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Modulation by Phenethyl Isothiocyanate and Budesonide of Molecular and Histopathologic Alterations Induced by Environmental Cigarette Smoke in Mice

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Abstract Our discovery that the perinatal period involves nucleotide modifications and gene overexpression in mouse lung prompted us to evaluate whether mice may become more susceptible to cigarette smoke when exposure starts immediately after birth. We previously showed that mainstream cigarette smoke is a quite potent carcinogen in neonatal mice. Further on, we showed that exposure of mice to environmental cigarette smoke (ECS), starting at birth, results in alterations of a variety of intermediate biomarkers. However, after 4 months of exposure to ECS followed by 7 months of recovery in filtered air, the lung tumor yield was rather low. In the present study, we evaluated the protective effects of the glucocorticoid budesonide and of the dietary agent phenethyl isothiocyanate in mice exposed to ECS for 9 months followed by 2 months of recovery. After weanling, the mice exposed to ECS since birth underwent a variety of alterations of molecular and cytogenetical end points, and 11 months after birth, they exhibited significant histopathologic changes, such as pulmonary anthracosis, emphysema, hemorrhagic areas, alveolar bronchiolarization, bronchial hyperplasia, and tumors, both benign and malignant. The carcinogenic response was less evident in dams exposed to ECS under identical conditions. Both phenethyl isothiocyanate and budesonide, administered daily with the diet after weanling, attenuated several alterations of ECS-related biomarkers and moderately protected the lungs from histopathologic alterations, including tumors. Thus, although not as efficiently as the bioassay in mainstream cigarette smoke–exposed mice, the model in neonatal mice is suitable to evaluate both ECS carcinogenicity and its modulation by chemopreventive agents.

Tobacco smoke is the major risk factor for human cancer, being responsible for the 85% to 90% of lung cancer, the most important death cause among neoplastic diseases in the world population, and for cancers in several other anatomic sites (1). Environmental cigarette smoke (ECS) or secondhand smoke, which is inhaled by involuntary smokers (or passive smokers), is mainly composed of sidestream smoke, released from the smoldering distal part of the cigarette, and, in minor proportion, of that portion of mainstream cigarette smoke (MCS) that is exhaled by active smokers. ECS, which is diluted in ambient air and undergoes aging processes, is classified as a lung carcinogen to humans (1, 2).

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The most obvious way to prevent lung cancer and other smoke-related diseases is either to refrain from smoking or to quit smoking or not to live in ECS-contaminated environments. The decline of smoking habits has already had a favorable public health effect in the male population of several countries (3). Growing importance is ascribed, as a complementary approach, to the possibility of preventing cancer by means of dietary and pharmacologic agents capable of reinforcing the host defense machinery. This strategy, which is referred to as cancer chemoprevention, has several targets in smoke-related carcinogenesis, such as (*a*) addicted active smokers who are unable to quit smoking; (*b*) ex-smokers, representing a broad proportion of the population where half of all new lung cancer cases are nowadays diagnosed (4); (*c*) passive smokers living in ECS-contaminated environments; and (*d*) transplacentally exposed individuals, in whom a variety of smoke-induced molecular alterations are detectable (5).

Twenty chemical components of cigarette smoke have been shown to induce lung tumors (6). Some of them, such as benzo(*a*)pyrene, as a prototype of polycyclic aromatic hydrocarbons, or 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), as a prototype of tobacco-specific nitrosamines, have extensively been investigated in laboratory animals (1, 6). Unfortunately,

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for a variety of reasons (1, 6), it is difficult to reproduce in rodents the carcinogenicity of cigarette smoke as a complex mixture, which hampers the evaluation of chemopreventive agents in preclinical models.

The most convincing medium-term bioassay for ECS tumorigenicity has been developed by Witschi et al. (7) and validated in four laboratories, including ours, during the last dozen years (8–14). This model involves the whole-body exposure of A/J mice or other mouse strains to high doses of ECS for 5 months followed by recovery in filtered air for an additional 4 months. However, the increase of benign lung tumor multiplicity is low (i.e., from an average of 1.1 lung adenomas per mouse in controls to an average of 2.8 in ECS-exposed mice; ref. 8). The narrow positivity window renders this model not particularly sensitive in evaluating the protective effect of chemopreventive agents. Of the pharmacologic and dietary agents evaluated by Witschi (15), only the glucocorticoid dexamethasone, in combination with myoinositol, significantly reduced the ECS-induced lung tumor multiplicity. No effect was produced by a variety of other promising chemopreventive agents, including green tea, acetylsalicyclic acid, *N*-acetylcysteine, 1,4-phenylenebis(methylene)selenocyanate, d-limonene, phenethyl isothiocyanate (PEITC), and a mixture of PEITC and benzyl isothiocyanate (15), Bowman-Birk protease inhibitor (16), and aerosolized epigallocatechin gallate (17), a major green tea component. However, *N*-acetylcysteine and β-carotene were found to inhibit the tumorigenicity of the ECS gas phase (18), which previously had been shown to be responsible for the lung tumorigenicity of the whole mixture (13, 14).

Recently, we showed that the simple transition from the fetal life to the neonatal situation, in an interval of very few hours, causes a tremendous oxidative stress in mouse lung, which results in a 2-fold increase of oxidatively generated DNA damage, a 5-fold increase of bulky DNA adducts, and overexpression of several genes (19). These genes, mainly having adaptive functions, are still silent immediately after delivery. The observed susceptibility of mouse lung during the perinatal period prompted us to evaluate whether mice may become more susceptible to the carcinogenicity of cigarette smoke when exposure starts at birth. The earliest study of this series (20) provided evidence that exposure of Swiss albino mice to high doses of MCS, starting within 12 hours after birth and continuing for 4 months, results in a potent carcinogenic response. In fact, carcinogenicity was characterized by (*a*) a short latency time, the earliest lung tumors being detectable after 75 days only; (*b*) a high incidence of preneoplastic and neoplastic lesions in the lung; (*c*) a high multiplicity of benign lung tumors; (*d*) occurrence of malignant lung tumors within 7 months of life; and (*e*) occurrence of malignant tumors in extrapulmonary organs (liver and urinary tract; ref. 20). These conclusions are being confirmed in further studies that are now in progress. The results of the histopathologic analyses thus far done using various mouse strains, including Swiss albino, H albino, and even the poorly sensitive DBA/2 and C57BL mice, exposed to MCS since birth for 2 to 4 months, show that as many as 143 of the 268 MCS-exposed mice thus far examined (53.3%) have lung tumors versus 4 of 106 sham-exposed mice (3.8%; ref. 20).[5] These data lend support to the conclusion that, under the described experimental conditions, MCS is a powerful carcinogen, thereby disproving the view, matured in 60 years of research, that cigarette smoke is a weak carcinogen in rodent models.

Thereafter, preliminarily to the chemoprevention study reported in the present study, we did a carcinogenicity study in CD-1 albino mice exposed to ECS since birth for a period of 4 months. After weanling, subgroups of sham-exposed mice and of ECS-exposed mice were sacrificed and evaluated for a variety of intermediate biomarkers in the respiratory tract and hematopoietic system (21) and in the cardiocirculatory system (22). Many biomarkers were significantly increased in ECS-exposed mice, including levels of bulky DNA adducts and oxidatively generated DNA damage in lung, heart, and aorta, amounts of thiobarbituric acid reactive substances in lung, loss of Fhit protein and apoptosis in pulmonary alveolar macrophages and bronchial epithelial cells, proliferation of bronchial epithelial cells, and cytogenetical damage in bone marrow and peripheral blood erythrocytes. Interestingly, the response to ECS, in terms of several biomarkers, was more pronounced in post-weanling females than in their dams, kept in the same cages (21, 22). Eleven months after birth and 7 months after stopping exposure to ECS, all surviving mice were sacrificed. Histopathologic analyses showed that the incidences of bronchial and alveolar hyperplasias and of lung adenomas were increased in ECS-exposed mice compared with sham-exposed mice. However, the tumorigenic response was rather weak (23).

For this reason, in the present study, which evaluated modulation of intermediate biomarkers, tumors, and other medium-term histopathologic alterations, we modified the experimental design by extending the time of exposure to ECS from 4 to 9 months and by treating not only the neonatal mice but also their dams until the end of the experiment. We tested budesonide and PEITC as chemopreventive agents after having evaluated their tolerability in a subchronic toxicity study. Budesonide is a glucocorticoid, a family of compounds that are effective cancer chemopreventive agents in animal models but can have side effects in humans (24). Several studies showed that budesonide, given either in diet or by aerosol, inhibits the formation of lung tumors in the lung of mice treated either with benzo(*a*)pyrene (24–30) or vinyl carbamate (31). In addition, budesonide was found to modulate gene expression during mouse lung tumorigenesis (28). PEITC is a naturally occurring isothiocyanate contained in watercress (*Nasturtium officinale*), which has been shown to inhibit lung tumors induced in mice and rats by NNK (32–36), whose metabolic activation is blocked by PEITC (34).

We report herein that ECS induces early alterations of a variety of biomarkers as well as a medium-term histopathologic alterations, also including lung tumors, which are attenuated in ECS-exposed mice treated, after weanling, with either PEITC or budesonide.

Materials and Methods

Mice

Swiss CD-1 albino mice were used in the subchronic toxicity study and in the chemoprevention study. Either pregnant or post-weanling mice, purchased from Harlan Italy, were housed in Makrolon cages on sawdust bedding and maintained on standard rodent chow (Teklad 2018, Harlan Italy) and tap water *ad libitum*. The cages were kept in

[5] R. Balansky et al., studies in progress.

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a cabinet where filtered air was circulated. The animal room had a temperature of 23 ± 2°C, a relative humidity of 55%, and a 12-h day/night cycle. Housing and all treatments of mice were in accordance with NIH guidelines and with our institutional guidelines.

Preliminary evaluation of subchronic toxicity with PEITC and budesonide

Both PEITC and budesonide were purchased from Sigma Chemical Co. To preliminarily assess the tolerability of these agents, 180 post-weanling mice were divided into nine groups, each composed of 10 males and 10 females. One group served as untreated controls. Four groups received PEITC with the diet, at the doses of 125, 250, 500, and 1,000 mg/kg diet, and four groups received budesonide at the doses of 0.3, 0.6, 1.2, and 2.4 mg/kg diet. The mice were inspected daily for general appearance and behavior and weighed at weekly intervals for 6 wk.

Design of the chemoprevention study

The 232 newborn mice born from 25 pregnant mice were divided into four experimental groups as follows: group A (71 mice, 34 males and 37 females), sham-exposed mice, kept in filtered air for up to 11 mo; group B (79 mice, 44 males and 35 females), mice exposed to ECS since birth for up to 9 mo followed by 2 mo in filtered air; group C (61 mice, 31 males and 30 females), mice exposed to ECS since birth and treated, after weanling, with PEITC at the maximum tolerated dose (see Results); and group D (57 mice, 36 males and 21 females), mice exposed to ECS since birth and treated, after weanling, with budesonide at the maximum tolerated dose.

When the infant mice became post-weanling, ~30 d after birth, they were housed separately, according to gender. The mice were weighed at periodic intervals. The 21 dams, staying in the same cages where their litters were accommodated until weanling, were thereafter kept in separate cages and continued to be either sham exposed (6 dams) or ECS exposed (15 dams) under identical conditions as their litters.

Fig. 1 shows a schematic diagram of the experimental design.

Evaluation of intermediate biomarkers

Forty-five days after birth and start of exposure to ECS [i.e., 15 d after weanling and start of treatment with either PEITC (group C) or budesonide (group D)], eight male mice from groups B to D were deeply anesthetized with diethyl ether and killed by cervical dislocation. Peripheral blood was collected from the lateral tail vein and smeared on duplicate slides. The left femur was collected, and bone marrow was smeared on duplicate slides. The right lung of each mouse was stored at −80°C for molecular analyses, whereas the left lung was fixed in formalin.

DNA was extracted from 50 to 100 mg lung tissue from the 32 mice, in the presence of an antioxidant (DTT), by using a commercially available kit using phenol-free reagents (GenElute DNA Miniprep kit, Sigma). Bulky lipophilic DNA adducts were extracted with butanol and detected by ^{32}P postlabeling, as described previously (37). DNA adducts, detected by ^{32}P imaging (InstantImager, Packard), were quantified by calculating the ratio between cpm detected in DNA adducts and cpm in normal nucleotides and expressed as adducts/10^8 nucleotides.

8-Oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) was evaluated by using a ^{32}P postlabeling procedure (38), following enrichment with trifluoroacetic acid and reaction with polynucleotide kinase and [γ-^{32}P]ATP (16 μCi/μL; specific activity, 325 Ci/mmol), and nuclease P1 digestion. ^{32}P-labeled 8-oxodGuo was purified by TLC in formic acid and then detected by ^{32}P imaging. 8-oxodGuo levels were quantified by calculating the ratio between cpm detected in the 8-oxodGuo–related spot and cpm detected in normal nucleotides and expressed as 8-oxodGuo/10^5 nucleotides. A DNA-free sample was used as a negative control, and four reference standards were used for 8-oxodGuo identification, as previously detailed (21).

For evaluating proliferation of bronchial epithelial cells, the proliferating cell nuclear antigen (PCNA) was analyzed by immunohistochemistry in 5-μm sections of lung, placed onto slides treated with poly-L-lysine (Poly-Prep Slides, Sigma Diagnostic). PCNA was detected by using the NCL-PCNA kit (Novocastra Laboratories), following the manufacturer's instruction. This kit uses an anti-PCNA monoclonal antibody (clone PC10) and avidin-biotinylated horseradish peroxidase complex technology (ABC technique). The slides were scored at a magnification of ×400 and 1,000 cells per mouse were examined.

Apoptosis of bronchial epithelial cells was evaluated by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) method using the TACS-XL Blue Label In Situ Apoptosis Detection kit (Trevigen), following the manufacturer's instruction. The slides were scored at a magnification of ×400 and 1,000 cells per mouse were examined.

The cytogenetical damage was evaluated in bone marrow polychromatic erythrocytes (PCE) and in peripheral blood normochromatic erythrocytes (NCE), as previously described (39). Briefly, bone marrow smears were air dried and stained with May-Grünwald-Giemsa, and 5,000 PCE per mouse were scored for the presence of micronucleated (MN) PCE. The PCE/NCE ratio, calculated by scoring 200 cells, was taken as an indicator of toxicity to bone marrow erythrocytes. Peripheral blood smears were dried and stained in the same way, and 50,000 NCE per mouse were scored for the presence of MN NCE.

Evaluation of histopathologic alterations

At periodic intervals (4, 5, 6, 7, and 8 mo), a total of 8 sham-exposed mice and of 16 ECS-exposed mice were sacrificed for evaluating the possible development of histopathologic alterations. Based on the indications emerging from these interim sacrifices (see Results), the experiment was stopped after 9 mo of exposure to ECS followed by 2 mo of recovery in filtered air. At that time, all surviving mice from the four experimental groups (see Results) and their dams were killed.

A complete necropsy was done. The lungs, liver, kidney, urinary bladder, and all other organs with suspected macroscopically visible lesions were collected. Each lung was cut into 15 to 20 sections, every 200 μm, whereas two standardized sections each were obtained from liver, kidney, and urinary bladder. In total, more than 8,000 slides were subjected to standard histopathologic analysis and examined as blind coded samples for the presence of neoplastic and nonneoplastic lesions.

Statistical analysis

The differences between groups about body weight, intermediate biomarkers, and multiplicity of lung tumors were analyzed by Student's *t* test for unpaired data. Comparisons between groups about survival and incidence of histopathologic lesions were made by χ^2 analysis.

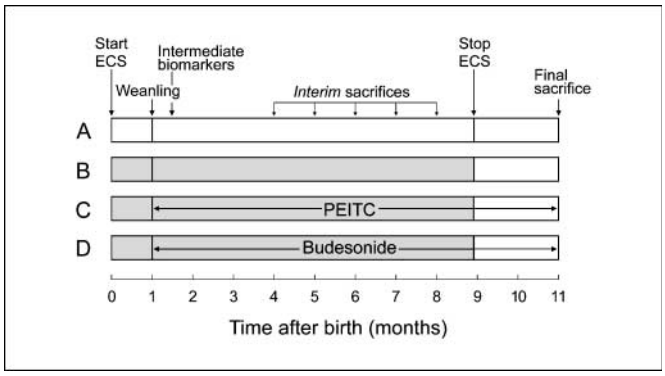

Fig. 1. Flow diagram showing an outline of the experimental design of the present study.

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Results

Preliminary toxicity study

Administration of the chemopreventive agents with the diet for 6 weeks at four doses each did not result into any apparent sufferance or alteration of behavior in any mouse. The body weights (mean ± SE within each group of 20 mice) in untreated mice, after 0, 1, 2, 3, 4, 5, and 6 weeks, were 21.6 ± 0.57, 24.9 ± 0.65, 26.1 ± 0.63, 27.2 ± 0.70, 27.1 ± 0.84, 31.7 ± 1.20, and 31.9 ± 0.86, respectively. Neither budesonide nor PEITC affected, at any dose, the body weights of mice throughout this period (data not shown). Therefore, both agents were subsequently used at the maximum doses tested (i.e., 2.4 mg/kg diet for PEITC and 1,000 mg/kg diet for budesonide).

Impairment of survival and body weight in ECS-exposed mice and effect of PEITC and budesonide

Besides the 32 mice sacrificed after 45 days for the evaluation of biomarkers and the 24 mice sacrificed at periodic interval for the evaluation of histopathologic alterations, a total of 28 premature deaths occurred before the end of the experiment. Mortality was very low in females, either sham exposed (two deaths) or ECS exposed (1) or exposed to ECS and treated with either PEITC (1) or budesonide (1). Among males, premature deaths affected sham-exposed mice (1 of 24, 4.2%), ECS-exposed mice (9 of 29, 31.0%), and ECS-exposed mice treated with either PEITC (5 of 23, 21.7%) or budesonide (8 of 28, 28.6%). Kaplan-Meier survival curves in the four experimental groups of male mice are shown in Fig. 2. The difference in survival between sham and ECS, irrespective of treatment with either budesonide or PEITC, became statistically significant on weeks 47 and 48. It should be noted that several deaths in males were clustered during the last 4 weeks of the experiment when exposure to ECS had already been discontinued.

Figure 3 shows the body weights measured starting 3 days after the 4th week, when the mice had terminated the weanling period and could be separated by gender, until the 48th week, when the experiment was terminated. Body weights were measured once per week until week 8 and every 4 weeks from week 8 to week 84. At all times, the body weight was

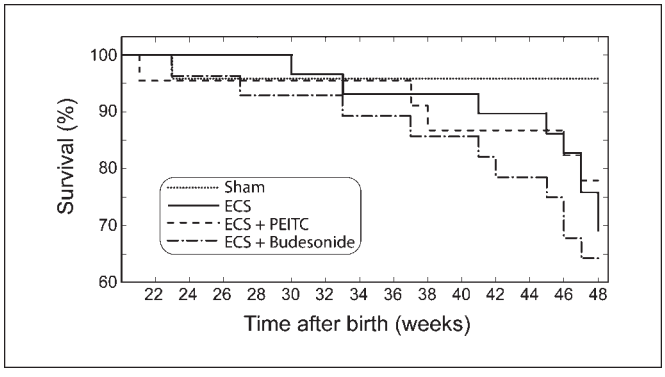

Fig. 2. Kaplan-Meier curves showing survival of male mice as related to exposure to ECS since birth until the 38th week and treatment with either PEITC or budesonide after weanling until the end of the experiment. The 32 mice sacrificed after 45 d for the evaluation of biomarkers and the 24 mice sacrificed at periodic intervals for the evaluation of histopathologic alterations were taken out from the initial numbers of mice within each experimental group.

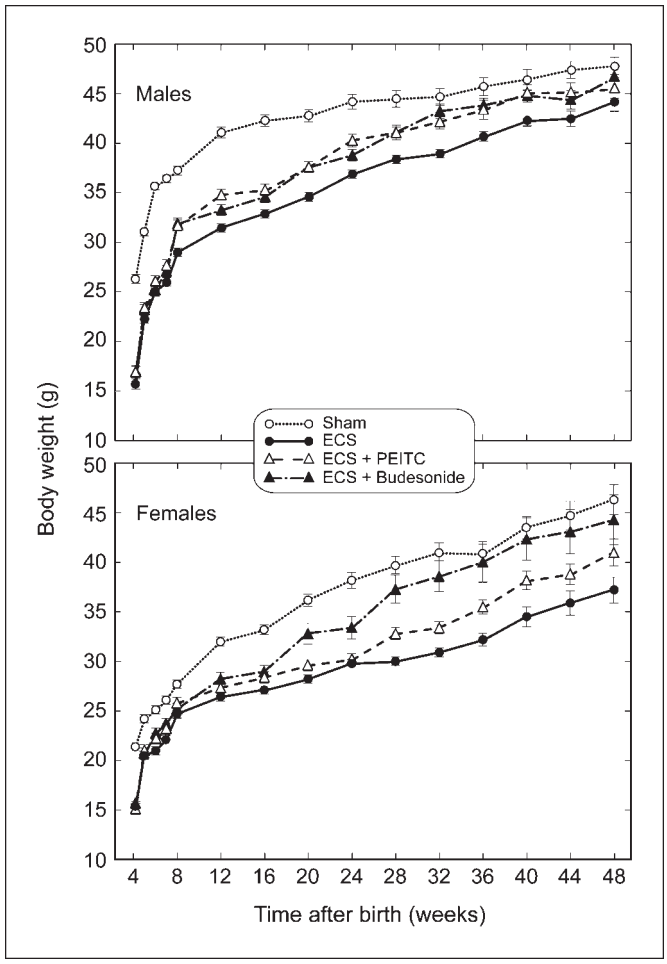

Fig. 3. Body weights of variously treated mice starting after weanling (4.2 wk), when the mice could be divided by gender, until the end of the experiment. The data are means of the mice surviving at the indicated time within each experimental group; *bars*, SE.

significantly lower in ECS-exposed mice than in sham-exposed mice of both genders. Starting from the 5th to 6th week, both PEITC and budesonide significantly attenuated the ECS-induced body weight loss to such an extent that, from the 12th week onwards, the body weights of the ECS-exposed mice treated with the two chemopreventive agents were at most times no longer significantly different from those of sham-exposed mice.

Alterations of intermediate biomarkers in ECS-exposed mice

Exposure of mice to ECS since birth, for 45 days, resulted in significant alterations of a variety of intermediate biomarkers (Table 1). A diagonal radioactive zone (data not shown) appeared in ^{32}P postlabeling autoradiographs of lung DNA from ECS-exposed mice, with a 3.7-fold increase of the signal compared with sham-exposed mice. Similarly, there was a 2.2-fold increase of 8-oxodGuo levels, showing induction by ECS of oxidatively generated DNA damage in the lung.

ECS stimulated the proliferation of bronchial epithelial cells, as shown by an 8-fold increase of PCNA. In parallel, there was a 5.5-fold increase of the apoptotic index in the same cells.

In addition, ECS produced a systemic cytogenetical damage. In fact, there was a 2.1-fold increase of MN frequency

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Table 1. Alterations of intermediate biomarkers in Swiss albino mice 45 d after exposure to ECS since birth and 15 d after treatment with either PEITC or budesonide

Biomarker	Treatment of mice			
	Sham	ECS	ECS + PEITC	ECS + budesonide
Whole lung				
Bulky DNA adducts/10^8 N	2.42 ± 0.26	9.05 ± 0.44*	6.83 ± 0.36*,†	8.07 ± 0.25*
8-oxodGuo/10^5 N	1.54 ± 0.11	3.35 ± 0.17*	2.43 ± 0.22*,†	1.53 ± 0.07‡
Bronchial epithelial cells				
PCNA (%)	0.1 ± 0.04	0.8 ± 0.06*	0.7 ± 0.07*	0.6 ± 0.08*,§
Apoptosis (%)	0.2 ± 0.03	1.1 ± 0.10*	0.7 ± 0.08*,†	0.6 ± 0.07*,‡
Bone marrow				
MN PCE (‰)	3.0 ± 0.27	6.3 ± 0.65*	5.9 ± 0.55*	4.6 ± 0.38*,§
PCE/NCE ratio	1.2 ± 0.05	0.9 ± 0.04*	1.0 ± 0.03‖	1.0 ± 0.04
Peripheral blood				
MN NCE (‰)	2.6 ± 0.49	5.6 ± 0.69‖	4.3 ± 0.53¶	3.8 ± 0.30§

NOTE: The results are mean ± SE of the data obtained in eight mice per group.
Abbreviation: N, nucleotides.
*$P < 0.001$, significantly different from sham.
†$P < 0.01$, significantly different from ECS.
‡$P < 0.001$, significantly different from ECS.
§$P < 0.05$, significantly different from ECS.
‖$P < 0.01$, significantly different from sham.
¶$P < 0.05$, significantly different from sham.

in bone marrow PCE, accompanied by a significant decrease of the PCE/NCE ratio, which is an indicator of toxicity toward bone marrow erythrocytes. Cytogenetical damage was also detected in peripheral blood, with a 2.2-fold increase of MN frequency in the NCE from ECS-exposed mice.

Modulation of intermediate biomarkers by PEITC and budesonide

Both PEITC and budesonide, administered during the 15 days following the weaning period, modulated ECS-related biomarkers to a variable extent, depending on the investigated end point. As shown in Table 1, administration of budesonide strongly inhibited the oxidatively generated DNA damage but did not significantly decrease the levels of bulky DNA adducts. In contrast, PEITC produced a moderate but statistically significant decrease of both ECS-induced nucleotide alterations.

The ECS-induced hyperproliferation of bronchial epithelial cells was slightly but significantly attenuated by budesonide. Both PEITC and budesonide were able to inhibit the ECS-induced increase of the apoptotic index in the same cells. These data prompted us to do further experiments with the goal of evaluation modulation of apoptosis by means of PEITC and budesonide in ECS-free mice. To this purpose, we applied the TUNEL method to lung samples of mice treated for 6 weeks with these two agents in the framework of the subchronic toxicity experiment. The results of these analyses showed that, at the same dose used in ECS-exposed mice, PEITC is able to significantly induce apoptosis. In fact, the apoptotic index (mean ± SE of the data in eight mice per group) was 0.16 ± 0.02 in untreated controls, 0.18 ± 0.06 and 0.28 ± 0.07 ($P < 0.05$ versus controls) in mice treated with PEITC at 250 and 1,000 mg/kg diet, respectively, and 0.14 ± 0.02 and 0.18 ± 0.04 in mice treated with budesonide at 0.6 and 2.4 mg/kg diet, respectively.

Supplementation of the diet with either PEITC or budesonide, during the 15 days after weanling, tended to attenuate the cytogenetical alterations produced by ECS and to normalize the PCE/NCE ratio. However, only budesonide was able to inhibit the ECS-induced increase of both MN PCE in bone marrow and MN NCE in peripheral blood (Table 1).

Medium-term histopathologic alterations in mice exposed to ECS since birth

At periodic intervals (4, 5, 6, 7 and 8 months), a total of 8 sham-exposed mice and 16 ECS-exposed mice were sacrificed. No histopathologic alteration was detected in sham-exposed mice at any time, excepting a marginal effect (mucous hypersecretion in the lung) observed in one of the two mice sacrificed after 8 months. In contrast, since the 4th month onwards and with growing intensity, ECS-exposed mice exhibited signs of pulmonary inflammation, atelectasia, emphysema, and vascular alterations (hyaline degeneration of arteries, venous stasis, and hemorrhagic areas). Only three cases of alveolar hyperplasia and one case of adenoma (after 5 months) were detected. Irrespective of exposure to ECS, no appreciable histopathologic alteration was observed in liver, kidney, or urinary bladder (data not shown).

After 9 months of exposure to ECS followed by 2 months of recovery in filtered air, all surviving mice were sacrificed. These included 52 sham-exposed mice (24 males and 27 females) and 47 mice exposed to ECS since birth (20 males and 27 females). The results of all histopathologic analyses are summarized in Table 2 (incidence data for all lesions

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detected in lung, liver, and kidney) and Table 3 (multiplicity data for lung tumors).

Compared with normal lung (Fig. 4A), exposure to ECS resulted in several significant alterations, including anthracosis (Fig. 4B), emphysema (Fig. 4C), alveolar bronchiolarization (Fig. 4D), and bronchial hyperplasia (Fig. 4E). Both incidence and multiplicity of alveolar adenomas (Fig. 4F) and total benign tumors were significantly increased in ECS-exposed females. Total malignant tumors, including adenocarcinomas (Fig. 4G), bronchioloalveolar carcinomas (Fig. 4H), and alveolar carcinomas (Fig. 4I), were significantly increased in both genders. By combining benign and malignant lung tumors, there was a 2-fold increase of incidence and a 1.5-fold increase of multiplicity in males (not significant) and a 8.2-fold increase of incidence and a 9.3-fold increase of multiplicity in ECS-exposed females ($P < 0.01$ for both incidence and multiplicity compared with sham-exposed mice). By combining the two genders, there was a 3.6-fold increase of incidence and a 3.2-fold increase of multiplicity of total lung tumors ($P < 0.01$ and 0.05, respectively). Note that positivities for pulmonary anthracosis, emphysema, and tumors were unrelated to each other.

"Spontaneous" liver steatosis was significantly decreased in ECS-exposed females, whereas glomerular sclerohyalinosis was significantly increased in males (Table 2). No alteration was detected in the urinary bladder (data not shown).

Modulation by PEITC and budesonide of histopathologic alterations in mice exposed to ECS since birth

Some indicators of toxicity were detected at the histopathologic analysis of both liver and kidney from ECS-exposed mice treated with either PEITC or budesonide. In fact, as shown in Table 2, in both males and females, the two agents significantly increased vascular hyaline degeneration of the liver as well as kidney tubular interstitial nephritis and urinary cysts. On the other hand, the chemopreventive agents significantly attenuated ECS-related liver steatosis (budesonide) and kidney glomerular sclerohyalinosis (both PEITC and budesonide).

In the lung, both PEITC and budesonide attenuated ECS-induced emphysema and alveolar bronchiolarization. The ECS-induced alveolar adenomas and total benign tumors were not affected by PEITC and were even slightly increased by budesonide in males, whereas malignant tumors were significantly decreased by PEITC in males. In both males and females, budesonide decreased the yield of malignant lung tumors, which was no longer significantly different from that of sham-exposed mice.

Histopathologic alterations in mice exposed to ECS during adulthood

Twenty-one dams generating the neonatally exposed mice, 6 of which kept in filtered air for 11 months (sham exposed) and 15 exposed to ECS for 9 months followed by 2 months in filtered air, were kept under identical conditions as their litters. As shown in Table 2, the only significant alterations observed in ECS-exposed dams were massive lung anthracosis and emphysema, both alterations being even more pronounced in dams than in their daughters. On the other hand, alveolar bronchiolarization and bronchial hyperplasia were less pronounced in dams than in their daughters, but these differences were not statistically significant. No tumor at all was observed in the 6 sham-exposed dams, and the occurrence of 3 lung tumors (2 benign and 1 malignant) in the 15 ECS-exposed dams (20%) was appreciable but did not reach the statistical significance threshold.

Discussion

The results of the present study show that mice exposed whole body to ECS since birth undergo a variety of alterations of early biomarkers as well as medium-term histopathologic alterations, also including benign and malignant lung tumors. To a variable extent and with different patterns, these alterations were modulated by the chemopreventive agents PEITC and budesonide.

The observed alterations of intermediate biomarkers in the respiratory tract and in the hematopoietic system included bulky DNA adducts and oxidatively generated DNA damage in the lung, proliferation and apoptosis of bronchial epithelial cells, and cytogenetical damage in bone marrow and peripheral blood erythrocytes. These findings support the conclusions of our previous studies showing the ability of ECS to induce genotoxic damage and other alterations of intermediate biomarkers in various cells, tissues, and organs of rodents exposed *in vivo* (5, 10, 21, 22, 40–52).

In addition, exposure of mice to ECS since birth for 9 months followed by an additional 2 months in filtered air produced significant histopathologic alterations in the respiratory tract, such as pulmonary anthracosis, emphysema and hemorrhagic areas, alveolar bronchiolarization, and bronchial hyperplasia. Moreover, ECS significantly increased the yield of benign lung tumors (in females only), of malignant lung tumors, and of total (benign + malignant) lung tumors. In agreement with the results of the carcinogenicity study in mice exposed to MCS since birth (20), females were found to be more susceptible than males to the carcinogenicity of ECS. On the other hand, ECS-exposed males suffered from a much higher premature mortality. The question of whether women and men differ in their susceptibility to smoke carcinogens has been debated for many years and is still controversial (53).

The tumorigenic response observed in the present study in neonatal mice exposed to ECS for a longer period of time is more convincing than that observed in our previous study, in which the neonatal mice had been exposed to ECS for 4 months only followed by 7 months in filtered air (23). In fact, in the previous study, the increase of lung tumor yield became statistically significant only in mice coexposed to ECS and light (23). In any case, carcinogenicity of ECS was much less striking than that obtained with MCS in neonatal mice (20).

It was of interest to evaluate whether starting exposure to ECS at birth gives indeed an advantage, in terms of induction of lung tumors, compared with mice in which exposure starts during adulthood. The dams, exposed to ECS under identical conditions as their litters, showed a particularly severe pulmonary anthracosis and emphysema, but all other histopathologic alterations, also including benign and malignant tumors, were less pronounced than in neonatally exposed mice. Although these conclusions need to be confirmed by testing a larger number of adult mice, they are consistent with our previous findings that neonatal mice are more susceptible than

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Table 2. Incidence of histopathologic alterations in mice as related to exposure to ECS and treatment with either PEITC or budesonide

Histopathologic alteration	Sham				ECS			
	M [24]	F [28]	M + F [52]	Dams [6]	M [20]	F [27]	M + F [47]	Dams [15]
Lung								
Anthracosis	0	0	0	0	10 (50.0)*	4 (14.8)†	14 (29.8)*	8 (53.3)†,‡
Emphysema	3 (12.5)	1 (3.6)	4 (7.7)	0	9 (45.0)†	6 (22.2)†	15 (31.9)§	9 (60.0)§,‖
Peribronchial lymphoid tissue	0	2 (7.1)	2 (3.8)	4 (66.7)	1 (5.0)	3 (11.1)	4 (8.5)	1 (6.7)
Phlogosis	0	1 (3.6)	1 (1.9)	0	1 (5.0)	1 (3.7)	2 (4.3)	0
Alveolar bronchiolarization	1 (4.2)	0	1 (1.9)	0	7 (35.0)§	9 (33.3)*	16 (34.0)*	1 (6.7)**
Bronchial hyperplasia	0	2 (7.1)	2 (3.8)	1 (16.7)	4 (20.0)†	12 (44.4)§	16 (34.0)*	4 (26.7)
Alveolar hyperplasia	0	0	0	0	0	1 (3.7)	1 (2.1)	1 (6.7)
Hemorrhage	0	4 (14.3)	4 (7.7)	2 (33.3)	3 (15.0)†	9 (33.3)	12 (25.5)†	5 (33.3)
Alveolar adenomas	1 (4.2)	0	1 (1.9)	0	0	4 (14.8)†	4 (8.5)	2 (13.3)
Endobronchial papillomas	0	0	0	0	1 (5.0)	0	1 (2.1)	0
Total benign tumors	1 (4.2)	0	1 (1.9)	0	1 (5.0)	4 (14.8)†	5 (10.6)	2 (13.3)
Alveolar carcinomas	0	0	0	0	3 (15.0)†	5 (18.5)†	8 (17.0)§	1 (6.7)
Bronchioloalveolar carcinomas	0	0	0	0	1 (5.0)	0	1 (2.1)	0
Papillary carcinomas	1 (4.2)	0	1 (1.9)	0	0	0	0	0
Adenocarcinomas	1 (4.2)	1 (3.6)	2 (3.8)	0	0	0	0	1 (6.7)
Total malignant tumors	2 (8.3)	1 (3.6)	3 (5.8)	0	4 (20.0)	5 (18.5)	9 (19.1)†	1 (6.7)
Total benign + malignant tumors	3 (12.5)	1 (3.6)	4 (7.7)	0	5 (25.0)	8 (29.6)§	13 (27.7)§	3 (20.0)
Liver								
Steatosis	5 (20.8)	12 (42.9)	17 (32.7)	2 (33.3)	1 (5.0)	3 (11.1)§	4 (8.5)†	1 (6.7)
Hepatitis	2 (8.3)	1 (3.6)	3 (5.8)	1 (16.7)	2 (10.0)	5 (18.5)	7 (14.9)	4 (26.7)
Hemosiderosis	0	2 (7.1)	2 (3.8)	1 (16.7)	0	3 (11.1)	3 (6.4)	4 (26.7)
Sinusoidal hyaline degeneration	0	0	0	0	0	0	0	0
Vascular hyaline degeneration	0	0	0	0	0	0	0	1 (6.7)
Glycogenic nuclei	2 (8.3)	0	2 (3.8)	1 (16.7)	1 (5.0)	0	1 (2.1)	2 (13.3)¶¶
Perivascular lymphoid tissue	0	0	0	2 (33.3)	0	0	0	0
Autolysis	1 (4.2)	0	1 (1.9)	0	0	0	0	0
Sclerohyalinosis	0	1 (3.6)	1 (1.9)	0	0	0	0	0
Distrophic calcifications	0	1 (3.6)	1 (1.9)	0	0	0	0	0
Edema/Sinusoidal congestion	0	0	0	0	0	0	0	0
Hepatocellular adenoma	0	0	0	0	1 (5.0)	0	0	0
Kidney								
Glomerular sclerohyalinosis	1 (4.2)	1 (3.6)	2 (3.8)	0	7 (35.0)§	4 (14.8)	11 (23.4)§	0
Tubular interstitial nephritis	1 (4.2)	0	1 (1.9)	0	3 (15.0)	1 (3.7)	4 (8.5)	5 (33.3)‡
Urinary cysts	1 (4.2)	1 (3.6)	2 (3.8)	0	0	0	0	0
Perivascular lymphoid tissue	0	1 (3.6)	1 (1.9)	1 (16.7)	0	1 (3.7)	1 (2.1)	1 (6.7)

NOTE: The numbers between square brackets indicate the number of mice per experimental group. The numbers between brackets indicate the percentage of positive mice.

*$P < 0.001$, compared with sham.

†$P < 0.05$, compared with sham.

‡$P < 0.001$, compared with daughters.

§$P < 0.01$, compared with sham.

‖$P < 0.01$, compared with daughters.

¶$P < 0.1$, compared with ECS alone.

**$P < 0.1$, compared with daughters.

††$P < 0.05$, compared with ECS alone.

‡‡$P < 0.1$, compared with sham.

§§$P < 0.01$, compared with ECS alone.

‖‖$P < 0.001$, compared with ECS alone.

¶¶$P < 0.05$, compared with daughters.

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Table 2. Incidence of histopathologic alterations in mice as related to exposure to ECS and treatment with either PEITC or budesonide (Cont'd)

ECS + PEITC			ECS + budesonide		
M [18]	F [29]	M + F [47]	M [18]	F [20]	M + F [38]
11 (61.1)*	13 (44.8)*	24 (51.1)*	14 (77.8)*	14 (70.0)*	28 (73.7)*
3 (16.7)¶	2 (6.9)	5 (10.6)	3 (16.7)¶	8 (40.0)*	11 (28.9)§
2 (11.1)	7 (24.1)	9 (19.1)†	4 (22.2)†	6 (30.0)†,¶	10 (26.3)§
0	2 (6.9)	2 (4.3)	0	1 (5.0)	1 (2.6)
1 (5.6)††	6 (20.7)§	7 (14.9)†,¶	3 (16.7)	2 (10.0)‡‡,¶	5 (13.2)†
4 (22.2)†	12 (41.4)§	16 (34.0)*	7 (38.9)*	10 (50.0)*	17 (44.7)*
2 (11.1)‡‡	2 (6.9)	4 (8.5)†	1 (5.6)	0	1 (2.6)
0¶	11 (37.9)†	11 (23.4)†	5 (27.8)	6 (30.0)	11 (28.9)§
1 (5.6)	3 (10.3)	4 (8.5)	5 (27.8)†,§§	2 (10.0)	7 (18.4)§
0	0	0	0	0	0
1 (5.6)	3 (10.3)	4 (8.5)	5 (27.8)†,††	2 (10.0)	7 (18.4)§
0	6 (20.7)§	6 (12.8)§	2 (11.1)	1 (5.0)	3 (7.9)†
0	0	0	0	0	0
0	0	0	0	0	0
0	0	0	0	0	0
0††	6 (20.7)†	6 (12.8)	2 (11.1)	1 (5.0)	3 (7.9)
1 (5.6)¶	9 (31.0)§	10 (21.3)†	6 (33.3)	3 (15.0)	9 (23.7)†
1 (5.6)	8 (27.6)	9 (19.1)	0†	0*	0*,¶
0	1 (3.4)¶	1 (2.1)	0	1 (5.0)	1 (2.6)
0	1 (3.4)	1 (2.1)	0	1 (5.0)	1 (2.6)
7 (38.9)*,‖‖	0	7 (14.9)†,‖‖	5 (27.8)§,§§	1 (5.0)	6 (15.8)†,‖‖
0	0	0	3 (16.7)†,¶	2 (10.0)‡‡,¶	5 (13.2)§,§§
1 (5.6)	0	1 (2.1)	4 (22.2)	0	4 (10.5)‡‡
0	0	0	0	0	0
0	0	0	0	0	0
0	0	0	0	0	0
0	0	0	0	0	0
0	1 (3.4)	1 (2.1)	0	0	0
0	0	0	0	0	0
0‖‖	0††	0‖‖	0‖‖	0¶	0§§
11 (61.1)*,§§	5 (17.2)†	16 (34.0)*,§§	13 (72.2)*,‖‖	6 (30.0)§,§§	19 (50.0)*,‖‖
7 (38.9)§,§§	1 (3.4)	8 (17.0)†,§§	7 (38.9)§,§§	1 (5.0)	8 (21.0)§,‖‖
0	0	0	0	0	0

their dams to ECS-induced alterations of early biomarkers (21, 22).

Budesonide and PEITC are among the most promising chemopreventive agents. They were tested at doses that had preliminarily been shown to produce no alteration in body weight gain and general appearance, at least for up to 6 weeks and in ECS-free mice. However, the histopathologic analyses done at the end of the experiment, after 10 months of treatment with these agents of ECS-exposed mice, showed some sign of toxicity in liver and kidney. The design of the study does not allow us to speculate whether similar alterations would have occurred in ECS-free mice. On the other hand, it is noteworthy that both PEITC and budesonide significantly attenuated the loss of body weight gain in ECS-exposed mice.

In the interpretation of the protective effects of PEITC and budesonide toward ECS-related alterations, it should be taken into account that the oral treatment regimen with these chemopreventive agents necessarily started after having terminated the weanling period (i.e., when the mice had already been exposed to ECS for 4-5 weeks). Apart from the high doses both of carcinogens and of protective agents that need to be used in animal models, this experimental design aims at mimicking the situation of an individual who is passively exposed to ECS early in life and, after weanling, is treated with either dietary principles or pharmacologic agents.

In this light, it is noteworthy that, after 15 days only of treatment, PEITC and budesonide were able to counteract some of the alterations of biomarkers induced in mice exposed to ECS since birth. In particular, as reported in a separate article (51),

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Table 3. Multiplicity of lung tumors in mice as related to exposure to ECS and treatment with either PEITC or budesonide

Histopathologic alteration	Sham				ECS			
	M [24]	F [28]	M + F [52]	Dams [6]	M [20]	F [27]	M + F [47]	Dams [15]
Alveolar adenomas	0.08 ± 0.08	0	0.04 ± 0.04	0	0	0.15 ± 0.07*	0.09 ± 0.04	0.13 ± 0.09
Endobronchial papillomas	0	0	0	0	0.05 ± 0.05	0	0.05 ± 0.05	0
Total benign tumors	0.08 ± 0.08	0	0.04 ± 0.04	0	0.05 ± 0.05	0.15 ± 0.07*	0.13 ± 0.05	0.13 ± 0.09
Alveolar carcinomas	0	0	0	0	0.15 ± 0.08*	0.22 ± 0.10*	0.19 ± 0.07†	0
Bronchioloalveolar carcinomas	0	0	0	0	0.05 ± 0.05	0	0	0
Papillary carcinomas	0.04 ± 0.04	0	0	0	0	0	0	0
Adenocarcinomas	0.04 ± 0.04	0.04 ± 0.04	0.04 ± 0.03	0	0	0	0	0.07 ± 0.07
Total malignant tumors	0.04 ± 0.04	0.04 ± 0.04	0.04 ± 0.03	0	0.20 ± 0.09	0.22 ± 0.10‡	0.21 ± 0.07*	0.07 ± 0.07
Total benign + malignant tumors	0.17 ± 0.10	0.04 ± 0.04	0.10 ± 0.05	0	0.25 ± 0.10	0.37 ± 0.12†	0.32 ± 0.08*	0.27 ± 0.15

NOTE: The numbers between square brackets indicate the number of mice per experimental group. The values are means ± SE within each experimental group.

*$P < 0.05$, compared with sham.

†$P < 0.01$, compared with sham.

‡$P < 0.1$, compared with sham.

§$P < 0.05$, compared with ECS.

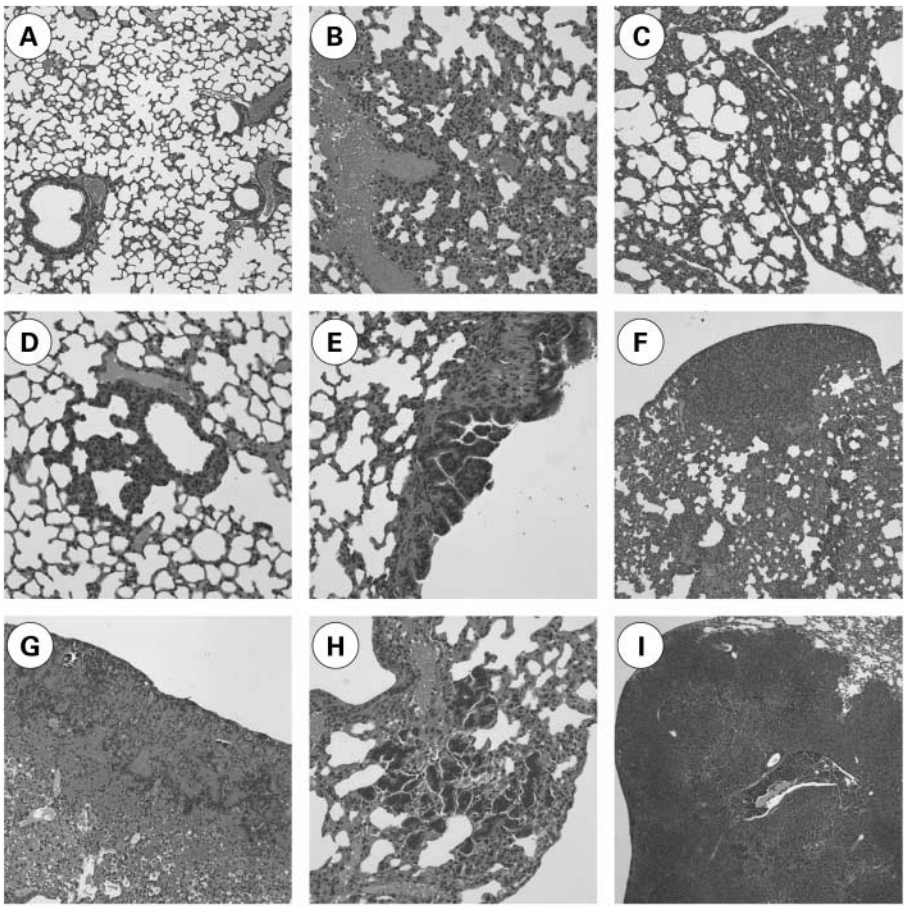

Fig. 4. Appearance of normal lung (*A*) and examples of histopathologic alterations in the lung, which were significantly increased in ECS-exposed mice compared with sham-exposed mice (see Tables 2 and 3). They include anthracosis (*B*), emphysema (*C*), alveolar bronchiolarization (*D*), bronchial hyperplasia (*E*), alveolar adenoma (*F*), adenocarcinoma (*G*), bronchioloalveolar carcinoma (*H*), and alveolar carcinoma (*I*). Stained with H&E. Original magnifications, ×4 (*I*), ×10 (*A, C, F*, and *G*), or ×20 (*B, D, E*, and *H*).

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Table 3. Multiplicity of lung tumors in mice as related to exposure to ECS and treatment with either PEITC or budesonide (Cont'd)

ECS + PEITC			ECS + budesonide		
M [18]	F [29]	M + F [47]	M [18]	F [20]	M + F [38]
0.06 ± 0.06	0.10 ± 0.06	0.09 ± 0.04	0.28 ± 0.11	0.10 ± 0.07	0.18 ± 0.06
0	0	0	0	0	0
0.06 ± 0.06	0.10 ± 0.06	0.09 ± 0.04	0.28 ± 0.11	0.10 ± 0.07	0.18 ± 0.06
0	0.24± 0.09	0.15 ± 0.06	0.11 ± 0.08	0.05 ± 0.05	0.13 ± 0.06
0	0	0	0	0	0
0	0	0	0	0	0
0	0	0	0	0	0
0[§]	0.24 ± 0.09	0.15 ± 0.06	0.11 ± 0.08	0.05 ± 0.05	0.13 ± 0.06
0.06 ± 0.06	0.35 ± 0.10[†]	0.23 ± 0.07	0.39 ± 0.14	0.15 ± 0.08	0.26 ± 0.08

both agents were able to revert ECS-induced DNA damage in pulmonary alveolar macrophages. Of the biomarkers investigated in the present study, budesonide had a quite powerful effect in protecting the lung from the oxidative DNA damage generated by ECS. This finding is likely to be related to the anti-inflammatory properties of this glucocorticoid, which by attenuating inflammation blocks a major source of oxidative damage. In addition, budesonide significantly attenuated ECS-related cytogenetical damage in bone marrow and peripheral blood and, in the bronchial epithelium, inhibited the ECS-induced hyperproliferation of cells and completely prevented stimulation of apoptosis. The major protective effects of PEITC were a reduction of bulky DNA adducts and 8-oxodGuo in the lung and an attenuation of apoptosis in bronchial epithelial cells. Inhibition of ECS-induced bulky DNA adducts by PEITC is likely to be related to the ability of this chemopreventive agent to stimulate the metabolic activation of smoke components (34).

The potent capacity of ECS to stimulate apoptosis in the respiratory tract of rodents has already been established in our previous studies in Sprague-Dawley rats (41), adult SKH-1 mice (43), and neonatal Swiss albino mice (21). The protective effect on ECS-induced apoptosis produced by both PEITC and budesonide deserves some comments. In fact, as inferred from literature data reviewed until the end of 2004 (54), the isothiocyanates PEITC (10 of 10 studies) and benzyl isothiocyanate (7 of 7 studies) and the glucocorticoids dexamethasone (15 of 15 studies) and budesonide (6 of 8 studies) displayed a proapoptotic role in various test systems. Due to this apparent discrepancy, we did further experiments with the goal of evaluating modulation of apoptosis by PEITC and budesonide in ECS-free mice. These experiments showed that PEITC is able per se to significantly stimulate apoptosis in mouse lung. It thus seems that PEITC acts as a proapoptotic agent in unexposed cells, whereas it is able to attenuate the potent apoptotic stimulus induced in ECS-exposed cells. This conclusion provides an example of the concept, highlighted in our previous articles (41, 54), that modulation of apoptosis by chemopreventive agents is a double-edged sword. In fact, on one hand, stimulation of spontaneous apoptosis represents a protective mechanism in carcinogenesis. On the other hand, inhibition by chemopreventive agents of the apoptotic process induced by ECS or other carcinogens reflects their ability to counteract upstream signals, such as genotoxic damage, redox imbalances, or other forms of cellular stress that trigger apoptosis.

Both PEITC and budesonide exhibited a moderate and nonsignificant protection of the lung from ECS-induced medium-term histopathologic alterations, such as emphysema and alveolar bronchiolarization. PEITC significantly inhibited the ECS-related formation of malignant lung tumors but only in males, whereas budesonide inhibited tumors in both genders but not to a significant extent. It is likely that some consistent trends could have reached the statistical significance threshold by using broader groups of mice, but the size of the experimental groups was driven by the capacity of the exposure chambers in the smoking machine.

In conclusion, the present study showed that the model using CD-1 albino mice exposed to ECS since birth has an appreciable sensitivity in revealing the pulmonary carcinogenicity of this complex mixture and its modulation by chemopreventive agents. However, the amplitude of the carcinogenic response is far lower than that observed by exposing mice to MCS (20).[5] Preliminary results suggest that the model in mice exposed to MCS since birth is also quite effective in detecting the cancer chemopreventive ability of PEITC and budesonide.[5] The results thus far generated by testing ECS and MCS in neonatal mice are not directly comparable because brands of cigarettes and exposure conditions are different and the daily exposure to MCS is shorter but more intense. In any case, the assumption that ECS may be more potently carcinogenic than MCS, which is based on the results of comparative experiments evaluating the carcinogenicity of cigarette smoke condensates on the mouse skin (55), needs to be reevaluated by using smoke inhalation systems.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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