Environmental Tobacco Smoke Suppresses Nuclear Factor- κ B Signaling to Increase Apoptosis in Infant Monkey Lungs

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Rationale: Exposure to environmental tobacco smoke in early life has adverse effects on lung development. Apoptosis plays an essential role in development; however, the molecular mechanisms of pulmonary apoptosis induced by environmental tobacco smoke is unknown.

Objectives: To investigate the mechanistic role of nuclear factor (NF)- κ B, a critical cell survival pathway, in the developing lungs exposed to environmental tobacco smoke.

Methods: Timed-pregnant rhesus monkeys and their offspring were exposed to filtered air or to aged and diluted sidestream cigarette smoke as a surrogate to environmental tobacco smoke (a total suspended particulate concentration of 0.99 mg/m³ for 6 h/d, 5 d/wk) from 45–50 d gestational age to 72–77 d postnatal age (n = 4/group).

Measurements and Main Results: NF- κ B–DNA binding activity, regulated anti-apoptotic genes, and apoptosis were measured in lung tissues. Exposure to environmental tobacco smoke significantly suppressed NF- κ B activation pathway and activity. Environmental tobacco smoke further down-regulated NF- κ B–dependent anti-apoptotic genes and induced activation of caspases, cleavage of cellular death substrates (poly(ADP)-ribose polymerase and caspase-activated DNase) and an increase in the rate of apoptosis in the lung parenchyma. No significant alterations were observed for activator protein 1, p53 or Akt activity.

Conclusions: Our results indicate that exposure to low levels of environmental tobacco smoke during a critical window of maturation in the neonatal nonhuman primate may compromise lung development with potential implications for future lung growth and function. These findings support our hypothesis that NF- κ B plays a key role in the regulation of the apoptotic process.

Keywords: apoptosis; environmental tobacco smoke; infant monkeys; lung development; NF- κ B

Exposure to environmental tobacco smoke (ETS) in children is a hazardous and widespread health problem (1, 2). The developing lungs are particularly vulnerable to ETS (3, 4), and exposure to ETS during the perinatal period adversely affects the overall growth and function of the respiratory system (5–10). ETS most likely disrupts normal development by altering specific cellular signaling; however, little information is available to define the precise effects of such exposure. As an essential physiologic process, apoptosis plays a critical role in development and tissue

homeostasis. Apoptosis is also involved in a wide range of pathologic conditions, including developmental defects (11). Tobacco smoke increases apoptosis in adult rat and mouse lungs (12, 13). Moreover, there is evidence that maternal exposure to passive smoking during pregnancy enhances apoptosis in the lungs of newborns (14), while the mechanisms by which ETS induces apoptosis have not yet been defined. At the molecular level, apoptosis is tightly regulated by pro- and anti-apoptotic factors, and alteration in gene expression of these factors can result in abnormal apoptosis. Therefore, the cellular signals that govern the expression of pro- and/or anti-apoptotic genes play critical roles in the modulation of the apoptotic process.

Nuclear factor- κ B (NF- κ B) is the major transcription factor that controls the apoptotic process (15–17). NF- κ B regulates the expression of a number of anti-apoptotic genes, including bcl-2 family members, cellular inhibitors of apoptosis (c-IAPs), and TNF receptor–associated factors (TRAFs) (17–19). It exists as a heterodimeric or homodimeric complex consisting of p50, p65/RelA, p52, c-Rel, and Rel-B subunits. In unstimulated cells, NF- κ B is maintained in the cytoplasm by the inhibitory protein I κ B, mainly I κ B α . Upon stimulation, I κ B is rapidly phosphorylated by I κ B kinases (IKK), and subsequently undergoes ubiquitination and degradation by proteasome. The released NF- κ B complex then translocates to the nucleus and activates target gene transcription (20, 21). Changes in NF- κ B target anti-apoptotic gene expression will abnormally regulate the apoptotic process.

Based on the central role of NF- κ B activity in cell survival, we postulated that modulation of NF- κ B signaling may be involved in ETS-induced apoptosis. To date, no studies have been done to examine the effects of ETS on NF- κ B signaling and its role in the regulation of apoptosis during lung development. Therefore, the present study was designed to investigate whether perinatal exposure to ETS modulates NF- κ B activation and NF- κ B–dependent anti-apoptotic gene expression in the lungs of infant nonhuman primates, a species whose lung development is closely aligned with that of humans. Preliminary results of this study were previously reported in an abstract (22).

METHODS

See the online supplement for further details concerning methods.

Animals and Exposure to ETS

Rhesus monkeys were obtained from the animal colony at the California National Primate Research Center at the University of California, Davis. The dams used in this study ranged in age from 5 to 9 yr and had prior successful pregnancies. Four timed pregnant dams were exposed to filtered air (FA) and four dams to aged and diluted sidestream smoke as a surrogate to ETS in a smoking apparatus built in our laboratory as described previously (23). Research cigarettes (1R4F) obtained from the Tobacco Research Institute at the University of Kentucky were used. Exposure to ETS began at Gestational Day 45–50 and continued for 6 h/d, 5 d/wk through 72–77 d postnatal age at a target concentration of 1 mg/m³ of total suspended particulates. The cumulative exposure was 128–134 d.

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Figure 1. Effect of environmental tobacco smoke (ETS) on NF- κ B activity. (A) Electrophoretic mobility shift assay of NF- κ B-DNA binding activity in the lungs of infant monkeys. Lane 1, competition assay; lanes 2–5, filtered air (FA); lanes 6–9, ETS. (B) Densitometry of NF- κ B-DNA binding activity. Values are expressed as means $\pm$ SE (n = 4). Exposure to ETS significantly decreased NF- κ B-DNA binding activity. *p < 0.05, compared with FA control. (C) Supershift assay of NF- κ B p65, p50, and c-Rel. A supershift band for p65 as well as a weak supershift band for p50 were noted in the NF- κ B-DNA complex.

Necropsy and Tissue Sampling

An overdose of pentobarbital (60 mg/kg) was administered by intravenous injection to each infant the day after the termination of ETS exposure. The superior and inferior lobes of the right lung were removed and frozen. The left lung (superior and inferior lobes) and the right middle lobe were fixed by airway infusion with 1% or 4% paraformaldehyde and stored in fixative. Tissue samples from each fixed lung lobe were embedded in paraffin, sectioned, and stained to examine airways and parenchyma.

Western Blot Analysis

Cytosolic and nuclear proteins were extracted and processed from frozen tissue samples containing both airways and lung parenchyma for Western blot analysis as previously described (24).

Quantitative Real-Time RT-PCR

Total RNA was isolated from lung tissues, and cDNA synthesis was performed. The levels of Bcl-2, XIAP, and GAPDH mRNA were quantitated with a LightCycler Instrument (Roche Diagnostics, Indianapolis, IN) (25). The primer sequence for each gene were the following: Bcl-2, sense 5'-ATG TGT GTG GAG AGC GTC AA-3' and antisense 5'-CAA AGG CAT CCC AGC CTC-3'; XIAP, sense 5'-GGT ATC CAG GGT GCA AAT ATC TG-3' and antisense 5'-CAG TAG TTC TTA CCA GAC ACT CCT CA-3'; GAPDH, sense 5'-GTC AAC GGA TTT GGT CGT ATT G-3' and antisense 5'-AAT TTG CCA TGG GTG GAA T-3'. The results were expressed as mean $\pm$ SE.

Electrophoretic Mobility Shift Assay

Nuclear proteins were used to determine NF- κ B, AP-1, and p53-DNA binding activity by electrophoretic mobility shift assay as previously described (24). Supershift assay was performed for NF- κ B p65, p50, and c-Rel subunits.

TUNEL Assay

In situ apoptosis detection was done in tissue sections using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) according to the manufacturer's instructions. Counting of TUNEL-positive cells was determined for both airways and lung parenchyma.

Statistical Analysis

Statistical analyses were performed using Statview statistical software (SAS Institute, Cary, NC). All data were presented as mean $\pm$ SE. Comparisons between ETS-exposed and filtered air-exposed groups were made by Student's *t* test. A value of p < 0.05 was considered significantly different.

RESULTS

Effect of ETS on NF- κ B Activity

NF- κ B is the major anti-apoptotic transcription factor whose activity is critical for cell survival. To examine the effect of ETS exposure on NF- κ B activity during perinatal development, we measured NF- κ B-DNA binding activity in the nuclear extract of lung tissues of infant monkeys using an electrophoretic mobility shift assay. Perinatal exposure to ETS significantly decreased NF- κ B-DNA binding activity in these animals (p < 0.05) (Figures 1A and 1B). To identify the subunits presented in the NF- κ B-DNA binding complex, supershift assay was performed. As shown in Figure 1C, a supershift for NF- κ B p65 was observed, indicating the presence of p65 in the complex. A weak supershift band was also noted for p50. In contrast, c-Rel was not observed in the supershift assay.

Effect of ETS on NF- κ B Activation Pathway

To better understand the underlying mechanism by which ETS inhibits NF- κ B activity, the NF- κ B activation pathway was examined. Figures 2A and 2B show that the cytoplasmic level of the inhibitory protein, I κ B α , was significantly elevated in infant monkeys exposed to ETS. The levels of phosphorylated I κ B α as well as phosphorylated IKK α / β were significantly decreased by ETS exposure. No significant change was observed for the level of I κ B β . In agreement with NF- κ B-DNA binding activity, these data clearly indicate the inhibitory effects of ETS on IKK activity, I κ B α phosphorylation and degradation, as well as NF- κ B activation.

Because of their molecular chaperone property, heat shock proteins (HSP), HSP70 in particular, can exert an inhibitory effect on NF- κ B as a partial substitute for I κ B. Therefore, we also

Figure 2. Effect of ETS on NF- κ B activation pathway. (A) Western blot analysis of I κ B α , I κ B β , phospho-I κ B α , phospho-IKK α , and phospho-IKK β . Lanes 1–4, FA; lanes 5–8, ETS. (B) Densitometry of Western blot for I κ B α , I κ B β , phospho-I κ B α , phospho-IKK α , and phospho-IKK β . There was a significant increase of I κ B α , as well as significant decreases of phospho-I κ B α , phospho-IKK α , and phospho-IKK β in infant monkeys exposed to ETS (filled bars). Open bars, FA. No significant change was observed for I κ B β . (C) Western blot analysis of HSP 70. (D) Densitometry of Western blot for HSP 70. Values are presented as means $\pm$ SE (n = 4). There was a significant increase of HSP70 in ETS-exposed infant monkeys. *p < 0.05, compared with FA control.

examined the effect of ETS on HSP70 induction. Our results clearly show the induction of HSP70 after ETS exposure. The level of HSP70 was significantly increased by 142% over the control value in ETS exposed infant monkeys (Figures 2C and 2D).

Effect of ETS on NF- κ B-Regulated Anti-Apoptotic Gene Expression

To explore the role of NF- κ B activity in the regulation of the apoptotic process, NF- κ B-dependent anti-apoptotic gene expression was measured using quantitative real-time RT-PCR. As expected, the expression of Bcl-2 and XIAP mRNA were significantly decreased after ETS exposure (Figure 3). Expression of NF- κ B-dependent anti-apoptotic gene products were also measured by Western blot analysis. Figure 4 shows that ETS significantly decreased the protein levels of Bcl-2, Bcl-xl, TRAF1, TRAF2, and XIAP by 55, 69, 43, 52, and 85%, respectively. The level of c-IAP1 was also reduced (by 29%) after ETS exposure, although this reduction was not statistically significant.

Effects of ETS on JNK/p38 MAPK/AP-1, p53, and Akt Activation

To examine the response of other apoptosis-related signal pathways after ETS exposure, the activation status of JNK/p38MAPK/AP-1, p53, and Akt were determined. Figure 5 shows that no significant changes were observed for the levels of phosphorylated (activated) JNK and p38 MAPK with ETS exposure. No significant changes were also noted for AP-1-DNA binding

activity as well as the expression of AP-1 target gene FasL. Similarly, the levels of p53, p53-DNA binding activity, and Bax expression were found not to be altered by ETS exposure (Figure 6). ETS exposure was also found to not change the level of phosphorylated Akt (data not shown).

Figure 3. Effect of ETS on the expression of Bcl-2 and XIAP mRNA. Quantitative detection of Bcl-2 and XIAP mRNA was performed using real time RT-PCR. Values for Bcl-2 and XIAP mRNA expression were normalized to the expression of GAPDH. Data are expressed as means $\pm$ SE (n = 4). Exposure to ETS (filled bars) significantly decreased the expression of Bcl-2 and XIAP mRNA. *p < 0.05, **p < 0.01, compared with FA control (open bars).

Figure 4. Effect of ETS on the expression of NF- κ B-dependent anti-apoptotic proteins. (A) Western blot analysis of Bcl-2, Bcl-xl, TRAF 1, TRAF 2, c-IAP1, and XIAP in the lungs of infant monkeys. Lanes 1–4, FA; lanes 5–8, ETS. (B) Densitometry of Western blot. Values are presented as means $\pm$ SE ($n = 4$). Exposure to ETS (filled bars) significantly decreased the expression of Bcl-2, Bcl-xl, TRAF1, TRAF2, and XIAP proteins. * $p < 0.05$, ** $p < 0.01$, compared with FA control (open bars).

Figure 5. Effect of ETS on JNK/p38/c-Jun/FasL pathway. (A) Western blot analysis of phospho-JNK, phospho-p38, and FasL. Lanes 1–4, FA; lanes 5–8, ETS. (B) Densitometry of Western blot. (C) Electrophoretic mobility shift assay of AP-1-DNA binding activity. Lane 1, competition assay; lanes 2–5, FA; lanes 6–9, ETS. (D) Densitometry of electrophoretic mobility shift assay. Values are presented as means $\pm$ SE ($n = 4$). No significant changes were noted for phospho-JNK, phospho-p38, FasL, or AP-1-DNA binding activity between FA control (open bars) and ETS (filled bars) groups.

Figure 6. Effect of ETS on p53 and Bax. (A) Western blot analysis of p53 and Bax in the lungs of infant monkeys. Lanes 1–4, FA; lanes 5–8, ETS. (B) Densitometry of Western blot. (C) Electrophoretic mobility shift assay of p53-DNA binding activity. Lane 1, competition assay; lanes 2–5, FA; lanes 6–9, ETS. (D) Densitometry of electrophoretic mobility shift assay. Values are presented as means $\pm$ SE ($n = 4$). No significant changes were noted for p53, Bax, or p53-DNA binding activity between FA control (open bars) and ETS (filled bars) groups.

Figure 7. Effects of ETS on caspase activation. (A) Western blot analysis of caspase 8, 9, and 3 in the lungs of infant monkeys. Lanes 1–4, FA; lanes 5–8, ETS. (B) Densitometry of Western blot. Values are presented as means $\pm$ SE ($n = 4$). There were significant increases of cleaved caspase 8, 9, and 3 in ETS-exposed infant monkeys (filled bars). * $p < 0.05$, ** $p < 0.01$, compared with FA control (open bars).

Effect of ETS on Caspase Activation

Apoptosis is orchestrated by the activation of the caspase cascade. To examine the effect of ETS on caspase activation, the initiator caspases (caspases 8 and 9) and the key effector caspase of the apoptotic machinery (caspase 3) were assessed by Western blot analysis. ETS exposure resulted in enhanced levels of cleaved caspase 8, 9, and 3, indicating the activation of these caspases. Compared with the filtered air control animals, infant monkeys exposed to ETS had the cleavage products of caspase 8, 9, and 3 increased by 171, 82, and 168%, respectively (Figure 7).

Effect of ETS on Caspase Substrates

Activation of the effector caspase cleaves a distinct subset of cellular substrates and eventually induces apoptotic changes. Among the major targets of caspase 3 are poly (ADP)-ribose polymerase (PARP) and DNA fragmentation factor (DFF). It was observed that the cleaved PARP, a nuclear protein cleaved by caspase to an 89-kD inactive form, was elevated after ETS exposure. The nuclear levels of caspase-activated DNase (CAD), or the DNA fragmentation factor 40 (DFF 40), was also increased in infant monkeys exposed to ETS (Figure 8). These results indicate ETS-induced caspase-mediated cleavage of the key cellular death substrates.

Effect of ETS on Apoptosis

To determine the effect of ETS on apoptosis within the lungs of infant monkeys, a TUNEL assay was used to localize and

quantify the apoptotic cells (Figure 9). In line with the changes in NF- κ B activity and apoptotic cascades, ETS induced increased apoptosis in the lung parenchyma of the infant monkeys (Figure 10). The majority of the apoptotic cells were alveolar epithelial and interstitial cells. Alveolar macrophages were also among the apoptotic cells observed. No significant increase of apoptotic cells was observed in the airways after ETS exposure.

DISCUSSION

ETS exposure during the developmental period has adverse effects on lung growth and function (5–10). It is well known that apoptosis plays an essential role in development and tissue homeostasis (11). Disruption of the normal pattern of apoptosis is implicated in many pathologic conditions, including developmental defects. There is evidence that maternal passive smoking results in enhanced apoptosis in the lung tissues of newborns (14). However, the mechanisms by which ETS induces apoptosis are unclear. In the present study, we clearly show that perinatal exposure to ETS suppressed the NF- κ B activation pathway and NF- κ B activity in the lungs of infant monkeys. The expression of NF- κ B-dependent anti-apoptotic genes, including Bcl-2, Bcl-xl, TRAF-1, TRAF-2, and XIAP, were down-regulated, which paralleled activation of the initiator caspases 8 and 9 and the effector caspase 3, as well as enhanced cleavage products of the key cellular death substrates and increased apoptosis within the lungs. No alterations were observed for other apoptosis-related signal pathways, including AP-1, p53, and Akt. These data indicate the role of NF- κ B activity in ETS-induced apoptosis in

Figure 8. Effect of ETS on cleaved PARP and CAD. (A) Western blot analysis of cleaved PARP and CAD in the nucleus of the lungs of infant monkeys. Lanes 1–4, FA; lanes 5–8, ETS. (B) Densitometry of Western blot. Values are presented as means $\pm$ SE ($n = 4$). The levels of cleaved PARP and CAD were significantly increased in infant monkeys exposed to ETS (filled bars). * $p < 0.05$, compared with FA control (open bars).

Figure 9. Effect of ETS on apoptosis in the lungs. TUNEL labeling of infant monkey lungs in airways (A and B) and lung parenchyma (C and D) for FA (A and C) and ETS (B and D). TUNEL-positive cells were identified as darkly stained cells, as indicated by arrows.

infant lungs. Based on these findings, a model of NF- κ B signaling in ETS-induced apoptotic process during lung development has been proposed (Figure 11).

The relevance of the changed apoptotic process in this study has to do with lung development; a significant abnormal elevation of apoptosis within the lung parenchyma during a critical period of lung development may have far-reaching consequences. We observed that epithelial and interstitial cells of the alveolar septa are the primary resident cells undergoing ETS-induced apoptosis (Figures 9 and 10). The formation and remodeling of new alveoli are processes driven at the cellular level and, therefore, could be interrupted and/or impaired due to ETS-induced apoptosis within the lung parenchyma. An increase in apoptosis is highly suggestive of altered lung growth, but further studies would be required for confirmation, especially in regards to whether such changes at this point in neonatal life would result in long-term, marked morphologic alterations in alveolar structure. This is an area of research that we plan to address in future experiments.

The cell signal pathways that regulate the apoptotic process have been investigated in the present study to elucidate the mechanisms of ETS-induced apoptosis. Previous studies have demonstrated the crucial role of NF- κ B activity in cell survival (16, 26–31). NF- κ B exerts its anti-apoptotic function through the transcription of a number of anti-apoptotic genes. Members of the Bcl-2 family (Bcl-2, Bcl-xl, Bfl-1/A1), IAP family (c-IAP1, c-IAP2, XIAP), and TRAF1/2 are among NF- κ B-dependent anti-apoptotic genes (17–19). The Bcl-2 family determines the release of apoptotic factors from mitochondria and the activation of caspase 9 (32, 33). c-IAPs directly bind and inhibit effector caspases such as caspase 3, as well as prevent activation of procaspase 9. In addition, c-IAPs can associate with TNFR1 signaling complex by interacting with TRAF2 and provide sufficient prox-

imity for inhibition of caspase 8, the initiator caspase in the death receptor apoptotic pathway (17, 34). Among the members of the IAP family, XIAP has the highest anti-apoptotic potency (35). TRAF1 and TRAF2 are adaptor proteins required for optimal NF- κ B activation, and their anti-apoptotic activity is most likely due to their ability to augment the activation of NF- κ B (17, 36). Down-regulation of anti-apoptotic proteins disrupts the balance between the pro- and anti-apoptotic factors, resulting in activation of caspases 8, 9, and 3. Activated caspase 3 cleaves a distinct subset of cellular death substrates such as PARP and DFF, inducing apoptotic changes (37–42). Results from this study show that perinatal ETS exposure suppressed NF- κ B activity and its dependent anti-apoptotic gene expression, leading to apoptotic changes. To our knowledge, this is the first report to reveal the role of NF- κ B activity in ETS-induced apoptotic process during lung development.

To date, several reports have found NF- κ B inhibitory effects of tobacco smoke or tobacco smoke components (43–47), but the underlying mechanisms remained unclear. In our study, examination of the NF- κ B activation pathway showed that ETS decreased the levels of phosphorylated IKK α / β , suggesting inhibition of these kinases. Since IKK activity is essential for I κ B α phosphorylation and subsequent degradation, ETS-induced suppression of IKK resulted in accumulation of I κ B α , as observed in Figure 2. These results provide a plausible mechanism for the inhibitory effect of ETS on NF- κ B activity. The components of ETS responsible for the inhibitory effect on NF- κ B are not clear at this moment. Factors worth consideration are reactive oxygen species (ROS), which are present at high levels in ETS. ROS have been viewed previously as inducers of NF- κ B activation (48, 49). However, recent evidence has also shown ROS are not activators, but rather, inhibitors for NF- κ B (50–52). ROS may oxidize NF- κ B subunits and, thus, impair their DNA binding activity and transcriptional activity (53, 54). In addition, ROS induced oxidative modification of a redox sensitive cysteine residue in IKK α and IKK β results in inactivation of IKK (51, 55, 56). Other possible candidates of ETS components that contribute to NF- κ B inhibitory effects are hydroquinone, aldehydes (e.g., acrolein), and nicotine (44–46, 57–59). Furthermore, previous studies have shown tobacco smoke activates heat shock transcription factor (HSF) and expression of heat shock proteins (HSP), HSP 70 in particular, which could interact with NF- κ B as a partial substitute for I κ B and contribute to stress-induced NF- κ B suppression (60–66). In line with these observations, we also noted coordinate induction of HSP70 and inhibition of

Figure 10. Apoptotic index (number of TUNEL-positive cells per total numbers of cells). Values are presented as means $\pm$ SE ($n = 4$). Exposure to ETS significantly increased apoptosis in the parenchyma of infant monkeys. * $p < 0.05$, compared with filtered air control.

Figure 11. Proposed model of NF- κ B signaling in ETS-induced apoptotic process in the lungs of infant monkeys. Through suppression of IKK activation and I κ B α degradation, as well as induction of HSP 70, ETS inhibits NF- κ B activity and downregulates the expression of NF- κ B-dependent anti-apoptotic genes, including members of Bcl-2, TRAFs, and c-IAPs, resulting in activation of initiator caspases and effector caspase. The effector caspase 3 cleaves the key death substrates such as PARP and ICAD/CAD, leading to the apoptotic process.

NF- κ B after ETS exposure. These data may provide an additional inhibitory mechanism of ETS on NF- κ B activity.

Since the apoptotic process may involve several signal pathways, we also examined the alteration of activator protein-1 (AP-1) and p53, two other transcription factors. The JNK/p38 MAPK/AP-1 pathway responds to oxidative stress and inflammatory cytokines and participates in the regulation of the apoptotic process by transcribing apoptosis regulators such as FasL (67–70). Examination of JNK and p38 activation, AP-1 DNA-binding activity, and FasL expression showed no significant changes in the lungs of infant monkeys after ETS exposure. Similarly, no significant changes were observed for p53 expression, p53–DNA binding activity, and p53 regulated pro-apoptotic factor Bax expression. No alteration was also observed for the activation of Akt. These results could preclude the involvement of these pathways in ETS-induced apoptotic process under our experimental conditions.

Although we have revealed the effects of ETS exposure on the NF- κ B signal pathway and its role in the apoptotic process, there are limitations to the present study. Due to the high cost and ethical issues inherent to animal studies involving nonhuman primates and complex long-term inhalation exposures, the number of animals per group was relatively small compared with other animal studies. This study only investigated the adverse effects of perinatal ETS exposure in which we did not address the separate effects of prenatal exposure and postnatal exposure. Lastly, although it was a work week–related strategy to expose the animals five days per week in this study, the impact of two day’s “rest” per week on the measured outcome is unknown. However, it is likely that a two-day-per-week “rest” exposure

regimen could actually underestimate the effects of ETS on the developing lung in real-world conditions.

In summary, we have shown for the first time that exposure to ETS during the perinatal period inhibits NF- κ B activity and down-regulates NF- κ B–dependent anti-apoptotic gene expression, resulting in activation of the initiator caspases as well as the effector caspase, leading to cleavage of the key death substrates and increased apoptosis in the lungs of infant monkeys (Figure 11). These data mechanistically link NF- κ B signaling and the apoptotic process. Moreover, our findings suggest that exposure to low levels of ETS during critical windows adversely affects the developing lung in the neonatal nonhuman primate with potential implications to affect long-term lung growth and function.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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