

Cigarette smoke extract induces oxidative stress and apoptosis in human lung fibroblasts

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Carnevali, Stefano, Stefano Petruzzelli, Biancamaria Longoni, Renato Vanacore, Roberto Barale, Monica Cipollini, Fabrizio Scatena, Pierluigi Paggiaro, Alessandro Celi, and Carlo Giuntini. Cigarette smoke extract induces oxidative stress and apoptosis in human lung fibroblasts. *Am J Physiol Lung Cell Mol Physiol* 284: L955–L963, 2003. First published January 24, 2003; 10.1152/ajplung.00466.2001.—Cigarette smoke is a mixture of chemicals having direct and/or indirect toxic effects on different lung cells. We investigated the effect of cigarette smoke on human lung fibroblasts (HFL-1) oxidation and apoptosis. Cells were exposed to various concentrations (1, 5, and 10%) of cigarette smoke extract (CSE) for 3 h, and oxidative stress and apoptosis were assessed by fluorescence-activated cell sorting and confocal laser fluorescence microscopy. Both oxidative stress and apoptosis exhibited a dose-response relationship with CSE concentrations. Lung fibroblasts also showed marked DNA fragmentation at the Comet assay after exposure to 10% CSE. Coincubation of HFL-1 cells with *N*-acetylcysteine (1 mM) during CSE exposure significantly reduced oxidative stress, apoptosis, and DNA fragmentation, whereas preincubation (3 h) with the glutathione-depleting agent buthionine sulfoximine (125 μ M) produced a significant increase of oxidative stress. Cigarette smoke is a potent source of oxidative stress, DNA damage, and apoptosis for HFL-1 cells, and we speculate that this could contribute to the development of pulmonary emphysema in the lungs of smokers.

Comet assay

CIGARETTE SMOKE is the major cause of pulmonary emphysema. Epidemiological and clinical studies have shown that smokers are significantly more likely to develop emphysema compared with nonsmokers, and the seriousness of the disease is directly correlated with the amount of cigarette smoking (2, 20, 34). Among the different toxic effects of cigarette smoke on human tissues, oxidation of structural and functional molecules and modulation of cell turnover play a major role (29). For instance, Kasahara and coworkers (18) hypothesized that cigarette smoke may act by decreas-

ing the expression of vascular endothelial growth factor (VEGF) and its type 2 receptor, thus resulting in lung septal endothelial cell death. Because fibroblasts play a pivotal role in remodeling of pulmonary tissue, we have exposed fibroblasts to cigarette smoke and have studied two important processes: oxidative stress and apoptosis.

Oxidative stress is defined as a disturbance in the oxidant-antioxidant balance, resulting in potential cell damage. It is involved in many biological and pathological processes, such as inflammation and carcinogenesis, and in the development of many pulmonary diseases (23). In response to oxidative stress, lung cells release inflammatory mediators and cytokines (TNF- α , IL-1, and IL-8) that are able to induce neutrophil recruitment and activation of transcription factors such as activator protein-1 and nuclear factor- κ B (28).

Apoptosis is a form of cell death that occurs under several physiological and pathological situations, and it represents a common mechanism of cell replacement, tissue remodeling, and removal of damaged cells. It is characterized by cell shrinkage, chromatin condensation, internucleosomal DNA fragmentation (18a, 24, 41), and formation of apoptotic bodies. Apoptosis may occur spontaneously or in response to specific stimuli such as heat stress, radiation, steroids, and oxidative stress. Because oxidative stress and apoptosis are implicated in numerous processes, including aging, inflammation, and carcinogenesis, it is reasonable to hypothesize a link between these two processes. The mechanisms by which oxidants can modulate the apoptotic pathways have been recently reviewed (7). The aim of this study was to evaluate the ability of cigarette smoke to induce fibroblasts oxidation and apoptosis.

MATERIALS AND METHODS

Chemicals. *N*-acetylcysteine (NAC) was obtained from Zambon Pharmaceutical (Vicenza, Italy), and DL-buthionine-(*SR*)-sulfoximine (BSO) was purchased from Sigma Chemical (Milan, Italy). Dichlorodihydrofluorescein diac-

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etate (H₂DCFDA), annexin V-FITC, propidium iodide, and Hoechst 33342 were purchased from Molecular Probe (Eugene, OR), whereas annexin V-phycoerythrin (PE) was purchased from Bender MedSystems (Vienna, Austria).

Cell culture. Human fetal lung fibroblasts (HFL-1, lung, diploid, human) were obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, penicillin/streptomycin, and amphotericin B. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂. Cells were passed weekly, and cells from passages 15–20 were used for experiments.

Preparation of cigarette smoke extract. Cigarette smoke extract (CSE) was prepared by a modification of the method of Carp and Janoff (6). In brief, two cigarettes without filters were combusted with a modified syringe-driven apparatus. The smoke was bubbled through 50 ml of serum-free DMEM, and the resulting suspension was adjusted to pH 7.4 and then filtered through a 0.20- μ m pore filter to remove bacteria and large particles. The resulting CSE was applied to fibroblast cultures within 30 min of preparation.

We also studied the effects of two volatile components of cigarette smoke, acetaldehyde and acrolein, which are present at high concentrations in cigarette smoke. In separate experiments, the cells were exposed for the same period of time as to CSE (3 h) to millimolar concentrations of acetaldehyde and micromolar concentrations of acrolein.

Measurement of oxidative stress, apoptosis, and necrosis by cytofluorimetric analysis. We measured cellular oxidative stress fluorometrically by monitoring the oxidation of intracellular H₂DCFDA. Apoptotic cells were detected by the calcium-binding protein annexin V. In particular, phosphatidylserine residues, which are normally located in the internal phospholipid layer, are actively translocated to the external layer and thus become detectable by annexin V. Lung fibroblasts were exposed to fresh medium (control) or to different concentrations of CSE for 3 h, and, after exposure, the cells were washed twice with HBSS. H₂DCFDA and annexin V were added, and cultures were then incubated for the following 20 min at 37°C. At the end, cells were trypsinized, washed, and resuspended in PBS, and fluorescence intensity was measured by a flow cytometric assay. Flow cytometric analysis was performed with a fluorescence-activated cell sorting (FACS) Calibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped with Cell Quest analysis system. Green (for H₂DCFDA) and red (for PE-annexin V) fluorescence was excited using the 488-nm line of an argon laser. Band pass filters of 530 and 585 nm were used in the experiments with H₂DCFDA and annexin V, respectively, for double-labeling two-color detection. The machine was optimized daily with calibrate beads (Becton-Dickinson); the amplification and the photomultiplier tube voltages were adjusted to position the beads in the predetermined scatter and fluorescence channel with compensation software. Events were collected with a suitable threshold that excluded cell debris. No initial collection window was set, and all the events were first acquired and then analyzed with Cell Quest software. A total of 10,000 events was analyzed for each sample, and in every experiment a control tube with cells alone was performed.

To distinguish between apoptosis and necrosis, in separate experiments, we exposed the cells to 10% CSE, and after double staining with annexin V-FITC (green fluorescence) and propidium iodide (red fluorescence), we analyzed the cells by FACS as previously mentioned.

Confocal laser fluorescence microscopy. Determination of oxidative stress and apoptosis at the cellular level was eval-

uated as previously described (21). In brief, fibroblasts were grown on gelatin-coated coverslips until they reached sub-confluence. The glass coverslips were adapted to Plexiglas chambers. After exposure to CSE, HFL-1 were incubated with H₂DCFDA and annexin V for 20 min at 37°C, washed twice with PBS, and observed under an inverted microscope (Nikon Eclipse TE 300) equipped with a laser confocal scanning system (Radiance Plus; Bio-Rad, Hercules, CA). The 488-nm and the 543-nm excitation wavelengths from an argon laser and the 530- and 590-nm emission filters were used to collect images from the green fluorescent probe (H₂DCFDA) and the red fluorescence probe (annexin V), respectively. To minimize photo-oxidation of the probe, the laser beam was attenuated to 50% of maximal illumination, and exposure of cells to light was limited to the image acquisition intervals (2 s every 3 min) via the acquisition software. Cells were viewed with a $\times 60$ objective lens. Control and treated dishes were scanned at the same setting parameters. Analysis of the fluorescence signal was performed with Adobe Photoshop (Adobe, San Jose, CA) for each cell and for each period of time over a similar region of interest. In three different experiments, the fibroblasts exposed to 10% CSE were double stained with annexin V-FITC and propidium iodide to distinguish between apoptotic and necrotic cells.

Nuclear staining with Hoechst 33342. The cells exposed 3 h to 10% CSE were washed with PBS and incubated with Hoechst 33342 (5 mg/ml), a DNA-binding fluorescence dye, for 30 min at 37°C. The morphological characteristics of apoptotic cells were identified with the aid of a fluorescence microscope. Cells with fragmented and/or condensed nuclei were classified as apoptotic cells.

Single cell gel electrophoresis (Comet assay). The alkaline single cell gel electrophoresis assay (Comet assay) was developed to assess DNA fragmentation typical of toxic DNA damage and of early-stage apoptosis (14, 15). This assay, when electrophoresis is performed at pH ≥ 13 , provides a sensitive means of analyzing alkali-labile sites and single- and double-strand DNA breaks in small number of cells. In the electrophoretic field, DNA fragments, if present, migrate toward the anode, thus forming a comet-shaped strip. Usually, the comet tail is measured and taken as an indicator of DNA damage. Moreover, the amount of migrated DNA can be measured fluorometrically, and the product of comet tail by the percentage of migrated DNA gives an additional estimate (moment) for evaluating the total DNA damage.

In the present study, lung fibroblasts were exposed to fresh medium (control) or to 10% CSE with or without 1 mM NAC. After 3 h, the cells were trypsinized and resuspended in PBS. The assay was performed basically according to Frenzilli et al. (13). Briefly, roughened slides were cleaned with 100% methanol and air-dried. Two solutions, 0.5% normal melting agarose (NMA) and 0.7% low melting agarose (LMA) were prepared in Ca²⁺-, Mg²⁺-free PBS. For the first layer, 0.1 ml of NMA was used, whereas 85 μ l of 0.7% LMA, together with 1×10^4 cells (10 μ l of cell suspension plus 75 μ l of LMA), were used for the second layer. Finally, a third layer of 85 μ l of LMA was added. Slides were immersed in ice-cold freshly prepared lysis solution (1% sodium sarcosinate, 2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris-HCl, 1% Triton X-100, and 10% DMSO, pH 10) to lyse the cells and to allow DNA unfolding. After 1 h at 4°C in the dark, slides were placed on a horizontal electrophoresis unit. The unit was filled with fresh buffer (1 mM Na₂-EDTA and 300 mM NaOH, pH 13) to cover the slides. The slides were allowed to set in the high-pH buffer for 20 min to allow DNA unwinding and expression of alkali-labile sites. Electrophoresis was conducted for 20 min

at 25 V (300 mA). Alkali and electrophoresis treatments were performed in an ice bath. Subsequently, slides were washed gently to remove alkali and detergent in a neutralization buffer (0.4 M Tris·HCl, pH 7.5) and stained with 100 μ l of ethidium bromide (2 μ l/ml). All the steps described above were conducted under yellow light or in the dark to prevent additional DNA damage.

Cell GSH modulation with NAC and BSO. Because the pro-oxidant effects of CSE may be dependent on intracellular GSH concentration (25), in separate experiments we exposed fibroblasts to 10% CSE in coinubation with or without the GSH precursor NAC (1 mM). Furthermore, since BSO is known to induce GSH depletion, fibroblasts were preincubated in parallel experiments for 3 h with BSO (125 μ M) and then exposed to 1% CSE with or without NAC at the same concentration used in the previous experiments. Intracellular GSH was measured on lysed cells with a commercially available assay kit (Glutathione Assay Kit; Calbiochem, San Diego, CA) according to the manufacturer's protocol. The detection limit of the assay was 5 μ M.

Statistical analysis. Results obtained by FACS were evaluated by Kolmogorov-Smirnov statistical analysis for comparisons between frequency distributions of fluorescence intensity of cells. Statistical difference between distribution of events was accepted with $D/s(n) > 8.50$.

Confocal microscopy results were expressed as fluorescence intensity/1,000 pixels, and the differences between treated cells and corresponding controls were tested with ANOVA. Statistical difference was accepted at $P < 0.05$.

Results obtained by Comet assay were evaluated by the nonparametric Kruskal-Wallis test for differences between the median values.

RESULTS

Effect of CSE. Owing to the reported CSE-induced autofluorescence in alveolar macrophages (35), we checked first whether autofluorescence due to CSE also occurred in HFL-1 cells. These experiments (triplicates) showed a slight and not significant increase of fluorescent cells after 10% CSE exposure [$D/s(n) < 8.5$] compared with unexposed cells. Moreover, cells exposed to 10% CSE and incubated with H₂DCFDA or annexin V showed a significant increase of fluorescence intensity compared with cells exposed to 10% CSE in the absence of either probes [$D/s(n) = 28.46$ and $D/s(n) = 24.01$, respectively].

FACS analysis of H₂DCFDA-labeled cells showed that treatment for 3 h of HFL-1 with various CSE concentrations (1, 5, and 10%) caused a dose-dependent increase in cellular oxidative stress. With CSE at the lowest concentration (1%) we were able to determine a slight but not significant increase in cellular oxidation (Fig. 1A), whereas the increase was statistically significant with 5% CSE [Fig. 1B, $D/s(n) = 29.08$] and with 10% CSE [Fig. 1C, $D/s(n) = 42.34$]. Similar results were obtained when cells exposed to CSE were assayed for apoptosis. The lowest dose of CSE (1%) increased cellular apoptosis, but the increase was not statistically significant (Fig. 2A). However, fibroblasts apoptosis drastically increased after exposure to 5% CSE [Fig. 2B, $D/s(n) = 33.90$] and 10% CSE [Fig. 2C, $D/s(n) = 47.39$]. Because annexin V does not discriminate between apoptotic and necrotic cells, we used

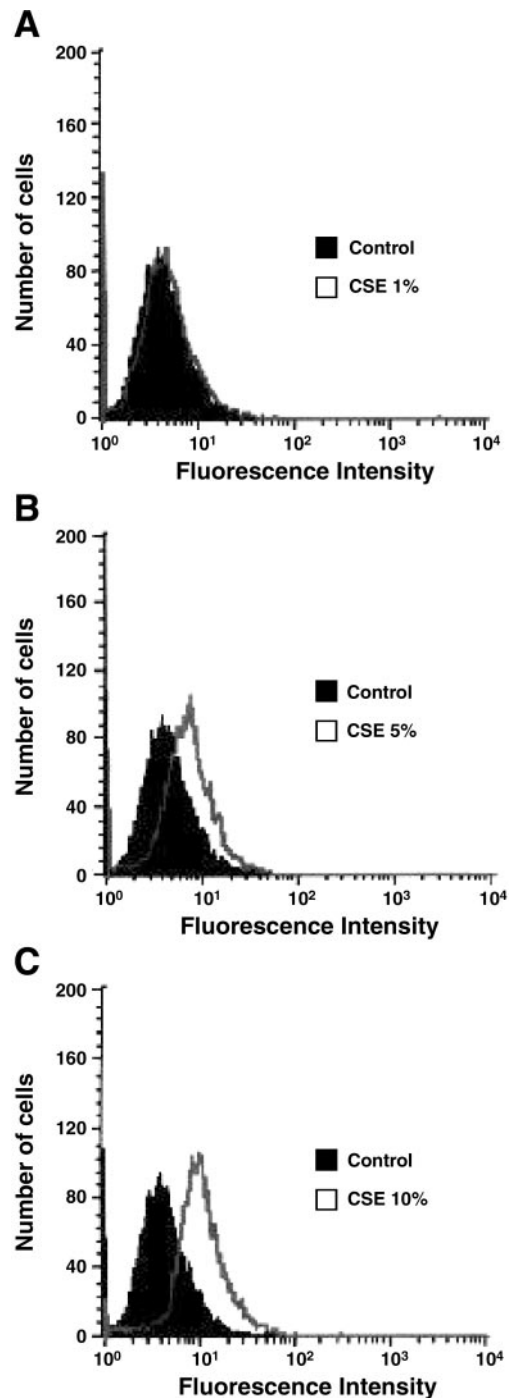


Fig. 1. Dose-response effects of cigarette smoke extract (CSE) on oxidation in cultured lung fibroblasts as evaluated by flow cytometry. The x-axes represent the intensity of intracellular dichlorodihydrofluorescein diacetate (H₂DCFDA) fluorescence. A slight, but not significant, increase was observed with 1% CSE (A). Significant increases of oxidative stress were observed in cells exposed to 5% (B) and 10% CSE (C). Data are representative of 5 independent experiments.

propidium iodide to identify necrotic cells in three separate experiments. As shown in Fig. 3 as a representative experiment, the percentage of apoptotic cells was much greater ($14.5 \pm 3.9\%$) than necrotic cells ($3.0 \pm 2.2\%$) after exposure to 10% CSE. To verify

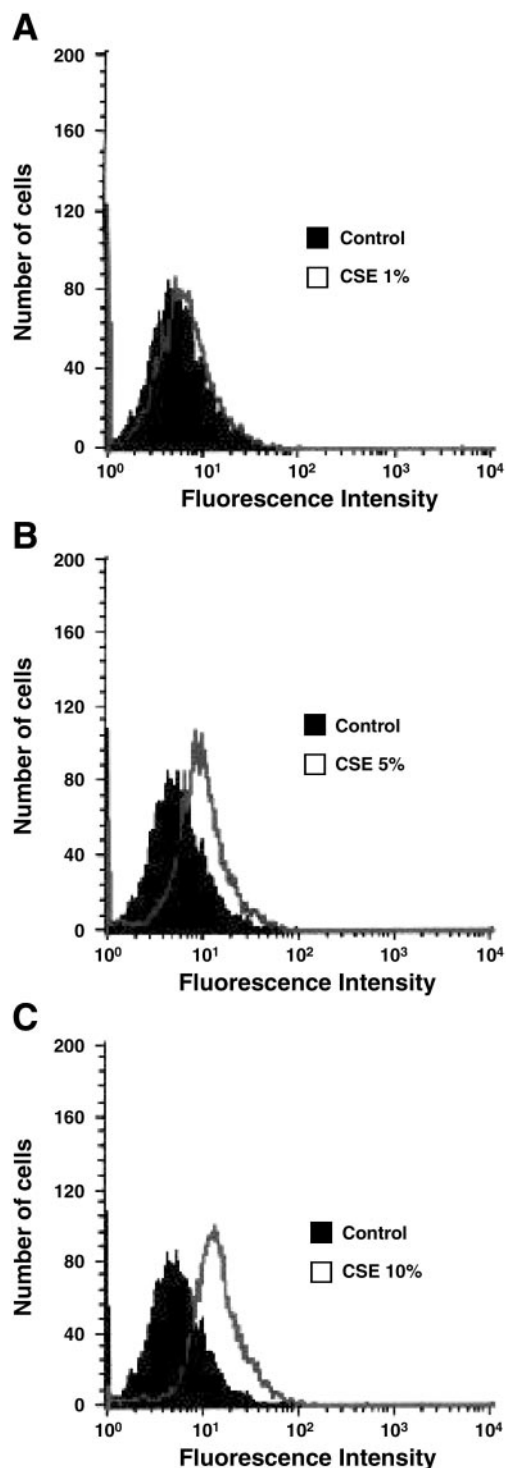


Fig. 2. Dose-response effects of CSE on apoptosis in cultured lung fibroblasts, as evaluated by flow cytometry. The x-axes represent the intensity of intracellular annexin V fluorescence. A slight but not significant increase of number of apoptotic cells was observed with 1% CSE (A). Significant increases of apoptosis were observed after exposure to 5% CSE (B) and 10% CSE (C). Data are representative of 5 independent experiments.

whether single components of CSE induce oxidative stress and apoptosis, we exposed cells to acetaldehyde (1 and 10 mM) and acrolein (10 and 100 μ M). Acetaldehyde was not able to induce either oxidative stress or

apoptosis at the used concentrations. However, acrolein induced both oxidative stress [$D/s(n) = 34.3$] and apoptosis [$D/s(n) = 25.4$] at high concentrations compared with controls (data not shown).

GSH modification with NAC and BSO. Fibroblasts were exposed for 3 h to 10% CSE with or without a concomitant nontoxic dose of NAC (1 mM). Figure 4 shows the ability of NAC to protect partially fibroblasts against the toxic effects of cigarette smoke, with a significant decrease of cellular oxidative stress [Fig. 4A, $D/s(n) = 18.19$]. Likewise, NAC also reduced apoptosis, as shown in Fig. 4B [$D/s(n) = 29.83$].

Because the lowest dose of CSE (1%) resulted in only a minimal increase in oxidative stress and apoptosis, we preincubated the cells for 3 h with the GSH-depleting agent BSO (125 μ M) to enhance the effects of CSE. The cells were then exposed to 1% CSE with or without 1 mM NAC. Figure 5A shows that GSH depletion was able to strengthen the effect of low-dose CSE (1%) with a significant increase in cellular oxidative stress [$D/s(n) = 46.27$]. Coincubation with NAC did not completely counterbalance the effect of BSO, but a significant decrease of oxidation was detectable [Fig. 5B, $D/s(n) = 18.58$]. We also evaluated whether pretreatment with BSO would result in an increase of apoptosis with 1% CSE. BSO pretreatment was not able to increase the intensity of annexin V fluorescence after exposure to 1% CSE (data not shown).

The exposure to CSE was associated with a significant, dose-dependent reduction of intracellular GSH (Fig. 6). Intracellular GSH increased after coincubation with 1 mM NAC and 10% CSE ($15.4 \pm 1.4 \mu$ M), though not significantly, compared with 10% CSE alone ($14.6 \pm 2.8 \mu$ M). To the same extent, GSH content after exposure to 1% CSE ($17.8 \pm 4.4 \mu$ M) was reduced by BSO preincubation ($14.9 \pm 1.9 \mu$ M), and

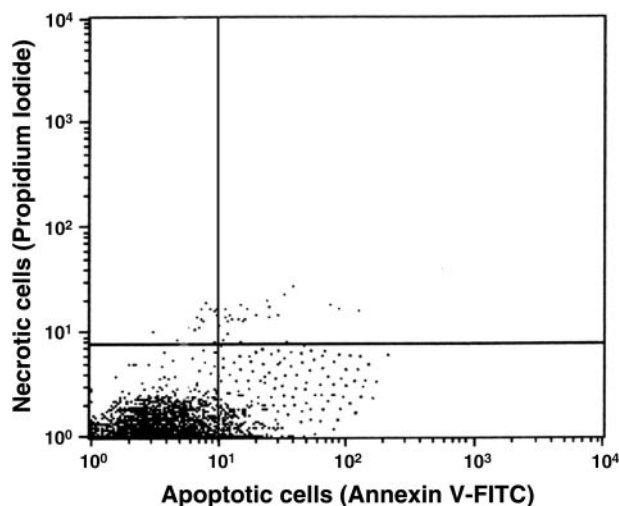


Fig. 3. Human fetal lung fibroblasts (HFL)-1 treated with 10% CSE for 3 h were double stained with annexin V-FITC and propidium iodide followed by flow cytometric analysis. X-axis, annexin V-FITC fluorescence; y-axis, propidium iodide fluorescence. There was a significant higher proportion of apoptotic cells compared with necrotic cells.

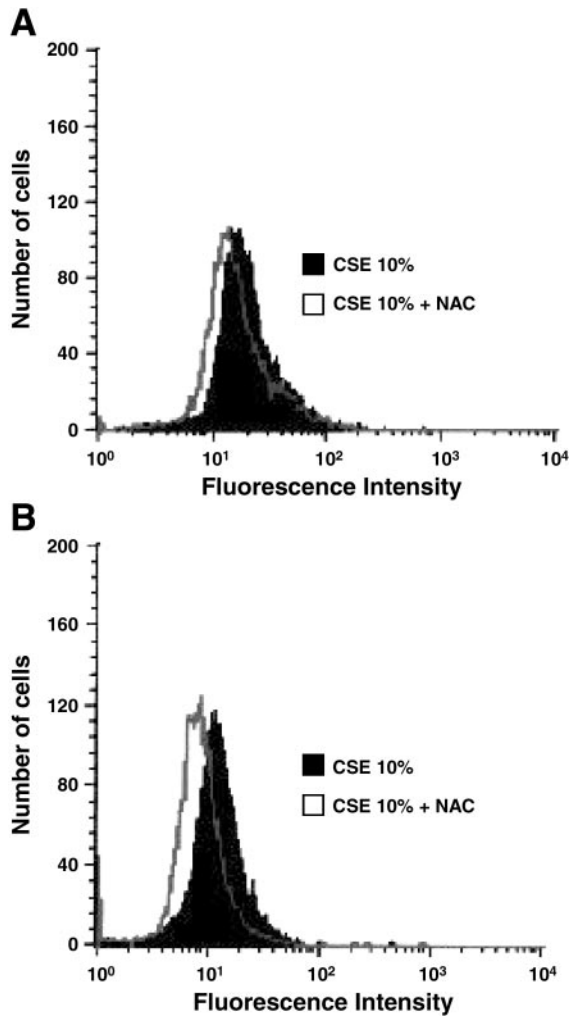


Fig. 4. Effect of *N*-acetylcysteine (NAC) on CSE-induced oxidative stress and apoptosis on HFL-1, as analyzed by flow cytometry. The *x*-axis denotes the intensity of intracellular H_2DCFDA (A) or annexin V fluorescence (B). When cells were coincubated with NAC, a significant decrease of both fibroblast oxidation (A) and apoptosis (B) was obtained. A and B represent 1 of 3 separate experiments.

this effect was partially reversed by coincubation with NAC ($15.6 \pm 3.8 \mu M$).

Monitoring of oxidative stress and apoptosis by confocal laser fluorescence microscopy. Results obtained by flow cytometry were confirmed at the single cell level (Fig. 7). As observed under light transmission microscopy, cells exposed to 10% CSE showed marked morphology changes (Fig. 7B) compared with controls (Fig. 7A). Coincubation with NAC was able to protect cells against the harmful effect of CSE (Fig. 7C), the cells looking like the controls. When we used confocal laser scanning microscopy, we observed a clear increase in fluorescence intensity, both with the oxidation probe (Fig. 7E) and with the apoptosis probe (Fig. 7H), within the cells exposed to 10% CSE compared with untreated cells (Fig. 7, D and G, respectively). It is interesting to note that the spots of red fluorescence (Fig. 7H) are present in the same cells but in different subcellular compartments from spots of green fluorescence in Fig.

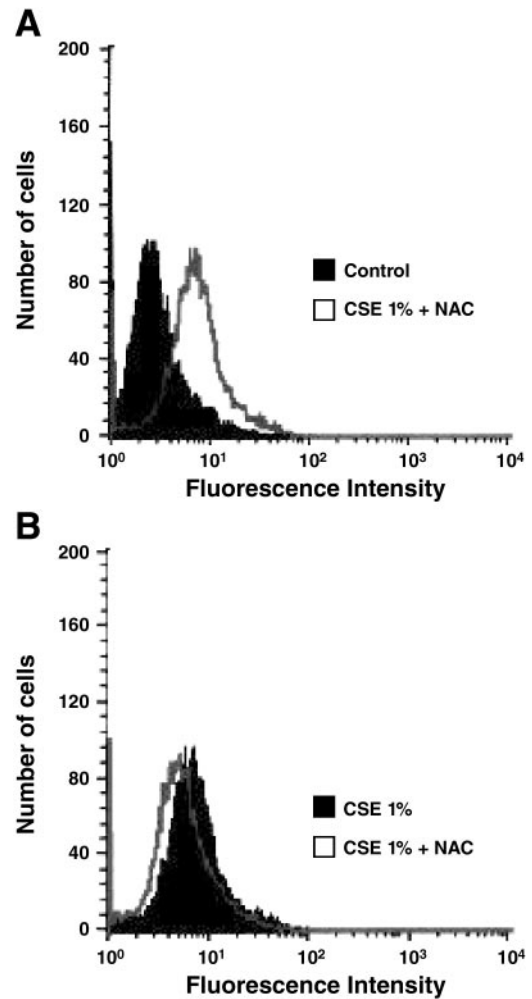


Fig. 5. Effect of buthionine sulfoximine (BSO) pretreatment on CSE-induced lung fibroblast oxidation, as analyzed by flow cytometry. Cells exposed to 1% CSE show a significant increase of cellular oxidative stress compared with control (A). When NAC was added to the cultures, we detected a significant decrease of cellular oxidative stress caused by BSO (B). This figure represents 1 of 3 separate experiments.

7E, suggesting that oxidation and apoptosis are concomitant but distinct phenomena. Moreover, NAC was able to decrease the fluorescence intensity of both oxidative stress (Fig. 7F) and apoptosis (Fig. 7I) induced

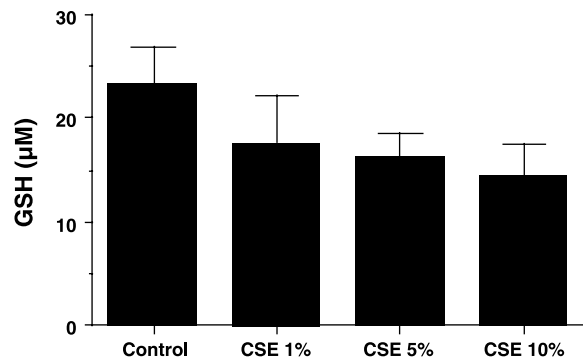
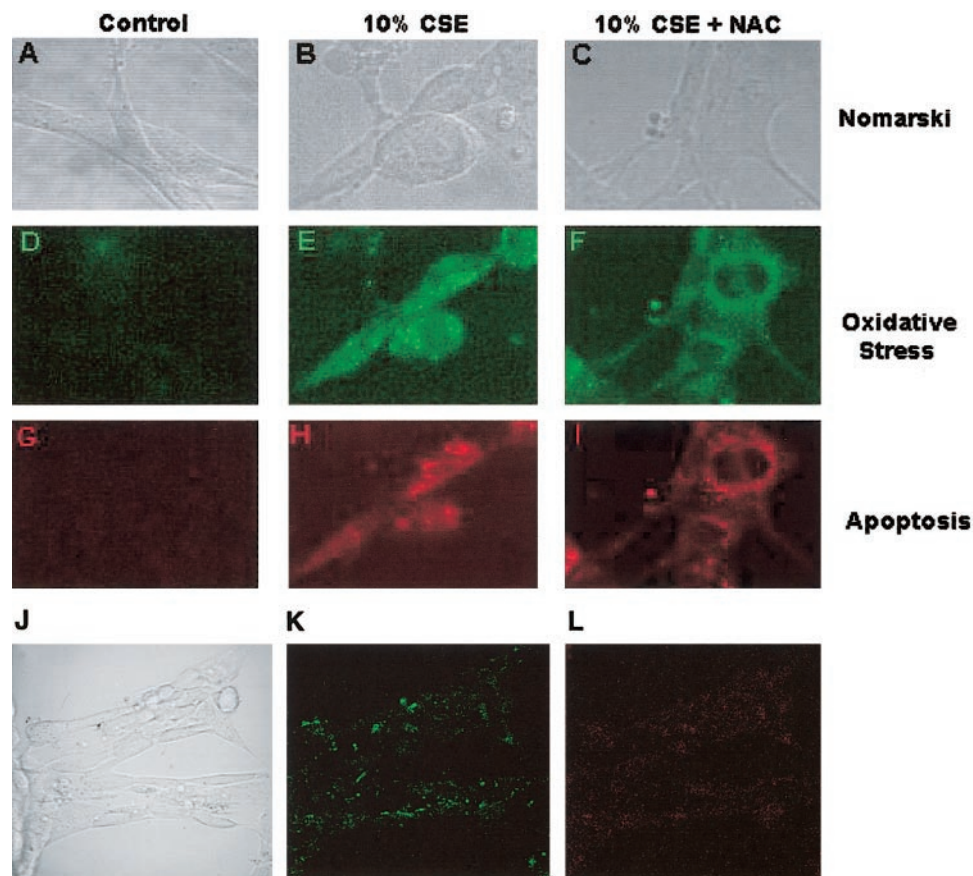


Fig. 6. Dose-response effects of CSE on intracellular GSH in cultured lung fibroblasts. Significant decrease of intracellular GSH was observed in cells exposed to 5 and 10% CSE. * $P < 0.05$.

Fig. 7. *A–I*: imaging of oxidative stress and apoptosis in lung fibroblasts exposed to CSE using confocal laser scanning microscopy. *A–C* show images of the cells with Nomarski microscopy. Marked morphology changes were observed when cells were exposed to 10% CSE (*B*) compared with nonexposed cells (*A*). NAC was able to protect cells against cigarette smoke with no evident alterations in cellular morphology (*C*). *D–F* show the results obtained with the oxidation probe H₂DCFDA (green). A great amount of H₂DCFDA linked to stressed cells (*E*) was observed, whereas no fluorescence is evident in control cells (*D*). When NAC was added to the cultures, there was an evident decrease of fluorescence intensity caused by CSE (*F*). *G–I* show the same cells fields after labeling of apoptotic cells with annexin V (red). No fluorescence was observed in control cells (*G*), whereas evident annexin V labeling was obtained after exposure to 10% CSE (*H*), indicating cell apoptosis. NAC coinubation significantly decreased the red fluorescence intensity caused by CSE (*I*). *J–L*: HFL-1 treated with 10% CSE for 3 h and double stained with annexin V-FITC and propidium iodide followed by confocal laser fluorescence microscopy analysis of apoptotic (*K*) and necrotic (*L*) cells. HFL-1 cells are shown under Nomarsky microscopy (*J*). There is a significant accumulation of annexin V in cell membranes (*K*), whereas the propidium iodide is not embedded in exposed cells (*L*).



by CSE. After the double staining with annexin V-FITC and propidium iodide, we observed a significant accumulation of annexin V in cell membranes (Fig. 7*K*), whereas there was labeling with propidium iodide (Fig. 7*L*).

To quantify the observed differences of oxidative stress and apoptosis showed in Fig. 7, we performed a computer analysis of fluorescent signals on an equivalent region of interest, and the results were expressed as fluorescence/1,000 pixels. CSE at 10% caused a significant increase in oxidative stress (***P* < 0.01, Fig. 8, solid bar) and apoptosis (***P* < 0.01, Fig. 8, open bar) compared with untreated cells. Coincubation with NAC significantly decreased both cellular oxidation (***P* < 0.01, Fig. 8, solid bar) and apoptosis (**P* < 0.05, Fig. 8, open bar).

Detection of apoptotic cells by Hoechst 33342. Quantitative detection of apoptotic cells was performed by manual counting of Hoechst 33342-positive cells. After CSE exposure (10%), nuclear staining with Hoechst 33342, which was consistent with apoptosis, was evident in ~16% of cells, a percentage significantly different compared with nonexposed cells (Fig. 9).

DNA damage. The detection of damaged cells assessed by the Comet assay, after 3-h treatment with 10% CSE, showed a marked increase in DNA fragmentation compared with controls, as revealed by the appearance of comet-like nuclei, where the head of the comet is the unfragmented portion of DNA and the tail

of the comet is the damaged DNA (Fig. 10*B*). The great majority of nuclei in control cells looked perfectly rounded (Fig. 10*A*), with only few cells showing DNA fragments moving as a comet tail. However, DNA frag-

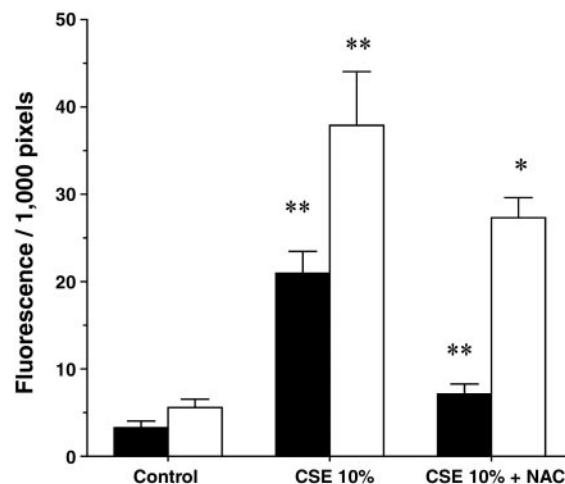


Fig. 8. Analysis of the fluorescence intensity obtained at confocal laser scanning microscopy. Analysis of fluorescence was performed for each cell for the same period of time over an equivalent region of interest and expressed as fluorescence/1,000 pixels. Oxidative stress, solid bars; apoptosis, open bars. Fluorescence significantly increased when cells exposed to 10% CSE were analyzed for oxidative stress and apoptosis (***P* < 0.01). NAC significantly decreased fluorescence signals both of cell oxidation (***P* < 0.01) and apoptosis (**P* < 0.05).

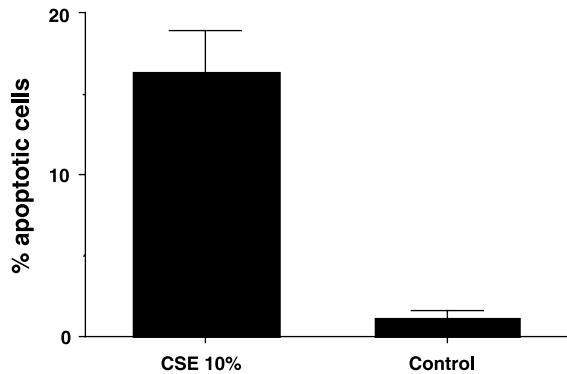


Fig. 9. Analysis of apoptotic cells by Hoechst 33342. Quantitative detection of apoptotic cells was performed by means of manual counting of fluorescent cells showing nuclear fragmentation and/or condensation. After CSE exposure (10%), the percentage of apoptotic cells was significantly higher ($16.3 \pm 2.6\%$) compared with nonexposed cells ($1.1 \pm 0.5\%$).

mentation after 10% CSE exposure was regularly obtained, with only 1–2% of treated cells still looking like controls. The amount of fragmented DNA moving in the comet tail is reflected by the length and the width of the tail (larger fragments move less than smaller fragments), and it was as high as 93% of total DNA. Coincubation of cells with NAC almost completely blocked DNA fragmentation observed following treatment with 10% CSE (Fig. 10C). Table 1 shows the medians of the tail length and of the tail moment (as defined as the product of the distance between the head and tail mass centers and the relative amount of DNA in the tail compared with the total DNA in each comet) (27).

DISCUSSION

Cigarette smoke is known to induce oxidative stress and inflammation in pulmonary tissues and cells, both in vitro and in vivo. It contains over 4,000 chemical species, including high concentrations of oxidants (9). The imbalance between oxidants and antioxidants is involved in the development of pulmonary emphysema (6, 36). However, since only some 15% of smokers develop emphysema, it is possible that not only the destruction but also the abnormal quantitative and qualitative repair of pulmonary tissue might be involved in the development of this disease (37).

Fibroblasts are the main cell type in connective tissue, and they play a major role in the repair of pulmonary tissue. Previous studies support the idea that fibroblasts are targets for a wide variety of stimuli

Table 1. Induction of DNA damage by cigarette smoke

Condition	Tail Length, μ	Tail Moment
Control	2.5 ± 10.3	7.3 ± 35.2
10% CSE	$40.3 \pm 16.8^*$	$614.1 \pm 742.3^*$
10% CSE + NAC	2.8 ± 8.0	3.2 ± 10.8

Values are mean \pm SE. Analysis by Kruskal-Wallis test shows a significant increase of tail length and tail moment when cells were exposed to 10% cigarette smoke extract (CSE) ($*P < 0.01$). N-acetylcysteine (NAC) abolished completely the effect of CSE with tail length and moment in the same order of magnitude of controls.

including cigarette smoke. For instance, cigarette smoke was able to inhibit fibroblast recruitment and proliferation (26) and to alter fibroblast-mediated collagen gel contraction in vitro (5). We used a human lung fibroblast cell line as a model to study some of the harmful effects of cigarette smoke. In this study, we showed that cigarette smoke is able to induce cellular oxidative stress and that the oxidation depends on the concentrations of CSE. We also demonstrated that cigarette smoke induces fibroblast apoptosis that parallels the oxidation, possibly because direct cigarette smoke oxidants and/or intracellular reactive oxygen species (ROS) generated by cigarette smoke can switch on apoptotic pathway(s) in fibroblasts. Assessment of apoptosis in our experiments was based on annexin V labeling and nuclear staining with Hoechst 33342. DNA fragmentation, which is involved in the development of programmed cell death as a part of total CSE-induced DNA damage, was studied by the Comet assay.

The Comet assay is currently used to show DNA fragmentation (30) to such an extent that warnings on the use of Comet assay as an indicator of genotoxic damage when apoptosis may also occur have been recently published (8). The oxidative and proapoptotic effects of cigarette smoke exposure on HFL-1 cells begin at very low CSE concentrations (1%) and reach statistical significance against controls at 5% concentration just after 3 h of incubation. This suggests that fibroblasts in the lung interstitium of smokers may be continuously challenged by compounds capable of interfering with their functions and/or lifespan. We speculate that the concomitant oxidation and apoptosis in human lung fibroblasts we observed in vitro after short-term exposure to CSE may lead, when repeated thousands of times in a smoker's life, to a defective tissue repair and contribute to the development of

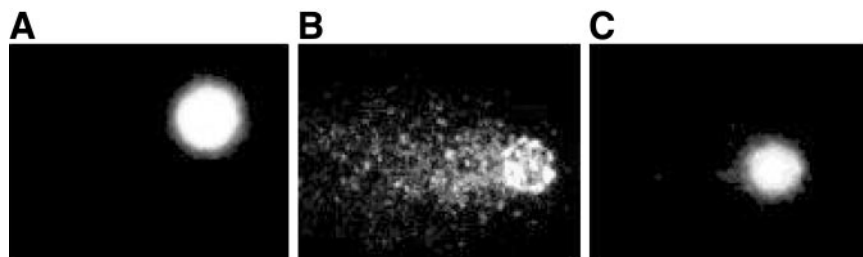


Fig. 10. Effects of CSE exposure on DNA fragmentation in human lung fibroblast. Fibroblasts were incubated for 3 h with fresh medium (control), 10% CSE, or 10% CSE in the presence of 1 mM NAC. DNA fragmentation was evaluated using the single cell gel electrophoresis assay, also known as Comet assay, as described in MATERIALS AND METHODS. Marked DNA fragmentation was observed in cells exposed to 10% CSE (B) compared with control (A). NAC was able to prevent almost completely the DNA damage caused by CSE (C).

pulmonary emphysema (31). The negative effect of CSE on fibroblast functions due to oxidation (26) may further hamper the efficacy of tissue repair. The increase of apoptotic changes, as revealed by the attachment of annexin V to the outer layer of the cell membrane of HFL-1 cells, occurred in our study just 3 h after CSE exposure. This is in agreement with very recent observations from Jungas and colleagues (17), who demonstrated activation of Bax 4 h after exposure of HeLa cells to H₂O₂.

We also evaluated the efficacy of GSH modification to modulate the toxic effects of cigarette smoke on human lung fibroblasts. To this end, we first used NAC, a potent antioxidant agent showing protective effects in several models of lung injury (3, 40), to enhance intracellular GSH content by increasing cysteine supply (10).

NAC can also block apoptosis induced by different agents (32), and the protective effect of NAC in nicotine-induced apoptosis in rat gingival fibroblasts has been reported (19).

In our experiments NAC was able to reduce the toxic effects of cigarette smoke with a significant and parallel decrease of both oxidative stress and apoptosis. These results lend further support to the hypothesis that cellular oxidative stress and apoptosis induced by cigarette smoke are closely related each other. Moreover, it is known that depletion of GSH can drastically reduce the defenses against oxidant-induced lung injury (25), and it also causes an increase of apoptotic cells (22). We then used a GSH-depleting agent such as BSO to modify intracellular GSH (4, 11, 12). Depletion of GSH caused by BSO significantly enhanced the effect of low doses of CSE. In fact, although 1% CSE resulted in only a slight increase of oxidative stress in fibroblasts, cellular oxidation dramatically increased when cells were preincubated with BSO. Depletion of GSH, together with ROS production, has been also suggested to be involved in apoptosis (12), and depletion of cellular thiols is known to induce apoptosis in T cells and fibroblasts (1, 33). Coincubation of HFL-1 with 1 mM NAC did not completely correct the 10% CSE-induced GSH depletion. Similarly, NAC did not reverse the BSO- and 1% CSE-induced GSH depletion, suggesting that the observed antioxidant effect of NAC may be due, at least in part, to a direct scavenging effect on CSE-derived oxidant radicals. Altogether, these results expand very recent observations by Ishii and coworkers (16), by showing that the proapoptotic influence of CSE on HFL-1 cells is associated with cell oxidation and that these effects can be observed as early as 3 h after CSE exposure.

These results support recent findings on the importance of resident cells in the development of emphysema. In a recent paper (18), cigarette smoke has been reported to alter VEGF maintenance of pulmonary endothelial cells, and this may result in emphysema due to pulmonary endothelial cell death, followed by progressive disappearance of the alveolar septa by apoptosis. This model of emphysema does not appear to involve inflammatory cells, and it indicates that paren-

chymal lung cells may enter a death cycle in emphysema before the actual damage to the parenchymal extracellular matrix. In this study, we demonstrated that fibroblasts undergo apoptosis after exposure to cigarette smoke, which parallels the increase in oxidative stress. We speculate that the development of pulmonary emphysema might be not only the result of an imbalance between oxidants and antioxidants, and/or between proteases and antiproteases, but also the consequence of fibroblast oxidation and apoptosis caused by cigarette smoke. Moreover, the protective effect of antioxidants such as NAC against the toxic effects of cigarette smoke underlines the importance of this class of molecules in the treatment of tobacco smoke-induced pulmonary diseases.

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