Wood smoke extract induces oxidative stress-mediated caspase-independent apoptosis in human lung endothelial cells: role of AIF and EndoG

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THE INHALATION OF TOXIC SMOKE generated from incomplete combustion of materials is associated with high mortality (25). Several in vivo studies have reported that inhaled toxic smoke increases oxidative stress in lung tissues (13, 14, 36) and causes oxidant lung injury (13, 14, 29, 36, 51), which manifests as pulmonary endothelial dysfunction leading to pulmonary edema (19, 24, 29). Smoke-induced pulmonary edema is often intractable and can evolve into acute respiratory distress syndrome (22). Although a link between toxic smoke and oxidant lung vascular injury has been indicated, the exact cellular mechanisms of smoke-induced injury to lung endothelial cells are completely unknown.

Toxic smoke, especially its particulate phase, contains high concentrations of free radicals, radical precursors, and long-lived metastable radical intermediates, all of which may generate reactive oxygen species (ROS) including superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radicals (40). To counteract the damaging effects of ROS, lung cells possess several antioxidant systems. For example, superoxide dismutase (SOD) catalyzes the conversion of O$_2^-$ to H$_2$O$_2$ and catalase catalyzes the breakdown of H$_2$O$_2$ to H$_2$O (12). In addition, the glutathione (GSH) system employs GSH as a substrate to detoxify H$_2$O$_2$ and other harmful peroxides, a reaction that involves glutathione peroxidase (GPx) (42). Furthermore, heme oxygenase-1 (HO-1), an inducible enzyme involved in the degradation of heme, acts as a stress-responsive protein with antioxidant capacity (33). ROS-related stimuli, such as cigarette smoke or hyperoxia, may increase intracellular ROS (9, 53), modify intracellular GSH content (1, 41), and alter expression of antioxidant enzymes (1, 17, 21) and HO-1 (30, 50) in various types of lung cells. Thus cellular oxidative stress occurs when the cell has an imbalance between the production of ROS and the antioxidant capacity of the system. Up to the present, whether and how toxic smoke causes oxidative stress in lung endothelial cells remains unclear.

Cellular oxidative stress, produced by stimuli such as cigarette smoke (9, 20) or hyperoxia (8, 53), accelerates apoptosis in lung cells. Apoptosis, programmed cell death, is significant in the development and homeostasis of cell populations (44). However, inadequate apoptosis has been implicated in the pathogenesis of lung injury with endothelial damage (7, 16, 31). Abundant evidence suggests that mitochondria play an important role in the regulation of apoptosis (37, 43). When the mitochondrial pathway is triggered, proapoptotic proteins, such as Bax, translocate to the outer mitochondrial membrane (37, 43). Abundant evidence suggests that mitochondria play an important role in the regulation of apoptosis (37, 43). When the mitochondrial pathway is triggered, proapoptotic proteins, such as Bax, translocate to the outer mitochondrial membrane (37, 43).
resulting in chromatin condensation and DNA fragmentation (37, 43). Both the caspase-dependent and caspase-independent pathways have been reported to mediate the apoptosis of endothelial cells (15, 52). Nevertheless, whether and how toxic smoke causes apoptosis in lung endothelial cells remains to be investigated.

This study was conducted, first, to investigate and characterize the oxidative stress and apoptosis caused by wood smoke extract (SE) in human pulmonary artery endothelial cells (HPAECs) and, second, to delineate the role of oxidative stress in triggering SE-induced HPAEC apoptosis. To accomplish these objectives, we measured cell viability, DNA fragmentation, several hallmarks of apoptosis, intracellular ROS levels, intracellular GSH levels, and expression of several antioxidant enzymes including Cu/Zn SOD, catalase, HO-1, and GPx in HPAECs exposed to SE. Inhibitors of caspases were used to study the apoptotic pathways. N-acetylcysteine (NAC), a well-studied ROS scavenger and GSH precursor (18), was employed to investigate the importance of oxidative stress in SE-induced apoptosis.

MATERIALS AND METHODS

Preparation of SE. The method for generating wood smoke has been described in detail in a previous study (23). After its generation, 3 liters of wood smoke were sucked through the experimental apparatus with a constant flow (0.3 l/min) and driven through a standard glass-fiber Cambridge filter by an air compressor. The smoke filter was quickly dried with a hot plate, and the raised weight in each filter was read at 530 nm with 690 nm as reference using a DIAS microplate spectrophotometer R1 (4-chloro-1-methyl-7-trifluromethyl-quinolinium methyl-sulfate) and reagent R2 (30% NaOH). A known concentration of GSH was used to generate a standard curve.

Cell viability assay. Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma) as described previously (45). Briefly, cells were incubated with or without SE for 24 h, and 100 μl of MTT (0.5 mg/ml in medium) were then added to each well. After 4 h of incubation with MTT at 37°C, the medium was carefully removed and cells were washed twice with PBS. The metabolized MTT were solubilized with 100 μl of DMSO, and the absorbance of the solubilized blue formazan was measured at 565 nm as reference using a DIAS microplate reader (Dynatech Technologies, Chantilly, VA). The reduced MTT optical density produced by SE exposure was considered as a decrease in cell viability. The cells incubated with control medium were considered 100% viable.

Annexin V-propidium iodide assay. Cells were simultaneously subjected to annexin V and propidium iodide (PI) assays. An annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Serotec, Oxford, UK) was used to bind phosphatidylserine, which is translocated to the outer leaflet of the plasma membrane during the early stages of cell apoptosis (38). Hence, apoptotic cells stain with annexin V-FITC only, whereas necrotic cells double stain for both annexin V-FITC and PI. For this purpose, cells were incubated with or without SE for 60 min. Cells were suspended in binding buffer at a final cell concentration of 1 × 10⁶ cells/ml and incubated with both annexin V-FITC and PI (1 μg/ml) for 20 min at 4°C in the dark. The suspension was then immediately analyzed using a FACSscan flow cytometer (Becton Dickinson).

Fluorescein diacetate-PI assay. The cell membrane permeability was assessed using fluorescein diacetate (FDA)-PI assay and flow cytometry as described previously (49). Briefly, cells were incubated with or without SE for up to 4 h. Before they were harvested, these cells were concurrently incubated with FDA (20 μg/ml; Molecular Probes) and PI (20 μg/ml; Molecular Probes) for 30 min. The cells were then centrifuged, and the supernatant containing PI and FDA were measured using a colorimetric assay (Bioxytech GSH-400; OxisResearch, Portland, OR). Briefly, cells were incubated with or without SE for up to 4 h. Cells were washed with PBS, and metaphosphoric acid (5%) was added to the cells, which were then scraped off. The mixture was centrifuged at 3,000 g for 5 min at 4°C, and the supernatant was measured at 400 nm after a chemical reaction with reagent R1 (4-chloro-1-methyl-7-trifluromethyl-quinolinium methyl-sulfate) and reagent R2 (30% NaOH). A known concentration of GSH was used to generate a standard curve.

Intracellular GSH levels. Intracellular GSH levels were measured using a colorimetric assay (Bioxytech GSH-400; OxisResearch, Portland, OR). Briefly, cells were incubated with or without SE for up to 4 h. Cells were washed with PBS, and metaphosphoric acid (5%) was added to the cells, which were then scraped off. The mixture was centrifuged at 3,000 g for 5 min at 4°C, and the supernatant was measured at 400 nm after a chemical reaction with reagent R1 (4-chloro-1-methyl-7-trifluromethyl-quinolinium methyl-sulfate) and reagent R2 (30% NaOH). A known concentration of GSH was used to generate a standard curve.

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Mitochondrial membrane potential assay. The JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carboxyanine iodide (JC-1; Molecular Probes) was used to measure mitochondrial membrane potential during cell apoptosis. JC-1 is able to enter mitochondria selectively. JC-1 is green at low concentration or low potential (<140 mV) when it is a monomer (35). However, JC-1 monomers convert to J-aggregates that emit red fluorescence aggregates at high potential (35). For this purpose, cells were cultured on glass coverslips coating with collagen IV (Sigma). Cells were incubated with 5 μM JC-1 for 30 min at 37°C in the dark, washed with PBS, and then incubated with or without SE for up to 60 min. Subsequently, cells were washed with PBS, fixed with 4% paraformaldehyde in PBS at 4°C for 15 min, and washed with PBS again. The glass coverslips containing cells were mounted using Vectashield mounting medium (Vector, Burlingame, CA), and the fluorescence intensities of both monomer and aggregated molecules were measured under a TCS SP2 confocal scanning laser microscope (Leica, Heidelberg, Germany) at excitation and emission wavelengths of 500 and 535 nm for red fluorescence and 550 and 700
nm for green fluorescence, respectively. According to the manufacturer’s suggestion, the JC-1 dye is retained well during typical paraformaldehyde fixation, but the staining pattern could potentially be altered and the appropriate control should be provided. Based on these suggestions, experiments of no-treatment time controls were conducted.

Quantification of DNA fragmentation. Cellular DNA fragmentation ELISA assays were performed with a kit according to the manufacturer’s protocol (Roche Molecular Biochemicals, Mannheim, Germany). Cells were incubated for 12 h at 37°C with the nonradioactive thymidine analog 5-bromo-2′-deoxyuridine (BrdU), which can be incorporated into genomic DNA. Subsequently, cells were incubated with or without SE for 6 h. Cells were then lysed, transferred to a microtiter plate coated with an anti-DNA antibody, and incubated for 90 min at room temperature. The plate was washed three times and heated for 5 min in a microwave oven. Anti-BrdU-peroxidase conjugate solution was added to each well, and the plate was incubated for 90 min at room temperature. After the plate was washed three times, substrate solution was added, and the plate was incubated in the dark on a shaker until color development was sufficient. The reaction was then stopped by addition of 25 μl of 0.56 M H2SO4. A microplate reader (Bio-Rad, Hercules, CA) was used to measure absorbance at 450 and 655 nm for each well.

Isolation of total RNA and real-time PCR. Total RNA was isolated using an RNeasy mini kit and an RNeasy-free DNase set (Qiagen, Valencia, CA). RNA (2 μg) was reverse transcribed using the SuperScript First-Strand synthesis system for RT-PCR kit (Invitrogen, Carlsbad, CA). A 1:5 dilution of the resulting cDNA was used as the standard, and 1:10 template dilution of the resulting cDNA was used to quantify the relative content of mRNA by real-time TaqMan-PCR. A melt analysis was run of all products to determine the specificity of the amplification using the Real Quant software (Roche). The primer sets were designed using Primer Express software (Real Quant, Roche) based on published sequences: human GAPDH sense primer, 5′-ACC ACA GTA CAT GCC ATC AC-3′; antisense primer, 5′-TCC ACC CTG TTG CTG TA-3′; human Cu/Zn SOD sense primer, 5′-GAGAAC TAT AGG AGT GAC-3′; antisense primer, 5′-CAA TTA CAC CAC AAG CCA AAC GAC-3′; human catalase sense primer, 5′-TCA GGA GGC GCC CAG TCG GTG TA-3′; antisense primer, 5′-TCA GGA GGC GCC CAG TCG GTG TA-3′; human HO-1 sense primer, 5′-TTG TCT ACC TCT ACC A-3′; antisense primer, 5′-TCA CCT TGC TGC GCC GCA TAC-3′; human endonuclease G (EndoG) sense primer, 5′-GAG CAG TCT CCG GCT GGT CAT AA-3′; human HPRT sense primer, 5′-GAG GCC GCG CCG CAG TCT CAG TA-3′; antisense primer, 5′-GAG CTT GGC CCT GGT CAT AA-3′. Parameters included an initial denaturation at 94°C for 180 s, followed by 40 cycles at 95°C for 30 s, 60°C for 25 s, 72°C for 30 s, and 1 cycle at 72°C for 7 min. Fluorescence data were acquired at the end of amplification. A melt analysis was run of all products to determine the specificity of the amplification using the Real Quant software (Roche). In addition, RT-PCR products (RT-PCR kit; Invitrogen) were obtained to confirm the results from real-time PCR; single PCR products were generated in each PCR. Briefly, RT-PCR products were separated by electrophoresis on 1% agarose gels containing ethidium bromide (1 μg/ml), and images were captured and quantified using a scanning densitometer. All values were normalized to the constitutive expression of the housekeeping gene GAPDH as described in an online data supplement (http://ajplung.physiology.org/cgi/content/full/00009.2005/DC1).

Western blot analysis for protein levels. The cell lysate was prepared using cell lysis buffer (Cell Signaling, Beverly, MA). Nuclear extracts were prepared using a method that has been reported previously (4) with modifications. Western blot analyses were performed as described previously (11). Briefly, the cell lysate (25–40 μg) or nuclear extracts (40 μg) were subjected to 12% SDSPAGE and transferred onto polyvinylidene difluoride membranes, which were then blotted. After being blocked with 5% skim milk in Tween 20-PBS, blots were incubated with various primary antibodies includ-
exposure for the subsequent studies of oxidative stress and apoptosis with respect to nonexposure control cells.

**SE induces oxidative stress and upregulates antioxidant enzymes.** Measurements using the HE/ETH and DCFH-DA/DCF assays and flow cytometry analysis indicated that SE exposure for up to 4 h caused an increase in intracellular levels of ROS, particularly $O_2^-\cdot$ (Fig. 2A) and $H_2O_2$ (Fig. 2B), in a time-dependent manner, as evidenced by increases in the percentage of ETH- and DCF-positive cells. The peak ETH fluorescence intensities measured for the control group at 0, 30, 60, and 240 min were 2.8 ± 0.2, 5.0 ± 0.2, 4.8 ± 0.3, and 5.1 ± 0.4, respectively, and those for the SE exposure group were 2.8 ± 0.2, 82.0 ± 7.2, 610.0 ± 20.0, and 600.0 ± 36.1, respectively. The peak DCF fluorescence intensities measured for the control group at 0, 30, 60, and 240 min were 6.9 ± 1.5, 8.0 ± 1.1, 8.9 ± 1.0, and 8.5 ± 1.5, respectively, and those for the SE exposure group were 7.1 ± 2.4, 386.7 ± 20.8, 396.7 ± 40.4, and 393.3 ± 25.2, respectively. Conversely, the colorimetric assay demonstrated that SE exposure for up to 4 h caused a decrease in intracellular GSH level in a time-dependent manner (Fig. 2C). Real-time PCR analysis revealed that SE exposure for 2 h significantly increased the expression of Cu/Zn SOD mRNA and HO-1 mRNA, whereas it failed to alter the expression of catalase and GPx mRNA (Fig. 3A). Western blot analysis demonstrated that SE exposure for up to 6 h caused comparable upregulation of both Cu/Zn SOD and HO-1 in a time-dependent manner (Fig. 3B).

**SE induces mitochondrial-to-nuclear translocation of AIF and EndoG and promotion of caspase-independent apoptosis.** Flow cytometry analysis revealed that SE exposure for 60 min caused marked increase in the percentage of annexin V-FITC-positive cells but not PI-positive cells (Fig. 4). Simultaneous staining with FDA and PI indicated that SE exposure for up to 4 h did not affect the percentage of FDA-positive cells and failed to increase the percentage of PI-positive cells (Fig. 5). SE exposure for up to 60 min also caused a loss of mitochondrial membrane potential, as demonstrated by the change in JC-1-derived fluorescence from red to green (Fig. 6A). Western blot analysis revealed that the level of cytosolic Bax (Fig. 7A) was increased after SE exposure for 1 or 2 h and returned to basal level at 4 h after exposure. However, the levels of procaspase-3 and caspase-9 were unaltered by SE exposure for up to 4 h, but they were decreased and increased, respectively, by $H_2O_2$ exposure, which was used as a positive control for caspase activation (Fig. 7, B and C). The colorimetric assay indicated that the specific caspase-3 activity was unaffected by SE exposure for 1 h, but it was increased by $H_2O_2$ exposure (Fig. 7D). In contrast, Western blot analysis demonstrated that SE exposure for up to 4 h increased the levels of AIF (Fig. 7E) and EndoG (Fig. 7F) in nuclear extracts. Confocal microscopic analysis showed the translocation of AIF and EndoG from mitochondria to the nucleus during apoptosis, as indicated by the yellow color resulting from the overlap of AIF or EndoG and nuclear staining (Fig. 6B). The DNA fragmentation assay revealed that SE exposure for 6 h significantly increased DNA fragmentation (Fig. 1B). Pretreatment with Ac-LEHD-CMK, a specific inhibitor of caspase-9, or Z-VAD-FMK, a broad-spectrum caspase inhibitor, failed to suppress SE-induced cell death (Fig. 1A) and DNA fragmentation (Fig. 1B), suggesting that SE induced caspase-independent apoptosis.

**Increased oxidative stress triggers SE-induced apoptosis.** NAC pretreatment successfully prevented the increase in $O_2^-\cdot$ (Fig. 8A) or $H_2O_2$ (Fig. 8B) in cells exposed to SE for 60 min and also prevented the reduction in GSH content (Fig. 8C) in cells exposed to SE for 4 h. The peak ETH fluorescence intensities were 3.7 ± 0.6, 590.0 ± 57.1, and 9.3 ± 0.4 for the control, SE exposure group, and SE exposure group with NAC pretreatment, respectively. The peak DCF fluorescence intensities were 9.6 ± 0.4, 403.3 ± 12.5, and 24.7 ± 4.1 for the control, SE exposure group, and SE exposure group with NAC pretreatment, respectively. Accompanying this prevention of oxidative stress and replenishment of GSH, NAC pretreatment also significantly decreased the nuclear level of AIF (Fig. 9A) and EndoG (Fig. 9B) in cells exposed to SE for 4 h, completely prevented the SE-induced increase in DNA fragmentation in cells exposed to SE for 4 h (Fig. 9C), and alleviated the SE-induced reduction in viability of cells exposed to SE for 24 h (Fig. 9D). In contrast, NAC pretreatment had no effect on the GSH level (Fig. 8C), DNA fragmentation (Fig. 9C), and the nuclear level of AIF (Fig. 9A) or EndoG (Fig. 9B) in cells with NAC pretreatment alone (without SE exposure).
DISCUSSION

Clinical and animal studies repeatedly have reported that inhaled toxic smoke causes pulmonary edema (19, 24, 29, 51). Although toxic smoke-induced lung vascular injury is known to be associated with oxidative stress, the exact cellular mechanisms are completely unknown. In this article, we report that SE induces an increase in intracellular oxidative stress, which triggers a consequent caspase-independent apoptosis in HPAECs.

Results of the first part of this study demonstrate that exposure of HPAECs to SE induced intracellular oxidative stress. SE caused increases in intracellular levels of ROS, particularly \( \cdot \)O\(_2\) and H\(_2\)O\(_2\), within the first 30 min after exposure, and these increases were time-dependent over the exposure for 4 h. The source of these increased intracellular ROS cannot be determined in this study, but xanthine oxidase, NADPH oxidase, and mitochondria may be possible origins (47). The increased ROS may serve as intracellular signaling to trigger various cellular responses, including transcription regulation of antioxidant enzymes (12). Indeed, we found that SE upregulated Cu/Zn SOD, did not alter the expression of catalase or GPx, and caused a depletion of the intracellular GSH level. Accordingly, it is plausible that, in our experimental model, the SE-induced upregulation of Cu/Zn SOD augmented the reduction of \( \cdot \)O\(_2\) to H\(_2\)O\(_2\). However, a further accumulation of intracellular H\(_2\)O\(_2\) ensued because SE did not correspondingly upregulate catalase and GPx to counterbalance this by reducing excess H\(_2\)O\(_2\). The increased H\(_2\)O\(_2\) may thus exhaust intracellular GSH when the GSH system attempts to detoxify them. The reduction in intracellular GSH level we observed during the entire experiment period would seem to reflect a decrease in antioxidant capacity (42). The excess intracellular H\(_2\)O\(_2\) may then work with \( \cdot \)O\(_2\) to form hydroxyl radicals, which will cause greater damage (40, 47). In the meanwhile, we also observed that SE upregulated HO-1, a ROS-sensitive stress protein that has been shown to be identical to heat shock proteins.
protein 32 (33). HO-1 is not an antioxidant enzyme per se, but it degrades heme to yield bilirubin, carbon monoxide, and free iron, all of which have antioxidant function (33). However, even after HO-1 has been called into action, the intracellular ROS level in SE-exposed cells was still higher than that in the nonexposed cells. These observations indicate that the increased intracellular ROS overwhelmed the overall antioxidant capacity of the cells, resulting in SE-induced intracellular oxidative stress.

The concept that toxic smoke may increase lung oxidative stress is not new, yet the supporting evidence has been mostly limited to in vivo studies. Toxic smoke is an oxidant irritant that has the ability to generate ROS when it reaches lung tissue (40). One in vitro study reported that ROS generated by wood smoke is able to cause lipid peroxidation in alveolar macrophages (26). In addition, toxic smoke produces an elevation of the biomarkers of oxidative stress such as lipid peroxidation and also decreases antioxidant enzyme activities in lung tissues (13, 14, 36, 51). Furthermore, toxic smoke may stimulate certain lung cells, such as polymorphonuclear leukocytes and alveolar macrophages, to release ROS (25). Together, these studies seem to suggest that toxic smoke increases lung oxidative stress by ROS generated from exogenous (smoke) and endogenous sources (lung cells). Thus the present study provides the first evidence to indicate that toxic smoke may actually induce intracellular oxidative stress by increasing ROS and by changing the antioxidant profile within the cells. This is another way of defining smoke-induced lung oxidative stress.

Other ROS-related stimuli have been demonstrated to alter intracellular antioxidant profiles. For example, cigarette smoke increases GPx expression, but it has no effect on Cu/Zn SOD and catalase expression in bronchial epithelium (17). Hyperoxia upregulates GPx, Cu/Zn SOD, and catalase in umbilical vein endothelial cells (21), but it has no effect on the mRNA levels of any of these enzymes in bronchial epithelial cells (39). Cigarette smoke causes a depletion of GSH in alveolar epithelial cells (41), but hyperoxia increases the GSH content in bronchial epithelial cells (39). The results of these studies and the present research support the notion that the changes in the intracellular antioxidant profiles of cells vary in terms of both different ROS-related stimuli and the type of cell.

We have further demonstrated that the exposure of HPAECs to SE accelerated apoptosis. The early event of SE-induced apoptosis is revealed by the translocation of phosphatidylserine to the outer leaflet of the plasma membrane (38). Negative...
results of staining for PI in these cells indicated that SE did not cause cell necrosis at this stage. Concurrent staining by FDA and PI revealed that SE exposure for up to 4 h did not affect cell membrane permeability. We also observed an increase in Bax protein, one of the best-studied proapoptotic factors from the Bcl-2 family (44). Bax is known to translocate from the cytosol to mitochondria in response to apoptotic stimuli and cause a destabilization of the outer mitochondrial membrane (37, 44), a result that is borne out by the SE-induced loss of mitochondrial membrane potential observed in this study. The subsequent releases of apoptogenic molecules from the outer mitochondrial membrane would then initiate the death path-

Fig. 5. Effects of SE on the simultaneous staining of fluorescein diacetate (FDA) and PI in HPAECs. Cells were incubated with medium alone (control) or 40 μg/ml SE for up to 4 h. Cells were concurrently subjected to the FDA assay and PI assay and were analyzed using flow cytometry. The histogram is a representative of 3 independent experiments.

Fig. 6. Confocal microscopic images showing mitochondrial membrane destabilization (A) and mitochondrial-to-nuclear translocation of apoptosis-inducing factor (AIF) or endonuclease G (EndoG) (B) in HPAECs exposed to SE. Cells were incubated with medium alone (control) or 40 μg/ml SE for 0–60 min. A: loss of mitochondria membrane potential is demonstrated by the change in JC-1-derived fluorescence from red (at high potential as J-aggregates) to green (at low potential as a monomer) in SE-exposed cells. B: both AIF and EndoG are indicated by rhodamine, showing red fluorescence, whereas histone H1 in nucleus is revealed by FITC, showing green fluorescence. The nuclear translocation is demonstrated by the overlap of either AIF or EndoG and nuclear staining, as revealed by the yellow color. In A or B, the images shown are representative of 3 independent experiments.
ways, resulting in DNA fragmentation (37, 43, 44). To that end, we found that SE exposure failed to activate the caspase cascade, but it did cause mitochondrial-to-nuclear translocation of AIF and EndoG, two apoptogenic molecules for the caspase-independent pathway (27, 37, 43, 46). In addition, SE-induced apoptosis was unaffected by a specific inhibitor of caspase-9 and also by a broad-spectrum caspase inhibitor. These observations suggest that the SE-induced apoptosis is mediated through the caspase-independent pathway but not the caspase-dependent pathway.

Compared to the caspase family, the discovery of AIF (46) and EndoG (27) as key executors of apoptosis is quite recent and their regulation is still obscure. In HeLa cells, the mitochondrial release of AIF and EndoG requires Bax-mediated...
permeabilization, but they act downstream of caspase activation, suggesting their release occurs in a caspase-dependent fashion (2). In our experimental model, although the role of Bax activation in their release remains to be elucidated, it is clear that their release is caspase independent. This notion is compatible with another recent conclusion that their release is Bax mediated and is responsible for the caspase-independent apoptosis in human T lymphocytes (6). In this study, confocal microscopic analysis reveals that the mitochondrial-to-nuclear translocation of AIF was faster than that of EndoG. In addition, the SE-induced nuclear accumulation of AIF peaked at the first hour after exposure, whereas that of EndoG peaked at the second hour after exposure. These observations raise the question of whether the mitochondrial-to-nuclear translocation of AIF and EndoG may be regulated by different mechanisms. Apart from its apoptogenic function, AIF is known to have marked NADH oxidase activity and thus may react rapidly with oxygen to form $\text{O}_2^\cdot$/$\text{H}_2\text{O}_2$, providing another source that generates ROS generation (32).

In addition, we have demonstrated a cause-effect relationship between SE-induced oxidative stress and apoptosis in HPAECs. It is quite well established that NAC functions as a ROS scavenger and a glutathione precursor (18). Indeed, we found that NAC pretreatment effectively reversed SE-induced increases in intracellular ROS and depletion of intracellular GSH. More importantly, NAC pretreatment also suppressed SE-induced nuclear translocation of AIF or EndoG and prevented the resulting enhancement of DNA fragmentation. These findings strongly suggest that intracellular oxidative stress is the causative factor responsible for SE-induced caspase-independent apoptosis. In this context, both increased ROS and depletion of GSH should be considered as the possible triggering factors, as has been suggested by other investigators (10). This apoptosis appears to make up a significant proportion, if not all, of the SE-induced cell death, because NAC pretreatment also prevented the reduction in cell viability. Other ROS-related stimuli have been demonstrated to promote cell apoptosis. For example, hyperoxia induces caspase-dependent apoptosis in rat fibroblasts (8) and lung epithelial cells (53), and cigarette smoke induces apoptosis in lung fibroblasts (9), alveolar type II cell-derived cells (20), and monocytes (3); all of these are prevented by NAC. However, a broad-spectrum caspase inhibitor prevents the apoptosis in lung fibroblasts (9) but not in monocytes (3), suggesting that cigarette smoke may cause both caspase-dependent and -independent apoptosis, depending on cell type.

In summary, although SE upregulates certain enzymes with antioxidant capacity such as Cu/Zn SOD and HO-1 in HPAECs, it nevertheless increases intracellular oxidative stress. The oxidative stress then triggers the mitochondrial release of AIF and EndoG, resulting in caspase-independent apoptosis. It is thus conceivable that lung endothelial cell apoptosis may contribute to acute lung injury caused by toxic smoke. Understanding the mechanism of smoke-induced pathophysiology is important in designing new therapeutic strategies. Accordingly, administration of antioxidants, overexpression of enzymes with antioxidant capacity by gene transfer or pharmacological activation, and modulations of the proapoptotic or apoptogenic molecules in the caspase-independent apoptotic pathway are possible target choices for potential therapeutic regimes.

Fig. 9. Effects of NAC on increased levels of AIF (A) and EndoG (B) in nuclear extracts, enhancement of DNA fragmentation (C), and reduction in cell viability (D) induced by SE in HPAECs. Cells were pretreated with NAC for 2 h and then incubated with medium alone (control) or SE for 4, 4, 6, and 24 h in A, B, C, and D, respectively. The SE concentration used in A–C was 40 $\mu$g/ml. Protein levels were measured using Western blot analysis and quantified by densitometry using histone H1 as an internal control. Cell viability and apoptosis were measured using the MTT and DNA fragmentation assays, respectively. Data are means ± SE from 3 independent experiments. *P < 0.05 vs. control condition. #P < 0.05 vs. SE exposure.
WOOD SMOKE-INDUCED OXIDATIVE STRESS AND APOPTOSIS

REFERENCES


