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NF- κ B Inhibition is Involved in Tobacco Smoke-Induced Apoptosis in the Lungs of Rats

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Abstract

Apoptosis is a vital mechanism for the regulation of cell turnover and plays a critical role in tissue homeostasis and development of many disease processes. Previous studies have demonstrated the apoptotic effect of tobacco smoke; however, the molecular mechanisms by which tobacco smoke triggers apoptosis remain unclear. In the present study we investigated the effects of tobacco smoke on the induction of apoptosis in the lungs of rats and modulation of nuclear factor-kappa B (NF- κ B) in this process. Exposure of rats to 80 mg/m³ tobacco smoke significantly induced apoptosis in the lungs. Tobacco smoke resulted in inhibition of NF- κ B activity, noted by suppression of inhibitor of κ B (I κ B) kinase (IKK), accumulation of I κ B α , decrease of NF- κ B DNA binding activity, and downregulation of NF- κ B - dependent anti-apoptotic proteins, including Bcl-2, Bcl-xl, and inhibitors of apoptosis. Initiator caspases for the death receptor pathway (caspase 8) and the mitochondrial pathway (caspase 9) as well as effector caspase 3 were activated following tobacco smoke exposure. Tobacco smoke exposure did not alter the levels of p53 and Bax proteins. These findings suggest the role of NF- κ B pathway in tobacco smoke-induced apoptosis.

Keywords

Nuclear factor-kappa B (NF- κ B); Apoptosis; Tobacco smoke; Lung

Introduction

Tobacco smoke is a proven etiologic factor in the development of various lung diseases, such as lung cancer and chronic obstructive pulmonary disease (WHO, 2002), that have an impairment in the balance between cell growth and cell death. Apoptosis is a genetically regulated physiologic program that deletes harmful or unwanted cells during development, tumorigenesis and immune response. Apoptosis also occurs under pathological situations such as growth factor withdrawal, stress stimuli and DNA damage. It represents one of the most important mechanisms for tissue homeostasis and development of many disease processes (Thompson, 1995). The morphological and biochemical characteristics of apoptosis include cell shrinkage, chromatin condensation, internucleosomal DNA fragmentation and formation of apoptotic bodies (Budihardjo, et al, 1999; Hengartner, 2000).

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Tobacco smoke induces apoptosis both *in vivo* and *in vitro* (D'Agostini, et al, 2001; Vayssier, et al, 2001; Wang, et al, 2000; Rajpurkar, et al, 2002). However, the molecular mechanisms by which tobacco smoke triggers apoptosis remain unclear. Tobacco smoke is a complex chemical mixture containing over 4,000 different compounds, including large quantities of oxidants and free radicals that induce oxidative stress and apoptosis (Pryor and Stone, 1993; Chow, 1993; Carnevali, et al, 2003; Buttke and Sandstrom, 1995; Tan, et al, 1998; Aoshiba, et al, 2001; Banzet, 1999). Moreover, in response to oxidative stress, lung cells release proinflammatory cytokines, such as tumor necrosis factor (TNF)- α , that are able to produce an inflammatory response as well as apoptosis. Due to the reactivity of tobacco smoke and the complexity of the cellular response to noxious stimuli, tobacco smoke-induced apoptosis likely involves cellular signal pathways that govern the responses to the stimuli and the expression of apoptosis regulating factors. Elucidation of these signal pathways is critical to shed light into the molecular mechanisms of tobacco smoke-associated pathogenesis.

Nuclear factor-kappa B (NF- κ B) is the major anti-apoptosis transcription factor that is vital for cell survival (Beg and Baltimore, 1996; Li, et al, 1999; Van Antwerp, et al, 1996; Wang, et al, 1999; Wang, et al, 1998). NF- κ B regulates the expression of a number of anti-apoptotic genes, such as cellular inhibitors of apoptosis (c-IAP) and Bcl-2 family members (e.g., Bcl-2, Bcl-xl) (Wang, et al, 1999; Wang, et al, 1998; Chu, et al 1997; Lee, et al, 1999). 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 (Barnes and Karin, 1997; Karin and Ben-Neriah, 2000). Previous studies have shown that NF- κ B signaling can be modulated by oxidative stress and inflammatory cytokines - factors that are contained in or produced by tobacco smoke. To date, however, little information is available with respect to the role of NF- κ B pathway in tobacco smoke-induced apoptosis, although some controversial *in vitro* studies for the effects of tobacco smoke on NF- κ B activity alone have been reported (Vayssier, et al, 1998; Moodie, et al, 2004; Sugano, et al, 1998; Gebel and Muller, 2001; Shishodia and Aggarwal, 2004; Ahn, et al, 2007).

The aim of the present study was to investigate the effects of tobacco smoke on apoptosis induction and NF- κ B signaling modulation with the goal of understanding tobacco smoke-associated disease pathogenesis. We found that tobacco smoke induced caspase activation and apoptosis in the lungs of rats exposed to tobacco smoke. We further demonstrated that tobacco smoke suppressed NF- κ B activation and NF- κ B-regulated anti-apoptotic protein expression. These findings suggest that modulation of NF- κ B activity and its dependent apoptotic regulator are involved in tobacco smoke-induced apoptosis.

Methods

Chemicals and reagents

An *in situ* cell death detection kit was purchased from Roche Applied Science (Indianapolis, IN). A diaminobenzide (DAB) substrate kit was obtained from Zymed Laboratories (South San Francisco, CA). [γ -³²P]dATP was purchased from Amersham Pharma Biotech (Piscataway, NJ). NF- κ B consensus oligonucleotides and T4 polynucleotide kinase were bought from Promega (Madison, WI). Antibodies to NF- κ B p65, p50, I κ B α , I κ B β , HSP70, Bcl-2, Bcl-xl, c-IAP1, c-IAP2, XIAP, p53, Bax, caspase 8, caspase 9, caspase 3 and actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to phospho- IKK α / β came from Cell Signaling Technology (Beverly, MA).

Animals

Twelve-week-old male spontaneously hypertensive (SH) rats (derived from Wistar-Kyoto rats by phenotypic segregation of the hypertensive trait and inbreeding) weighing 260~310 g were purchased from Charles River Laboratories (Raleigh, NC). Selection of this strain of rat was based on previous studies done in our laboratory that demonstrated that these rats are highly sensitive to the pulmonary effects of tobacco smoke exposure (Smith, et al, 2002; Zhong, et al, 2005). All rats were allowed to acclimate for one week prior to the onset of experimental exposure. The rats were housed in polypropylene cages, maintained on a 12-hour light/dark cycle, and provided water and rat chow *ad libitum*. Animals were handled in accordance with standards established by the U.S. Animal Welfare Acts as set forth in the National Institutes of Health Guidelines and by the University of California, Davis, Animal Care and Use Committee.

Tobacco smoke exposure

Rats were exposed to a mixture of sidestream and mainstream cigarette smoke in a smoking apparatus built in our laboratory (Zhong, et al, 2005). The cigarettes were humidified 1R4F research cigarettes (Tobacco Health Research Institute, Lexington, KY). An automatic metered puffer was used to smoke the cigarettes under Federal Trade Commission conditions (35 ml puff, 2 second duration, 1 puff per minute). The smoke was collected in a chimney, diluted with filtered air, and delivered to whole-body exposure chambers. The exposures were characterized for the three major constituents of cigarette smoke, including nicotine, carbon monoxide, and total suspended particulates (TSP). Animals were exposed for 6 hours/day, 3 days/week for a total of 14 weeks. This exposure regimen was selected based on earlier studies that demonstrated rats better tolerated exposure to a high level of particulate matter smoke for prolonged periods of time with exposure being 3 days/week rather than 5 days/week. Carbon monoxide was measured every 30 minutes, TSP every 2 hours, and nicotine per day (approximately midway through the exposure period). Experiments consisted of animals exposed to filtered air (FA, control) only, a low concentration of tobacco smoke (30 mg/m³) and a high concentration of tobacco smoke (80 mg/m³). Tobacco smoke characteristics for the 30 mg/m³ exposure were the following: carbon monoxide (129 ± 7 ppm), TSP (31 ± 2 mg/m³), and nicotine (1.5 ± 0.5 mg/m³). Tobacco smoke characteristics for the 80 mg/m³ exposure were the following: carbon monoxide (217 ± 24 ppm), TSP (81 ± 9 mg/m³), and nicotine (8.3 ± 3.3 mg/m³).

Tissue Preparation

Animals were anesthetized with an overdose of sodium pentobarbital. The trachea was cannulated and the chest cavity opened by a midline incision. The right lung was frozen in liquid nitrogen and stored at -80°C until use. The left lung was inflation fixed by intratracheal instillation of 4% buffered zinc formalin (Z-Fix) at 30 cm of water pressure for 1 hour and stored in 70% ethanol before processing. Transverse slices were cut immediately cranial and caudal to the hilus of the left lung, dehydrated in a graded ethanol series, and embedded in paraffin for use in *in situ* apoptosis detection.

This tissue sampling strategy allowed for examination of the major axial airway path from the second to fourth generations of intrapulmonary airways, distal airways as well as the surrounding lung parenchyma. Tissue sections were prepared using a Sorvall HM 355 rotary microtome (Carl Zeiss, Inc., Thornwood, NY). Sections 5 µm thick were placed on Superfrost Plus glass slides.

Preparation of lung cytosolic and nuclear protein extract

The method for preparing lung cytosolic and nuclear protein extracts has been previously described (Zhou, et al, 2003; Zhong, et al, 2006). Briefly, immediately following deep anesthesia of the rats, the right lung was removed, frozen in liquid nitrogen, and stored in -80°C . The lungs were homogenized in ice-cold Tris-HCl buffer (25 mM Tris, 1 mM EDTA, 10% glycerol, and 1 mM DTT, pH 7.4) with a glass homogenizer. The homogenate was centrifuged at 10,000g for 20 minutes at 4°C . The supernatant containing cytosolic protein was aliquoted and stored at -80°C . The crude nuclear fraction in the pellets was washed three times with homogenate buffer containing Triton X-100, followed by washing one time without Triton X-100. Nuclear protein was extracted with buffer C (20 mM HEPES, 25% glycerol, 0.42 M NaCl, and 1mM EDTA) by centrifugation at 50,000g for 30 minutes. Protein concentration was measured by a modified Bradford assay according to the manufacture's instruction (Bio-Rad, Hercules, CA) with bovine serum albumin used as a standard.

TUNEL assay

Tissue sections were stained with hemotoxylin and eosin for morphological examination. Structures examined included the major axial airway, distal airways and lung parenchyma. In situ apoptosis detection was accomplished with terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) according to the manufacturer's instructions. Tissue sections were deparaffinized in xylene and hydrated in a graded ethanol series. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 30 minutes at room temperature. Following nonspecific blocking, sections were incubated with TUNEL reaction mixture for 1 hour at 37°C . After washing, sections were subsequently incubated with converter-POD for 30 minutes. Diaminobenzidine (DAB) was used as the chromogen substrate, and sections were counter-stained with nuclear fast red. DNase I was used as a positive control to induce DNA strand breakage prior to the TUNEL labeling procedure. Negative control for TUNEL labeling consisted of omission of the transferase enzyme. For quantification of TUNEL signals, apoptotic index, expressed as the percentage of apoptotic nuclei of the total number of nuclei counted, was determined for the main axial airway path, distal airways down to the terminal bronchioles, and the lung parenchyma. Terminal bronchioles were defined as the last conducting airway to an alveolar duct. Examination of the lung parenchyma was done by selecting nonoverlapping fields initiated by random start and subsequently stage movement across parenchymal tissue in a zigzag pattern. In each region, 500 to 1,000 cells per lung were counted.

Western Blot Analysis

Western blot analyses were used for the measurement of the protein levels of NF- κB p65, p50, I $\kappa\text{B}\alpha$, I $\kappa\text{B}\beta$, HSP70, Bcl-2, Bcl-x1, c-IAP1, c-IAP2, XIAP, p53, Bax, phospho-IKK α/β caspase 8, caspase 9 and caspase 3. Fifty μg cytosolic or nuclear proteins were loaded and separated on 10–12% SDS-PAGE, followed by transblotting to an ImmunBlot PVDF membrane (Bio-Rad, Hercules, CA). The membrane was subsequently probed with primary antibody at a dilution of 1:1000. Horseradish peroxidase-conjugated secondary antibody was added at a 1:3000 dilution. The blots were subsequently developed using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Piscataway, NJ). Following exposure on an autoradiography film, immunoreactive protein bands were quantified by densitometry. β -actin was used as a loading control.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed to determine NF- κB –DNA binding activity. Double-stranded DNA containing NF- κB consensus sequence labeled with [γ - ^{32}P]dATP using T4 polynucleotide kinase was used as probes. The binding reaction of nuclear proteins to the probe was assessed

by incubating mixtures containing 5 µg nuclear protein, 0.5 µg poly (dI•dC) and 40,000 cpm ³²P- labeled probe in the binding buffer (7.5mM HEPES, pH 7.6, 35mM NaCl, 1.5mM MgCl₂, 0.05mM EDTA, 1mM DTT, and 7.5% glycerol) for 30 minutes at 25°C. For the competitive assay, excessive unlabeled oligonucleotides were incubated with proteins prior to the addition of radiolabeled probe. Protein-DNA binding complex was separated by 5% polyacrylamide gel electrophoresis and autoradiographed overnight.

Statistical Analysis

Statistical analyses were performed using Statview statistical software (SAS Institute, Cary, NC). All data are presented as mean± SE. Comparisons among tobacco smoke-exposed and FA-exposed groups were made by ANOVA followed by Fisher's protected least significant difference post hoc multiple comparisons. A value of p<0.05 was considered significantly different.

Results

Effect of tobacco smoke on the induction of apoptosis

A TUNEL assay was used for localization and quantification of apoptotic cells within the main axial airway path, distal airways and lung parenchyma of rat lung to examine the effect of tobacco smoke on the induction of apoptosis. These studies demonstrated the induction of apoptosis in rat lungs following 14 weeks of tobacco smoke exposure (Fig. 1). The majority of apoptotic cells in the main airway path were epithelial cells, especially suprabasal epithelial cells in areas of squamous metaplasia (Fig. 1A and 1B). In distal airways, the apoptotic cells were primarily epithelial cells. In lung parenchyma, the apoptotic cells included epithelial cells and alveolar macrophages. The apoptotic indices in the main axial airway path and distal airway were significantly higher for rats exposed to 80 mg/m³ tobacco smoke compared to rats exposed to FA (Fig. 1C). The apoptotic index in lung parenchyma was not increased following tobacco smoke exposure, and the apoptotic index was not statistically different between rats exposed to 30 mg/m³ tobacco smoke and rats exposed to FA (Fig. 1C).

Effect of tobacco smoke on NF-κB activity

NF-κB is the key transcription factor in the regulation of apoptosis. To examine the effect of tobacco smoke exposure on NF-κB activity, EMSA was performed to measure NF-κB -DNA binding activity in the nuclear extracts of lung tissues. As shown in Fig. 2, exposure to 30 mg/m³ tobacco smoke did not change NF-κB -DNA binding activity significantly within the lung tissues compared to FA exposure. In contrast, exposure of animals to 80 mg/m³ tobacco smoke resulted in a significant decrease of NF-κB -DNA binding activity. In addition to NF-κB -DNA binding activity, the level of NF-κB subunits, including p65 and p50 in the nucleus, were measure by Western blot analyses. Consistent with the EMSA results, the nuclear protein levels of both NF-κB p65 and p50 were significantly reduced in the lungs of rats exposed to 80 mg/m³ with respect to FA control animals (Fig. 3). There was no difference between rats exposed to 30 mg/m³ and FA control animals.

Effect of tobacco smoke on NF-κB activation pathway

In order to better understand the underlying mechanisms of tobacco smoke on NF-κB activity, the NF-κB activation pathway was examined. Fig. 4B demonstrates that the levels of the inhibitor of NF-κB (IκB)-α in cytoplasm were significantly elevated in rats exposed to 80 mg/m³ tobacco smoke, whereas the cytoplasmic levels of IκB-β was not changed by tobacco smoke exposure, regardless of concentration. Examination of IκB kinases (IKK) revealed that 80 mg/m³ of tobacco smoke suppressed the levels of IKKα and IKKβ phosphorylation (activation).

No significant changes were observed for the levels of IKK α and IKK β activation from rats exposed to 30 mg/m³ tobacco smoke.

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, the effect of tobacco smoke on HSP70 induction was examined. The level of HSP70 was significantly greater (152%) in animals exposed to 80 mg/m³ tobacco smoke compared to animals exposed to FA (Fig. 4D).

Effect of tobacco smoke on NF- κ B –regulated anti-apoptotic protein expression

To further explore the role of NF- κ B in tobacco smoke-induced apoptosis, the expression of NF- κ B –regulated anti-apoptotic proteins Bcl-2, Bcl-xl, c-IAP1, c-IAP2, and XIAP were measured. In line with the NF- κ B activity described above, exposure to 80 mg/m³ tobacco smoke significantly down regulated the expression levels of Bcl-2, Bcl-xl, c-IAP1, c-IAP2 and XIAP by 49.7%, 67%, 75.9%, 57.1%, and 63.2%, respectively (Fig. 5B). In contrast, exposure to 30 mg/m³ tobacco smoke did not significantly alter the expression of these apoptotic regulators.

Effect of tobacco smoke on p53, and Bax expression

p53 is a well-known apoptosis regulator that controls the transcription of pro-apoptotic factors such as Bax. To explore the possible role of p53 in tobacco smoke induced apoptotic process, the effect of tobacco smoke on p53 and its regulated pro-apoptotic protein, Bax, was also examined. No significant alterations were observed for p53 or Bax expression in the lungs of rats exposed to tobacco smoke (Fig. 6B).

Effect of tobacco smoke on caspase activation

Apoptosis is executed through the activation of caspases. To further verify the effect of tobacco smoke in the induction of apoptosis, the levels of cleaved caspases 8, 9, and 3 in rat lungs were determined. In agreement with the apoptotic results described above, exposure of rats to 80 mg/m³ tobacco smoke resulted in significantly increased levels of cleaved caspases 8, 9, and 3, indicating the activation of these caspases (Fig. 7B). Compared to FA control animals, the cleaved caspases 8, 9, and 3 were elevated by 98.1%, 113.9%, and 84.7 %, respectively. Exposure of 30 mg/m³ tobacco smoke did not result in caspase activation.

Discussion

In the present study we demonstrated that exposure of rats to 80 mg/m³ tobacco smoke for 14 weeks induced caspase activation and induction of apoptosis in rat lungs. These effects paralleled suppression of NF- κ B DNA binding activity and downregulation of NF- κ B-dependent anti-apoptotic proteins including Bcl-2, Bcl-xl, c-IAP1, c-IAP2 and XIAP. These data indicate the involvement of NF- κ B signal pathway in tobacco smoke-induced apoptosis. We also examined a lower concentration of tobacco smoke (30 mg/m³) to determine if a dose response was present. We found that this lower concentration did not have any inductive effect for the pathways examined.

Apoptosis is executed via the activation of an extrinsic death receptors and/or intrinsic mitochondrial pathway(s) in response to various stimuli such as FasL, TNF, and oxidative stress (Budihardjo, et al, 1999; Hengartner, 2000). *In vivo* and *in vitro* studies have demonstrated that tobacco smoke induces apoptosis in various cells and tissues (D'Agostini, et al, 2001; Vayssier, et al, 2001; Wang, et al, 2000; Rajpurkar, et al, 2002). Consistent with these studies, we found that exposure of rats to tobacco smoke activated caspases 8, 9, and 3,

and induced apoptosis. Our data suggest that both the death receptor pathway and the mitochondrial pathway are activated in tobacco smoke-induced apoptosis.

To elucidate the mechanisms of tobacco smoke-induced apoptosis, the cell signal pathways regulating the apoptotic process have been investigated in the present study. Previous studies have demonstrated the crucial role of NF- κ B activity in anti-apoptosis and cell survival (Beg, et al, 1995; Beg and Baltimore, 1996; Horwitz, et al 1997; Li, et al, 1999; Li, et al 2000; Tanaka, et al, 1999; Li, et al, 1999; Udolph, et al, 2000; Van Antwitz, et al, 1996; Chaisson, et al, 2002).. The anti-apoptotic activity of NF- κ B depends on the regulation of gene expression. Multiple studies demonstrate that NF- κ B induces the expression of anti-apoptotic proteins. Members of the anti-apoptotic Bcl-2 family (Bcl-2, Bcl-xl, Bfl-1/A1) and cellular inhibitors of apoptosis (c-IAPs: c-IAP1, c-IAP2, XIAP) are among the NF- κ B –dependent anti-apoptotic proteins (Wang, et al 1999; Wang, et al, 1998; Chu, et al, 1997; Lee, et al, 1999). It is well established that the pro-apoptotic Bcl-2 members promote mitochondrial release of pro-apoptotic factors (e.g. cytochrome c), whereas the anti-apoptotic Bcl-2 member prevents this process. The balance between these two groups of Bcl-2 members determines the release of apoptotic factors from mitochondria and activation of caspase 9 (Hengartner, 2000). 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 the TNF receptor 1 signaling complex through an interaction with TNF receptor associated factor 2 (TRAF2), providing sufficient proximity for inhibition of caspase 8 (Wang, et al 1998, Deveraux, et al, 1998). Thus, c-IAPs can inhibit apoptosis induced by both the death receptor and mitochondria-dependent pathways. In agreement with these lines, we found in the present study that tobacco smoke suppressed NF- κ B activity with downregulation of Bcl-2, Bcl-xl, c-IAP1, c-IAP2 and XIAP, leading to activation of caspases 8, 9, and 3 and induction of apoptosis. Therefore, our data provide the evidence of the role of NF- κ B activity in TS-induced apoptosis.

Several previous *in vitro* studies have reported the NF- κ B inhibitory effect of tobacco smoke or its components, such as nicotine and hydroquinone (Vayssier, et al, 1998; Moodie, et al, 2004; Sugano, et al, 1998), but the molecular mechanisms remained unclear. To unravel the underlying mechanism(s) by which tobacco smoke inhibits NF- κ B activity, we examined the NF- κ B activation pathway. Our study shows that tobacco smoke decreased the levels of phosphorylated IKK α / β , indicating the inhibition of these kinases' activity. Since IKK activity is essential for I κ B- α phosphorylation and subsequent degradation, tobacco smoke-induced inhibition of IKK resulted in accumulation of I κ B α , as observed in Fig 4B. These results offer a plausible mechanism for the inhibitory effect of tobacco smoke on NF- κ B activity. The precise factor(s) responsible for the inhibitory effect of tobacco smoke on NF- κ B are not currently known. Reactive oxygen species (ROS), which are present in high amounts in tobacco smoke, may play an inhibitory role. ROS have been previously viewed as inducers of NF- κ B activation (van den Berg, et al, 2001; Schreck, et al, 1991). However, there are many dissenters who believe that ROS are not activators but rather inhibitors for NF- κ B (Bowie and O'Neill, 2000; Korn, et al, 2001, Zhang and Chen, 2004). ROS may oxidize NF- κ B subunits and, thus, impair their DNA binding activity and transcriptional activity (Molitor, et al, 1991; Nishi, et al, 2002). In addition, IKK α and IKK β contain a redox sensitive cysteine residue that is sensitive to oxidative modification. Oxidative modification of this residue by ROS results in inactivation of IKK (Rossi, et al, 2000; Kapahi, et al, 2000). Beside ROS, other possible candidates of tobacco smoke components that contribute to NF- κ B inhibitory effects include hydroquinone, aldehydes (e.g., acrolein), and nicotine. It has been observed that hydroquinone inhibits NF- κ B activation in mouse macrophages and human lymphocytes (Pyatt, et al, 1998; Kim, et al, 2005). Studies have also found the inhibitory effects of nicotine on NF- κ B activity (Sugano, et al, 1998; Zhang, 2001). A recent study showed the direct modification of IKK β and inhibition of NF- κ B activity by acrolein (Valacchi, et al, 2005). These findings support our results that tobacco smoke suppresses IKK activation and subsequent NF- κ B inhibition. Moreover,

previous studies have shown that tobacco smoke activates heat shock transcription factor (HSF) and expression of heat shock proteins (HSP), HSP70 in particular (Pinot, et al, 1997; Vayssier, et al, 1998). HSP70 could act as molecular chaperone to interact with NF- κ B, as a partial substrate for I κ B. Strong evidence suggests activation of HSF and induction of HSP70 contribute to stress response-induced NF- κ B suppression (Feinstein, et al, 1996; Rossi, et al, 1997; Wong, et al, 1997). Consistent with these observations, we also noted induction of HSP70 and inhibition of NF- κ B following tobacco smoke exposure. These data may be indicative of additional inhibitory mechanisms of tobacco smoke on NF- κ B activity.

Besides NF- κ B activity, we have also explored the possible involvement of other signal pathways in the regulation of tobacco smoke induced apoptosis. It has been established that p53 acts as a potent inducer of apoptosis through the action of its target genes, such as Bax. In our study, the expression levels of p53 and Bax showed no significant changes following tobacco smoke exposure. These data could preclude the involvement of p53 in tobacco smoke-induced apoptosis under our experimental conditions. Supporting our findings are reports that tobacco smoke-induced apoptosis in rat alveolar macrophages and human umbilical venous endothelial cells is independent of p53 protein (Aoshiba, et al, 2001; Wang, et al, 2001). In previous study we have illustrated the elevated levels of phosphorylated JNK and p38 MAPK, nuclear c-JUN and AP-1 DNA binding activity (Zhong, et al, 2005). Increased levels of FasL, the c-JUN regulated apoptosis inducer, was also observed in present study (data not shown).

In contrast to the apoptotic effect of tobacco smoke, we have previously shown that tobacco smoke resulted in striking (30 to 40-fold) increases in airway epithelial cell proliferation through MAPK/AP-1 pathways in the same model (Zhong, et al), indicating tobacco smoke exposure has opposite effects on cell turnover. Due to the fact that tobacco smoke results in MAPK/AP-1 activation and NF- κ B inhibition simultaneously, and that both MAPK/AP-1 and NF- κ B pathways play dual roles in the regulation of cell proliferation and apoptosis, the effects of tobacco smoke on cell growth and death should be considered within the context of a complex network of signaling pathways that respond simultaneously. In our current study it is obvious that tobacco smoke induced 3 to 4-fold increases in apoptosis in the airway (Fig. 1C) cannot offset the striking cell proliferation, and therefore skew the impairment in balance between cell growth and cell death towards hyper-proliferation. Further studies are warranted to examine the consequence of longer exposure whether these rats develop hyperproliferative lung diseases such as lung cancer or fibrosis, or apoptotic disease such as emphysema..

In conclusion, we have demonstrated that tobacco smoke inhibits NF- κ B activity and downregulates NF- κ B-dependent anti-apoptotic proteins, resulting in caspase activation of both mitochondrial and death receptor pathways and induction of apoptosis in the lungs of rats exposed to tobacco smoke (Fig. 8). These findings suggest the role of NF- κ B pathway in tobacco smoke-induced apoptosis, thus providing new insights into the molecular mechanisms of this important biological process.

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Fig. 1. Results of terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) of rat lungs. A: H & E staining of an area of squamous metaplasia in the main airway path of rats exposed to 80 mg/m³ tobacco smoke. B: TUNEL labeling in the main airway path. Arrows indicate TUNEL-positive cells. C: Apoptotic index (the percentage of apoptotic nuclei of the total number of nuclei counted) in airways and parenchyma. Values represent mean $\pm$ SE (n=6). **p<0.01, compared with FA. Magnification bar represents 50 microns.

Fig. 2. Effect of tobacco smoke on NF- κ B activity. A: Electrophoretic mobility shift assay to show NF- κ B-DNA binding activity in rat lungs. Lane 1, competition assay; lanes 2–5, filtered air (FA); lanes 6–9, 30 mg/m³ tobacco smoke; lanes 10–13, 80 mg/m³ tobacco smoke. B: Densitometry of NF- κ B -DNA binding activity. Data are expressed as means $\pm$ SE (n=4). **p<0.01, compared with FA.

Fig. 3.

Effect of tobacco smoke on the expression of NF- κ B subunits. A: Western blot analysis of NF- κ B p65 and p50 in the nuclei of rat lungs. Lanes 1–3, filtered air (FA); lanes 4–6, 30 mg/m³ tobacco smoke; lanes 7–9, 80 mg/m³ tobacco smoke. B: Densitometry of Western blot results for NF- κ B subunits. Values are presented as means $\pm$ SE (n=3). *p<0.05, compared with FA.

Fig. 4.

Effect of tobacco smoke on the NF-κB activation pathway. Western blot analysis of IκB-α, IκB-β, p-IKKα, p-IKKβ (A) and HSP70 (C). Lanes 1–3, FA; lanes 4–6, 30 mg/m³ tobacco smoke; lanes 7–9, 80 mg/m³ tobacco smoke. Densitometry of Western blot results for IκB-α, IκB-β, p-IKKα, p-IKKβ (B) and HSP70 (D). Values are presented as means ± SE (n=3).

**p<0.01, compared with FA.

Fig. 5. Effect of tobacco smoke on the expression of NF- κ B – dependent anti-apoptotic proteins. A: Western blot analyses of Bcl-2, Bcl-xl, c-IAP1, c-IAP2 and XIAP in rat lungs. Lanes 1–3, FA; lanes 4–6, 30 mg/m³ tobacco smoke; lanes 7–9, 80 mg/m³ tobacco smoke. B: Densitometry of Western blot results. Values are presented as means $\pm$ SE (n=3). *p<0.05, compared with FA ; **p<0.01, compared with FA.

Fig. 6.

Effect of tobacco smoke on the expression of p53 and Bax. A: Western blot analyses of p53 and Bax in the lungs of rats. Lanes 1–3, FA; lanes 4–6, 30 mg/m³ tobacco smoke; lanes 7–9, 80 mg/m³ tobacco smoke. B: Densitometry of Western blotting results. Values are presented as means $\pm$ SE (n=3).

Fig. 7.

Effect of tobacco smoke on caspase activation. A: Western blot analyses of caspases 8, 9 and 3 in the lungs of rats. Lanes 1–3, FA; lanes 4–6, 30 mg/m³ tobacco smoke; lanes 7–9, 80 mg/m³ tobacco smoke. B: Densitometry of Western blot results. Values are presented as means ± SE (n=3). *p<0.05, compared with FA; **p<0.01, compared with FA.

Fig. 8.

Proposed model of the role of NF- κ B pathway in tobacco smoke-induced apoptosis in the lungs of rats. Through suppression of IKK and induction of HSP70, tobacco smoke inhibits NF- κ B activity and downregulates NF- κ B -dependent anti-apoptotic proteins, including members of Bcl-2 and c-IAPs, leading to caspase activation of both mitochondrial (caspase 9) and death receptor (caspase 8) pathways as well as the effector caspase (caspase 3), eventually inducing apoptosis.