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Thirdhand smoke: a new dimension to the effects of cigarette smoke on the developing lung

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Rehan VK, Sakurai R, Torday JS. Thirdhand smoke: a new dimension to the effects of cigarette smoke on the developing lung. *Am J Physiol Lung Cell Mol Physiol* 301: L1–L8, 2011. First published April 8, 2011; doi:10.1152/ajplung.00393.2010.—The underlying mechanisms and effector molecules involved in mediating in utero smoke exposure-induced effects on the developing lung are only beginning to be understood. However, the effects of a newly discovered category of smoke, i.e., thirdhand smoke (THS), on the developing lung are completely unknown. We hypothesized that, in addition to nicotine, other components of THS would also affect lung development adversely. Fetal rat lung explants were exposed to nicotine, 1-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridinyl)-4-butanal (NNA), or 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), the two main tobacco-specific *N*-nitrosamine constituents of THS, for 24 h. We then determined key markers for alveolar paracrine signaling [epithelial differentiation markers surfactant phospholipid and protein synthesis; mesenchymal differentiation markers peroxisome proliferator-activated receptor γ (PPAR-γ), fibronectin and calponin], the BCL-2-to-Bax ratio (BCL-2/Bax), a marker of apoptosis and the involvement of nicotinic acetylcholine receptors (nAChR)-α3 and -α7 in mediating NNA's and NNK's effects on the developing lung. Similar to the effects of nicotine, exposure of the developing lung to either NNK or NNA resulted in disrupted homeostatic signaling, indicated by the downregulation of PPAR-γ, upregulation of fibronectin and calponin protein levels, decreased BCL-2/Bax, and the accompanying compensatory stimulation of surfactant phospholipid and protein synthesis. Furthermore, nAChR-α3 and -α7 had differential complex roles in mediating these effects. NNK and NNA exposure resulted in breakdown of alveolar epithelial-mesenchymal cross-talk, reflecting lipofibroblast-to-myofibroblast transdifferentiation, suggesting THS constituents as possible novel contributors to in utero smoke exposure-induced pulmonary damage. These data are particularly relevant for designing specific therapeutic strategies, and for formulating public health policies to minimize THS exposure.

nicotine; 1-(*N*-methyl-*N*-nitrosamino)−1-(3-pyridinyl)−4-butanal; 4-(methylnitrosamino)−1-(3-pyridyl)−1-butanone; lung development; surfactant

A MYRIAD OF WELL-DESCRIBED short- and long-term effects of tobacco smoke exposure on developing lung structure, physiology, and pathophysiology are known, which can have life-long consequences (5). These include suppression of alveolar-ization, functional residual capacity, tidal flow volume, and increased predisposition to asthma. The underlying mechanisms and effector molecules involved in mediating in utero smoke exposure-induced effects on the developing lung remain incompletely understood. While the underlying molecular mechanisms involved in these processes were being uncovered, a novel type of tobacco exposure, i.e., thirdhand smoke (THS) exposure has recently been described (2, 5–7, 9, 14, 18).

THS consists of residual tobacco smoke pollutants that remain on surfaces and in dust after tobacco has been smoked and includes secondary pollutants that result from chemical reactions of primary pollutants with each other and with oxidants in the environment (14). However, it must be emphasized that although the term THS is new, the chemical aging of tobacco smoke, the evidence it leaves behind in the indoor environments, and the smelly clothes and distaste from non-smokers have long been recognized. The constituents of THS that have been identified so far include nicotine, tobacco-specific nitrosamines (TSNAs), 3-ethenylpyridine, phenol, cresols, naphthalene, formaldehyde, and heavy metals, among others. A recent study by Sleiman et al. (14) revealed that the nicotine adsorbed on indoor surfaces can react with environmental gaseous nitrous acid and ozone to yield potentially bioactive residues such as 1-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridinyl)-4-butanal (NNA), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and *N*′-nitrosonornicotine.

Nicotine is the most extensively studied component of cigarette smoke, and there is evidence demonstrating its important role in smoke exposure-induced lung damage in the developing lung (4, 5). However, virtually nothing is known about the effects of the constituents of THS on the developing lung. We hypothesized that, in addition to nicotine, other components of THS would also affect lung development adversely. In this regard, using a lung explant model system, we have studied the effects of nicotine, as well as that of NNA and NNK, the two most abundant TSNAs formed when nicotine emitted in mainstream (MSS) and/or sidestream smoke (SSS) reacts with the environmental nitrous acid and ozone, on the growth and differentiation of the developing lung (14). It is important to note that TSNAs such as NNK and NNA are recognized lung carcinogens (1), but their effects on lung development and differentiation in the developing lung are completely unknown. This study was focused on the effects of NNA and NNK as surrogates for THS exposure within the context of our experimental design.

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METHODS

Animals. Time-mated Sprague-Dawley rats [*embryonic day* (e) 0 = day of mating] were obtained from Charles River Breeders (Holister, CA). The experiments described were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Los Angeles Biomedical Research Institute Animal Care Committee.

Reagents. Nicotine bitartrate and nicotinic acetylcholine (nACh) receptor antagonists (α-bungarotoxin and mecamylamine) were acquired from Sigma Chemical (St. Louis, MO). NNK and NNA were ordered from Toronto Research Chemicals (Toronto, ON, Canada).

Explant culture. Explants derived from three to five litters of rats were used for each experiment. Lungs were harvested from e19 fetal rats under sterile conditions. The lung tissue was chopped into $\sim$1-mm cubes and incubated in 0.5 ml of Waymouth's MB-252/1 medium containing penicillin (100 U/ml)-streptomycin (100 U/ml) and Fungizone (2.5 μg/ml) and cultured in six-well plates while rocking on an oscillating platform (3 cycles/min) in 5% CO_2-95% air at 37°C. The explants were initially allowed to attach for $\sim$1–2 h and were then treated with either nicotine, NNA, or NNK (all at 1×10^{-5}, 1×10^{-8}, or 1×10^{-11} M), for 24 h, followed by analyses as described below. In some experiments, e19 lung explants were treated with nicotine (1×10^{-8} M) for 24 h following 1-h pretreatment with the nAChR α7 antagonist α-bungarotoxin (1×10^{-7} M) or the nAChR α3 antagonist mecamylamine (1×10^{-7} M).

Triolein uptake assay. The triolein uptake assay was performed as previously described (16).

Choline incorporation assay. The choline incorporation assay was performed as previously described (7).

Protein expression by Western blotting. Western blotting was performed as previously described (7). The specific primary antibodies used included peroxisome proliferator-activated receptor γ (PPAR-γ; sc-7196, 1:200), surfactant protein (SP)-B (sc-133143 1:250), SP-C (sc-13979, 1:300), BCL-2 (sc-492, 1:300), Bax (sc-7480, 1:1,000),

fibronectin (sc-9068 1:500), acetylcholine receptor α3 (AChRα3; sc-5590, 1:200) and AChRα7 (sc-5544, 1:200), all of them acquired from Santa Cruz, CA; calponin (c2687, 1:3,500) was acquired from Sigma, St. Louis, MO. The densities of the specific protein bands were quantified by use of a scanning densitometer (GS-800 Calibrated Densitometer, Bio-Rad). The blots were subsequently stripped and reprobed with anti-GAPDH (1:10,000, MAB374, Millipore) antibody to confirm equal loading of the samples.

Statistical analyses. Experiments were repeated three or more times to confirm the validity of the observations. The significance of differences within groups was determined by one-way ANOVA with Newman-Keuls post hoc test. The null hypothesis was rejected when $P < 0.05$ was obtained.

RESULTS

Initially, we determined and compared the effects of NNK and NNA with those of nicotine on triolein uptake and choline incorporation into disaturated phosphatidylcholine, two important functional markers for alveolar lipofibroblast (LIF) and alveolar epithelial type II (AEII) cell maturation, respectively, and hence lung maturation. Choline incorporation into disaturated phosphatidylcholine, a critical component of surfactant phospholipid synthesis (Fig. 1*A*), increased significantly when e19 fetal rat lung explants were treated with nicotine (1×10^{-8} M), NNK (1×10^{-8} and 1×10^{-5} M), or NNA (1×10^{-11} and 1×10^{-8} M) for 24 h. Similarly, compared with the control, treatment with nicotine, NNK, or NNA (1×10^{-8} and 1×10^{-5} M) for 24 h increased triolein uptake, the effect being most pronounced with NNA (1×10^{-5} M) (Fig. 1*B*). Then we examined the effects of nicotine, NNK, and NNA on alveolar epithelial (SP-B and -C), and fibroblast (PPAR-γ, fibronectin, and calponin) differentiation markers, which have been shown
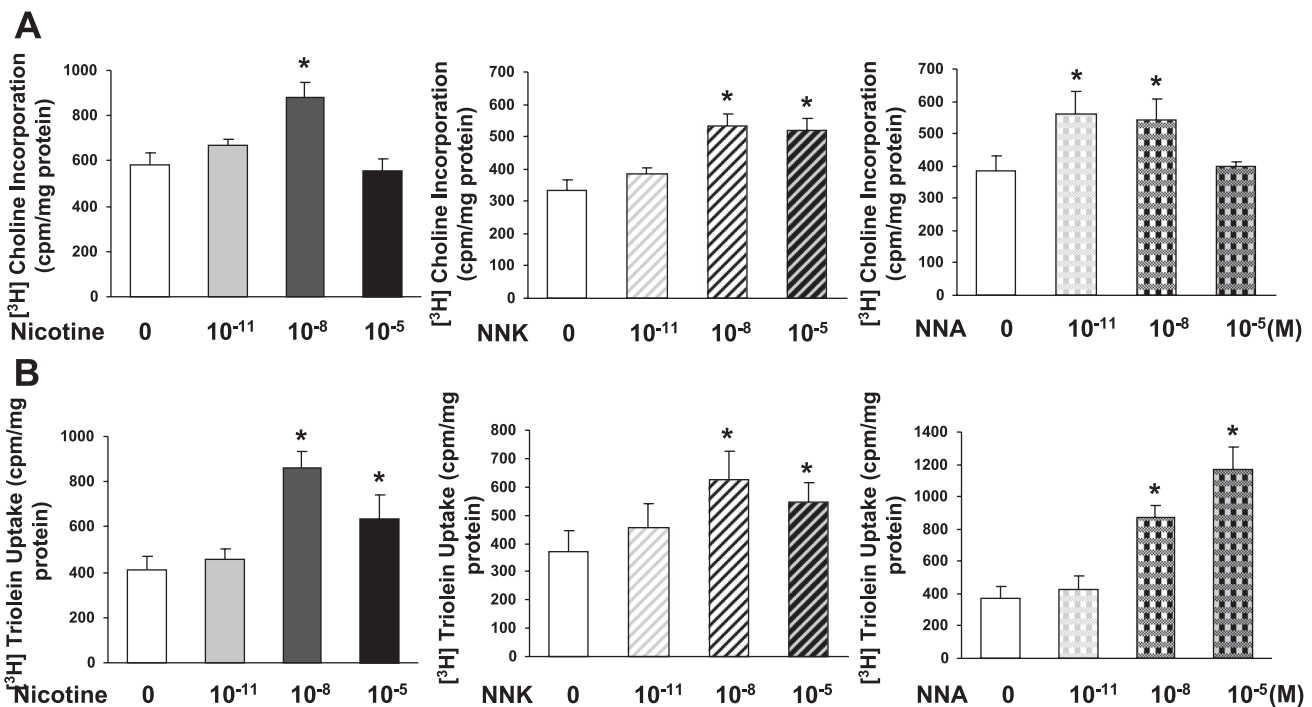

Fig. 1. Effect of 24-h treatment of *embryonic day 19* fetal rat lung explants with nicotine,, or 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), or 1-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridinyl)-4-butanal (NNA) (1×10^{-11}, 1×10^{-8}, and 1×10^{-5} M) on choline incorporation into disaturated phosphatidylcholine (*A*) and triolein uptake (*B*). There were significant dose-dependent increases in both choline incorporation and triolein uptake by nicotine, NNK, and NNA treatment (*$P < 0.05$ vs. control; $n = 6$).

to be key indicators of fetal lung maturation and injury (6, 7, 9, 17). Western blot analysis of protein extracts from e19 lung explants treated with nicotine, NNK, and NNA for 24 h resulted in dose-dependent effects on SP-B and -C levels (Fig. 2, *A* and *B*); whereas nicotine or NNK (1×10^{-8} and 1×10^{-5} M for both) increased SP-B levels, NNA either increased (1×10^{-11} M) or decreased (1×10^{-5} M) SP-B levels and had no effect at 1×10^{-8} M. There was no effect of nicotine on SP-C levels at any concentration examined, whereas NNK increased SP-C levels at all concentrations examined and NNA decreased SP-C levels at 1×10^{-8} and 1×10^{-5} M. In regard to the effect of these agents on fibroblast differentiation markers, PPAR-γ protein levels decreased and fibronectin and calponin protein levels increased with all three agents (Fig. 3). The effects of NNK and NNA are similar to the previously described effects of nicotine, suggesting that both NNK and NNA exposures also likely result in LIF-to-myofibroblast (MYF) transdifferentiation, as has been shown previously with nicotine (5–7, 9). Furthermore, Western blot analysis of the protein lysates of treated explants showed a decreased BCL-2-to-Bax ratio with all agents (Fig. 4), indicating an increase in apoptosis with nicotine, as well as with NNK and NNA. Since nAChR α_3 and α_7 have been shown to be involved in nicotine's effect on the developing lung, we next examined the involvement of these receptors in mediating NNK's and NNA's effects on the developing lung. First, we confirmed that both nAChR $\alpha3$ and $\alpha7$ were well expressed in the e19 lung

and observed that, similar to the effect of nicotine on the expression of these receptors, both NNK and NNA treatments for 24 h significantly increased the nAChR $\alpha3$ and $\alpha7$ protein levels (Fig. 5, *A* and *B*). Treatment with either α-bungarotoxin, an nAChR $\alpha7$ antagonist, or mecamylamine, an nAChR $\alpha3$ antagonist, did not block the NNK-mediated increase in surfactant phospholipid synthesis and SP-B protein levels (Fig. 6*A*), suggesting that NNK's effect on surfactant synthesis is independent of nAChR $\alpha3$ and $\alpha7$ stimulation. In contrast, treatment with mecamylamine blocked NNA-mediated increases in both surfactant phospholipid synthesis and SP-B protein levels (Fig. 6*B*), whereas bungarotoxin only blocked the SP-B increase and not choline incorporation, suggesting a complex role of nAChR $\alpha3$ and $\alpha7$ in the NNA-mediated increase in surfactant synthesis. However, both α-bungarotoxin and mecamylamine blocked NNK- and NNA-mediated increases in calponin protein levels and increased lung cell apoptosis (Fig. 7, *A* and *B*), suggesting possible roles of nAChR $\alpha3$ and $\alpha7$ in these responses.

DISCUSSION

The experimental model of THS-induced breakdown in lung development used in this study was designed to compare the effects of NNK and NNA on alveolar epithelial-mesenchymal paracrine mechanisms necessary for normal lung development (17) with those previously shown for nicotine (5–7, 9), namely,
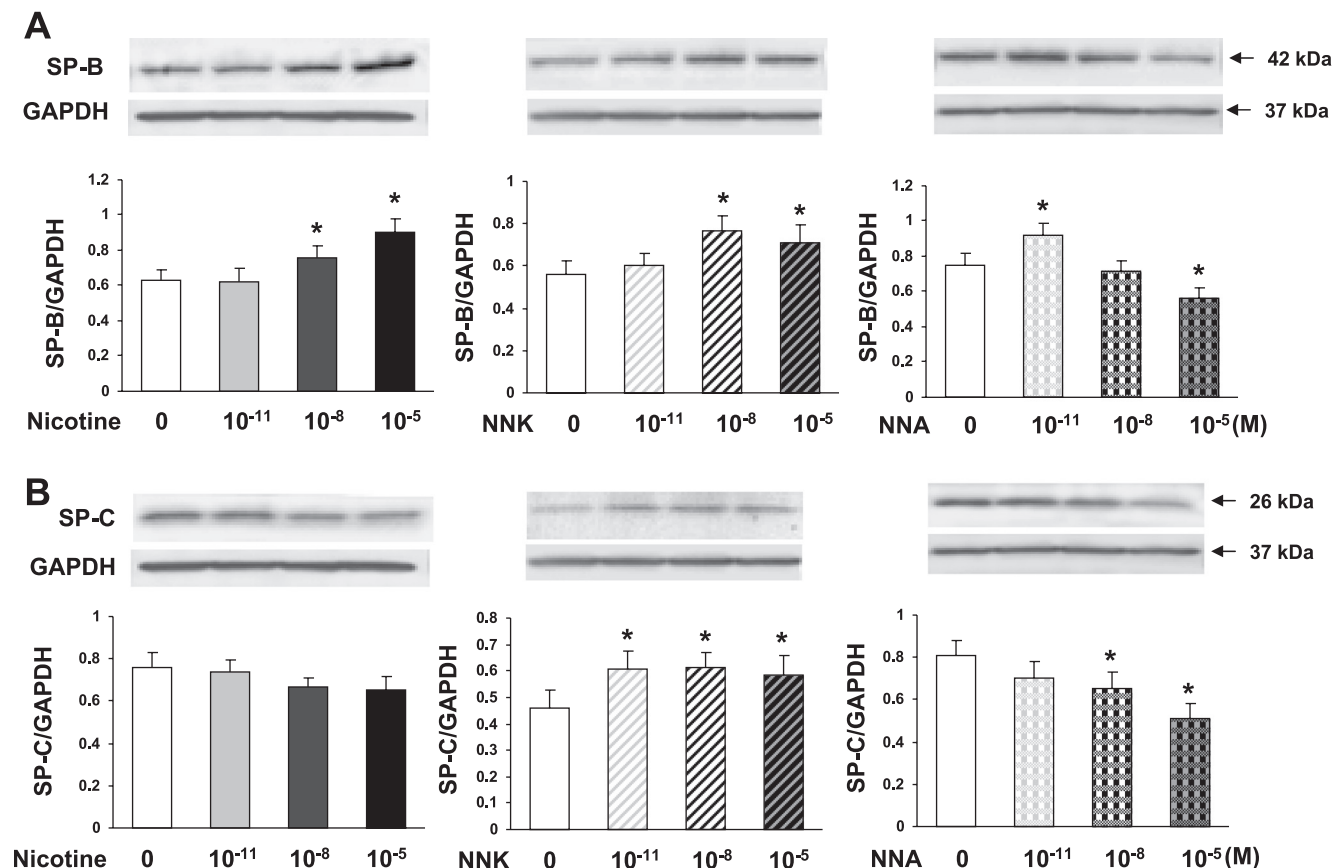

Fig. 2. Effect of 24-h treatment of *embryonic day 19* fetal rat lung explants with nicotine, NNK, or NNA (1×10^{-11}, 1×10^{-8}, and 1×10^{-5} M) on surfactant protein- B (SP-B) (*A*) and SP-C (*B*) protein levels. There were significant increases in SP-B levels by nicotine, NNK, and NNA treatments. However, SP-C levels did not change with nicotine, decreased with NNA, and increased with NNK (*$P < 0.05$ vs. control; $n = 3$).
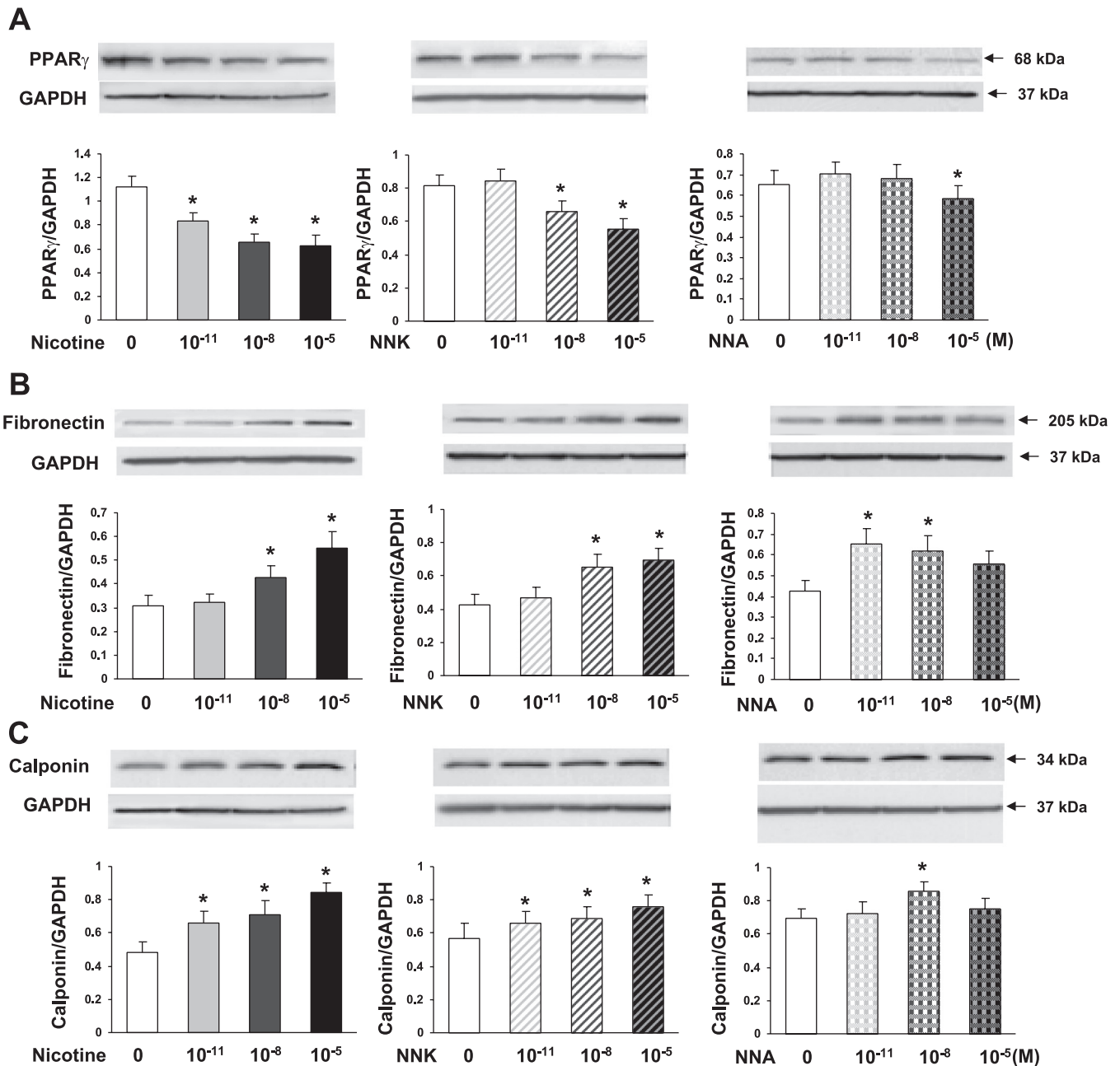
Fig. 3. Effect of 24-h treatment of *embryonic day 19* fetal rat lung explants with nicotine, NNK, or NNA (1×10^{-11}, 1×10^{-8}, and 1×10^{-5} M) on peroxisome proliferator-activated receptor-γ (PPAR-γ) (*A*), fibronectin (*B*), and calponin (*C*) protein levels. PPAR-γ levels decreased significantly with nicotine (1×10^{-11}, 1×10^{-8}, and 1×10^{-5} M), NNK(1×10^{-8}, and 1×10^{-5} M) and NNA (1×10^{-5} M). In contrast, fibronectin and calponin levels increased with nicotine, NNK, and NNA (*$P < 0.05$ vs. control; $n = 3$).

alveolar interstitial fibroblast PPAR-γ signaling, which both downregulates the default mesenchymal Wnt signaling and its downstream targets, such as fibronectin and calponin, and stimulates surfactant synthesis by the ATII cells. The net result of this signaling pathway on the mesenchyme is to induce the LIF phenotype. LIFs produce growth factors that stimulate ATII cell surfactant synthesis, which is primarily composed of disaturated phosphatidylcholine and SP-B and SP-C, and promote ATII cell proliferation. Therefore, the overall effect of nicotine, NNK, and NNA was the disruption of these homeostatic signaling mechanisms, indicated by the downregulation of PPAR-γ, upregulation of fibronectin and calponin protein

levels, decreased BCL-2-to-Bax ratio (BCL-2/Bax), and the accompanying compensatory stimulation of surfactant synthesis. Overall, the nicotine/NNK/NNA-induced breakdown in LIF-ATII cross-talk, characterized by LIF-to-MYF transdifferentiation (5–7, 9, 17), as evidenced by the downregulation of PPAR-γ signaling and increased fibronectin and calponin production, was observed in this model. And since LIFs promote ATII proliferation, but MYFs cannot (18), the observed increase in apoptosis is also consistent with the working model of failed alveolar development. Furthermore, we found differential and very complex roles of nAChR α3 and α7 in mediating NNK's and NNA's effects on the developing lung.
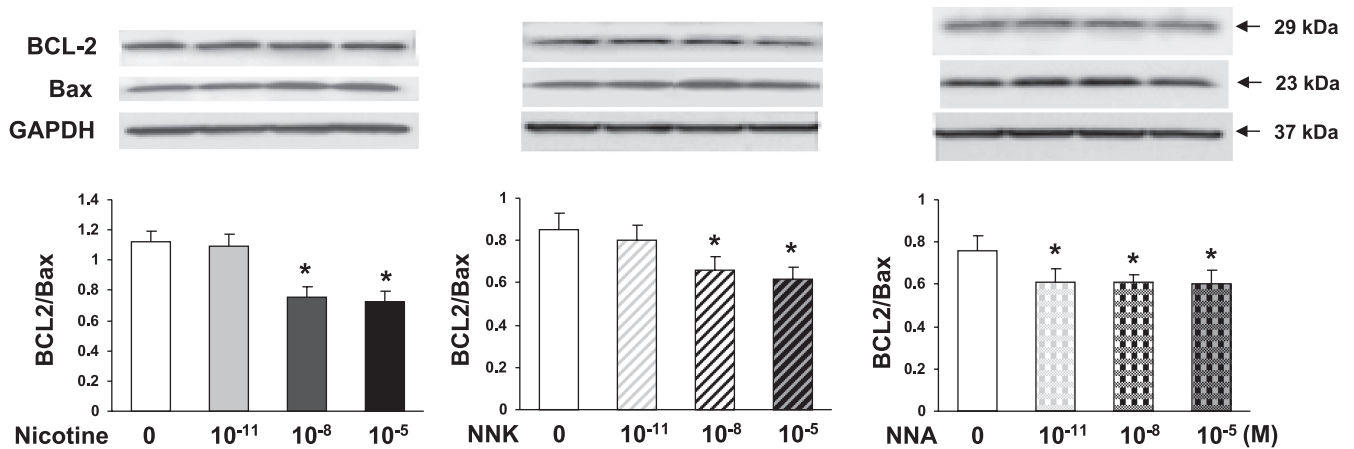
Fig. 4. Effect of 24-h treatment of *embryonic day 19* fetal rat lung explants with nicotine, NNK, or NNA (1×10^{-11}, 1×10^{-8}, and 1×10^{-5} M) on BCL-2-to-Bax ratio (BCL-2/Bax). BCL-2/Bax decreased significantly with nicotine, NNK, or NNA treatment (*$P < 0.05$ vs. control; $n = 3$).

Currently, there is virtually no realization that THS is a danger to human health. A recent study by Winickoff et al. (19) showed that only 65.2% of nonsmokers and 43.2% of smokers believe that THS is harmful to children. Thus there is a critical need to validate these projections in real-life situations in the field.

Depending on the combustion temperature, up until now only two major types of cigarette smoke had been identified:

1) MSS that is inhaled by the smokers and is generated at 1,200–1,600°C because of much higher utilization of ambient oxygen, and *2*) SSS, which is generated at much lower temperatures (600–900°C) by the smoldering end of the cigarette. Secondhand smoke (SHS) is a mixture of ~85% SSS and ~15% of smoke exhaled by the smoker. As alluded to above, another form of cigarette smoke has recently been recognized, i.e., THS (2, 14, 18). THS is derived from the deposition of the
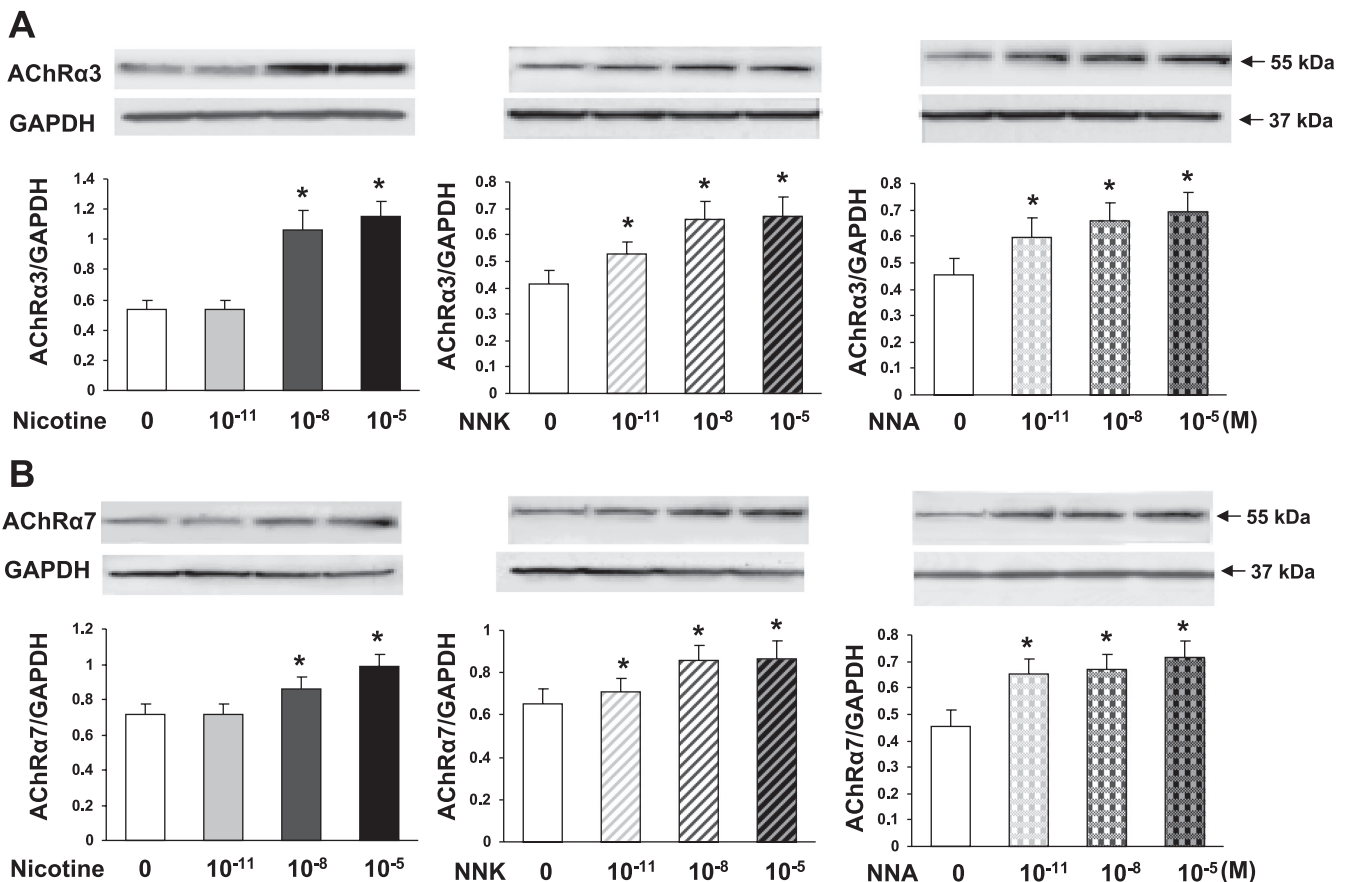

Fig. 5. Effect of 24-h treatment of *embryonic day 19* fetal rat lung explants with nicotine, NNK, or NNA (1×10^{-11}, 1×10^{-8}, and 1×10^{-5} M) on nicotinic acetylcholine receptor α3 (AChRα3) (*A*) and AChRα7 (*B*). There were significant increases in both AChRα3 and AChRα7 levels with nicotine, NNK, and NNA treatments (*$P < 0.05$ vs. control; $n = 3$).

Fig. 6. Effect of bungarotoxin (Bung) or mecamylamine (Mecl) pretreatment (1×10^{-7} M for both) on NNK- and NNA (1×10^{-8} M for all)-induced changes in surfactant phospholipid and surfactant protein B synthesis in *embryonic day 19* fetal rat lung explants. NNK-induced increase in surfactant phospholipid and SP-B was not blocked by either Bung or Mecl pretreatment (*A*), whereas NNA-induced increase in SP-B was blocked by either treatment and increase in surfactant phospholipid synthesis by only Mecl (*B*) (*$P < 0.05$ vs. control; #$P < 0.05$ vs. NNA; $n = 6$ for choline incorporation; $n = 3$ for Western blotting).
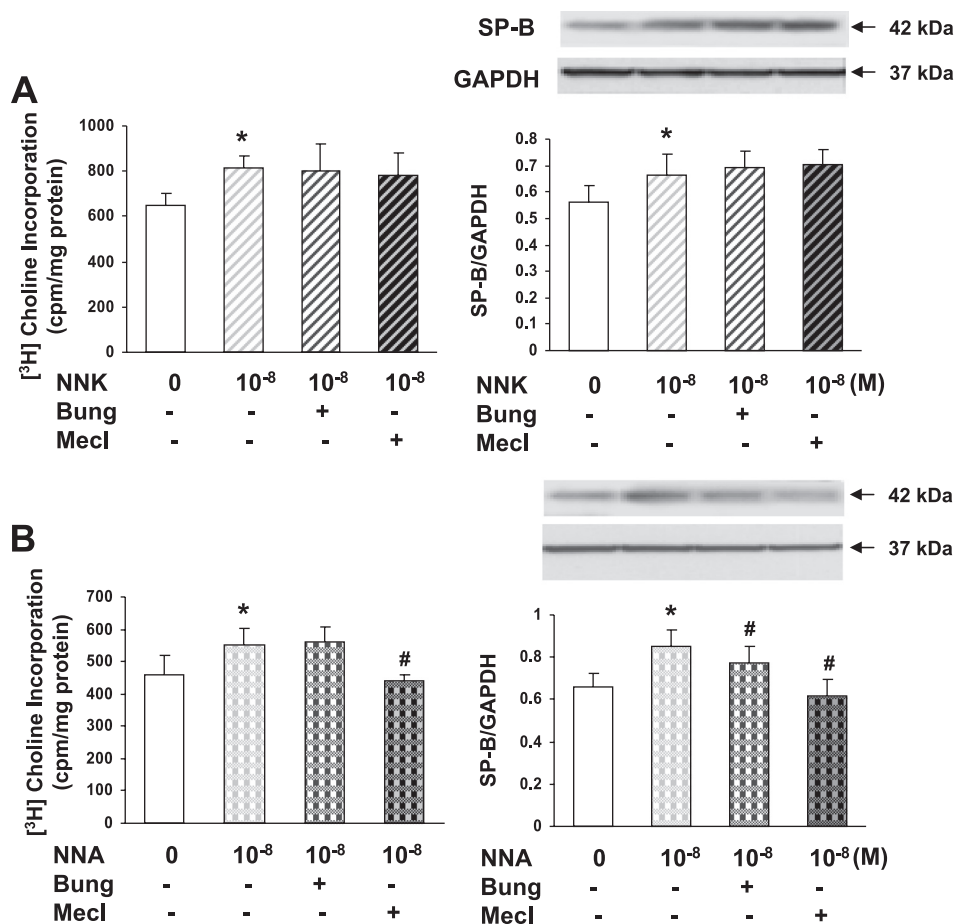
tobacco toxins from SSS and SHS, over and over again, on the surfaces of a particular compartment. Examples of repositories of THS are *1*) the upholstery of car seats, as well as the dashboard and window glass; *2*) in a family room: the indoor walls, curtains, carpet, upholstery of sofas and chairs, throw blankets, pillows, skin, hair, and clothing, etc. THS is a stealth toxin because it is present in the households of smokers where small children and elderly people live; the hotel rooms, casinos, and cars owned by smokers; and where the unsuspecting vulnerable populations may be exposed to the toxicants without realizing the dangers.

In comparing the toxicities of the various types of cigarette smoke, the secondary analysis by Schick and Glanz (10–12) of the secret unpublished documents on animal research of Philip Morris Tobacco showed that, per gram of tobacco, SSS is ~4 times more toxic than MSS and that aged SSS is ~2–4 times more toxic than fresh SSS (10–12). Furthermore, SHS can become ~6–12 times more toxic than the smoke inhaled by the smokers themselves. Aging of cigarette smoke potentially makes THS even more toxic in pragmatic terms and extent for potential human exposure. Because THS is essentially aged SHS that is adherent to surfaces and has smaller sized ultrafine particles but much larger sized molecular weight moieties with greatly heightened asthma hazard index values (13), it is likely to be much more toxic than MSS and fresh SSS. Nicotine is also now known to be oxidized in the presence of ozone and nitrous acid and to produce formaldehyde, *N*-dimethylforma- mide, nicotinaldehyde, and nitrosamines, all of which are

potential pulmonary toxins. On the basis of the calculations from the data of Sleiman et al. (14), it is expected that, under steady-state conditions, both NNA and NNK are likely to be present in the environment at much lower concentrations compared with those of nicotine. It is estimated that NNK concentrations will be 1,000-fold lower than those of nicotine, and that NNA concentrations will be 7-fold higher than those of NNK.

In addition to exposure to volatile components (re)emitted from adsorbed THS through inhalation, there are several other potential exposure routes for the less volatile components present in THS. Because of their low vapor pressure, direct inhalation of these components is less likely. Dermal contact with surfaces contaminated with THS, as well as ingestion of dust(s) loaded with THS components are the most likely major pathways for the exposure. In this context, children and pregnant mothers in busy households are particularly susceptible to THS exposure because they could breathe near, touch, and ingest materials from contaminated surfaces. For example, the dust ingestion rate in infants is more than twice that of adults, and urine cotinine levels of children in homes with strict nonsmoking policies are approximately six times lower than in homes without such policies (3). Infants and young children have been estimated to be manifold more sensitive than adults to pollutants in house dust because of such factors as increased respiration relative to body size and immature metabolic capacity (8). Therefore, in a way, THS is a hidden toxin present in the
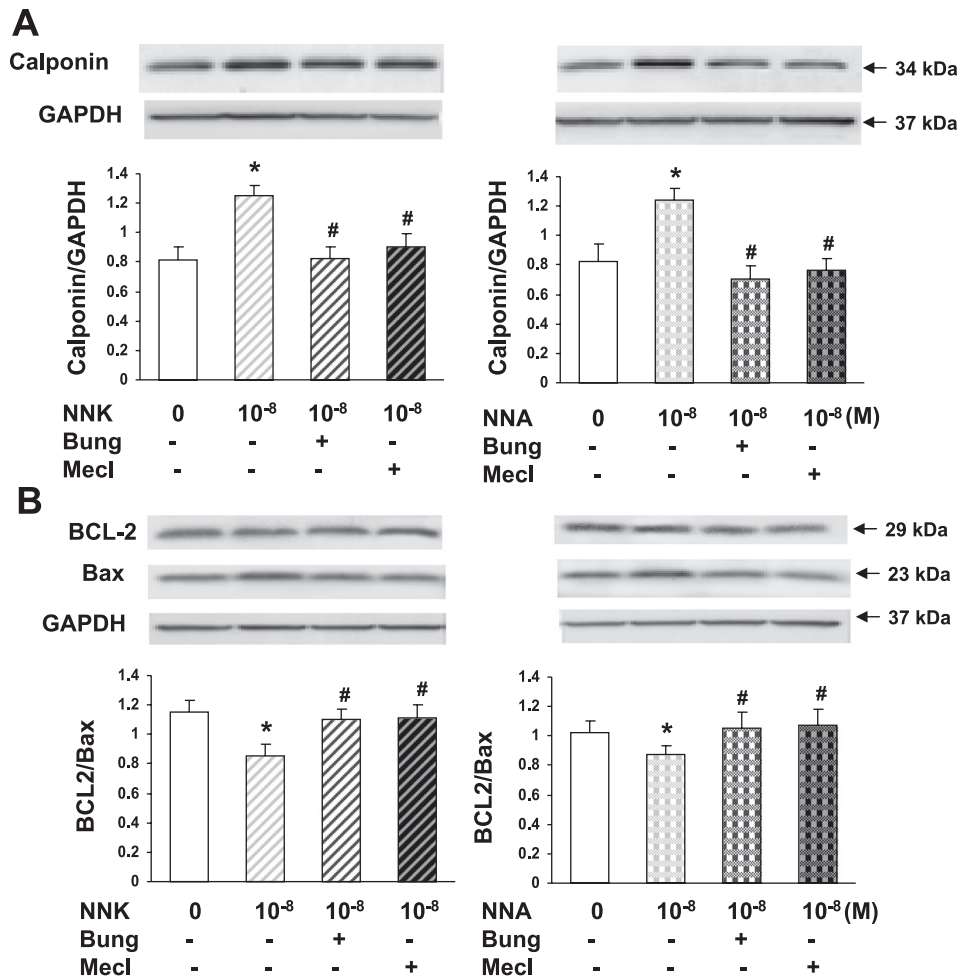
Fig. 7. Effect of Bung or Mecl pretreatment (1×10^{-7} M for both) on NNK- and NNA (1×10^{-8} M for all)-induced changes in calponin (*A*) and lung cell apoptosis (*B*) in *embryonic day 19* fetal rat lung explants, as determined by BCL2/Bax. Both NNK- and NNA-induced increase in calponin and decrease in BCL-2/Bax (*B*) was blocked by both Bung and Mecl pretreatments (*$P < 0.05$ vs. control; #$P < 0.05$ vs. NNK or NNA; $n = 3$).

households of smokers where pregnant women and small children live without realizing that they are being exposed to such dangerous toxicants. The same risk exists for adult workers who clean and change bed sheets in hotel rooms where cigarette smoking is allowed, all over the world: a problem of global proportions!

We must state that a comprehensive assessment of the risks of THS on the developing lung, the molecular pathways, the specific cell-types involved, and the levels of different THS constituents required in mediating these risks requires further extensive characterization of levels of THS constituents in the environment, and the realistic exposure of the developing fetus and growing child to these constituents. However, our limited but alarming and concerning data clearly highlight the potential risks and long-term consequences of THS exposure. Furthermore, because of the differences in the timing of alveolar development between the rat and human lung, the timing of exposure to THS will be particularly relevant in determining the effects of THS exposure on the developing human lung and the extrapolation of our data to humans. But it is important to underscore the fact that there is clear evidence that prenatal exposure to tobacco smoke components plays a much greater role in altered lung function in offspring than postnatal or childhood exposures (15).

DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the author(s).

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