation of exposure, phosphotyrosine proteins, iNOS, NF-κB, c-fos, MEK1 and ERK2 were significantly increased in a dose-dependent manner. Injury caused by the cigarette smoke included lung inflammation, emphysema dilatation and peribronchiole fibrosis in the terminal bronchioles and alveolar duct area. These lung lesions showed yellow-brown positive staining indicating the expression of iNOS, NF-κB, c-fos, phosphotyrosine, MEK1 and ERK2. The same areas also showed purple-red positive staining indicating lipid peroxidation. These findings suggest that cigarette smoke exposure can induce the expression of iNOS and c-fos via tyrosine kinase activation and MEK1/ERK2 signaling as a result of oxidant stress.

Experimental evidence of lipid peroxidation following cigarette smoke exposure has been reported previously (Chow 1982; Church and Pryor 1985; Lentz and DiLuzio 1974). We also monitored lipid peroxidation immunohistochemically and found an increase in peroxidation (purple-red staining) in the terminal bronchioles and alveolar ducts of lungs exposed to smoke. Gas-phase cigarette smoke, which contains NO_x and ROS (Pryor and Prier 1983), is highly oxidizing and capable of initiating peroxidation. Indeed, we found

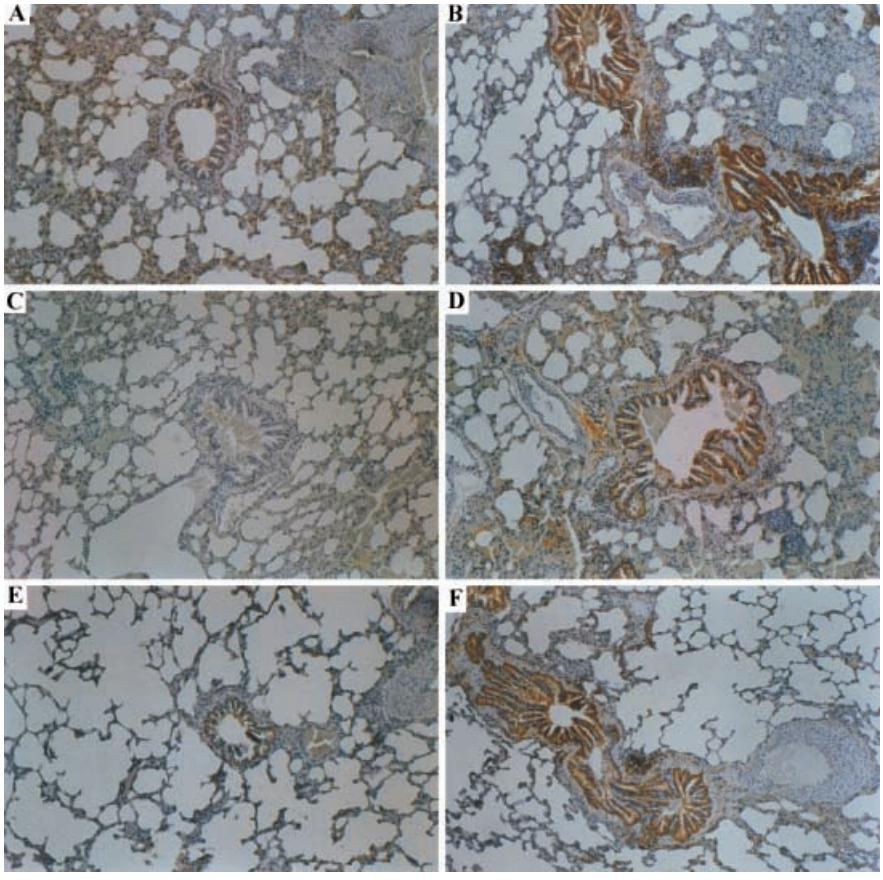


Fig. 5A–F Immunohistochemical evaluation of the effect of gas-phase cigarette smoke on the expression of c-fos (**A**,**B**), iNOS (**C**,**D**) and NF-κB (**E**,**F**) indicated as yellow-brown staining. Positive staining is apparent in the terminal bronchioles and alveolar ducts of lung from unexposed rats (**A**,**C**,**E**), and from rats exposed to the smoke from 15 cigarettes (**B**,**D**,**F**) (×100)

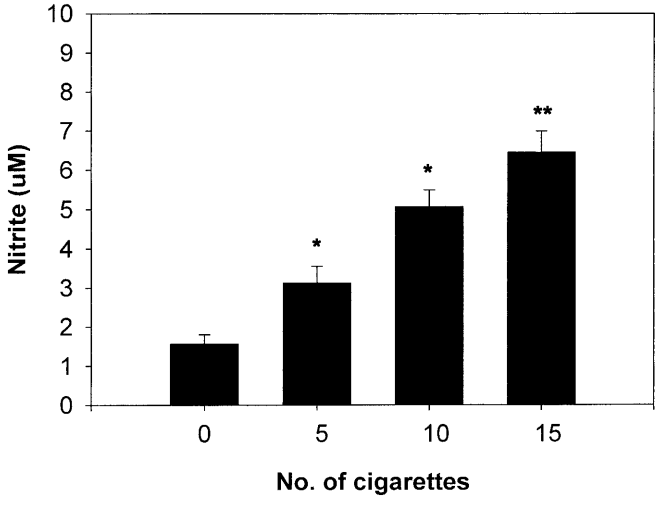


Fig. 6 Serum concentration of nitric oxide in rats exposed to gas-phase cigarette smoke in relation to the number of cigarettes. The rats were exposed to the smoke in a chamber for 1 h per day for 5 days per week over 2 months. Values are means ($\pm$SD, $n=6$). *$P<0.01$, **$P<0.001$ vs control

NO_x in the exposure chamber generated from the gas-phase smoke and in the serum of smoke-exposed rats. NO_x can react with other gaseous components of cigarette smoke to form peroxide radicals. NO_x can also react with hydrogen peroxide to produce superoxide and hydroxyl radicals. Finally, NO_x can cause oxidative damage to biological target molecules (Church and Pryor 1985) and this in turn can cause lipid peroxidation in the lung. We believe that lipid peroxidation by NO_x and ROS plays a significant role in the toxicity of cigarette smoke.

Cigarette smoking is a major cause of human lung injury, although the pathogenic mechanism remains unestablished. Increasing evidence suggests that free radicals are involved in many of the chronic diseases associated with smoking (Pryor 1982; Slaga et al. 1981; Ts'o et al. 1977). Molecular toxicological investigations have demonstrated that cigarette smoke can cause DNA single strand breaks (Fielding et al. 1989; Leanderson and Tagesson 1992; Nakayama et al. 1985; Spencer et al. 1995), elevated expression of heme oxygenase (Muller and Gebel 1994) and altered regulation of the c-fos gene at the transcriptional level (Muller 1995). However, there have been no previous studies of cigarette smoke-induced transduction signaling proteins associated with lung injury or carcinogenesis.

Our data strongly suggest that exposure of rats to gas-phase cigarette smoke leads to protein tyrosine phosphorylation, and MEK1/ERK2 and c-fos expression. The induction of c-fos expression but not of c-jun and c-myc expression by cigarette smoke in rats is similar to the findings in 3T3 cells (Muller 1995). The expression of c-fos has been identified as a signal for cell

proliferation (Pardee 1989) and can explain the lung injury caused by oxidative stress induced by cigarette smoke (Muller and Gebel 1994). In our study, no effects of smoke on PKC induction were observed in rat lungs, but a significant increase in protein tyrosine phosphorylation was observed. Hence, induction of c-fos is regulated by tyrosine kinase but not by PKC in smoke-exposed rat lung. On the other hand, the expression levels of MEK1/ERK2 (but not of MEKK-1, JNK or p38 kinase) increased in cigarette smoke-exposed lungs. Thus, it can be concluded that cigarette smoke-induced lung injury in the terminal bronchioles and alveolar ducts may be via ROS and NO_x generation which leads to the induction of c-fos through tyrosine kinase and MEK1/ERK2 proteins.

NF-κB proteins are ideal mediators of the genetic programs underlying inflammation since they are activated by inflammatory signals and activate the genes involved in inflammation (Thanos and Maniatis 1995). Most of the genes known to be activated by NF-κB are indeed involved in immune and inflammatory responses or, more generally, in responses to stress. Our results showed that the protein expression of NF-κB and target iNOS were significantly increased after cigarette smoke exposure for 1 h per day for 5 days per week over 2 months. The distribution of NF-κB and iNOS expression in rat lung was consistent with the area of the lesions caused by cigarette smoke. It has been reported that oxidative stress activates NF-κB in several cell phenotypes (Baldwin 1996). Activation of NF-κB in cigarette smoke-exposed rat lung may be because of ROS and NOx are generated following oxidant stress. Moreover, oxidant stress has been shown to be capable of stimulating PKC and tyrosine kinases, and activating NF-κB through MAPK signaling (Kleinert et al. 1996). In our study, no effect on PKC expression was noted in cigarette smoke-exposed rat lungs, but tyrosine phosphorylation increased. Thus, it can be stated that activation of NF-κB is regulated by tyrosine kinase but not by PKC in cigarette smoke-exposed rat lungs. These effects also increase the expression of iNOS that could be related to the inflammatory response to cigarette smoke in the terminal bronchioles and alveolar ducts of rats. These results suggest a signaling pathway for the induction of c-fos by MEK1/ERK2 and iNOS by NF-κB in the pathogenetic events in the lung following cigarette smoke exposure.

In conclusion, our results indicate that the effects of cigarette smoke in lung injury, including inflammation, dilatation and emphysema, may be via ROS and NO_x generation, which, in turn, leads to the expression of iNOS and c-fos through the MEK1/ERK2 signaling pathway. All these transduction signals may be regulated by tyrosine kinases which catalyse tyrosine phosphorylation of proteins and activate MEK1/ERK2 and/or NF-κB signaling.

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