Induction of DNA double-strand breaks in A549 and normal human pulmonary epithelial cells by cigarette smoke is mediated by free radicals

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Abstract. DNA double-strand breaks (DSBs) are potentially mutagenic/carcinogenic lesions. Induction of DSBs triggers phosphorylation of histone H2AX on *Ser*-139. Phosphorylated H2AX (γH2AX) can be detected immunocytochemically, and the intensity of γH2AX immunofluorescence (IF), reflecting the number of γH2AX-IF foci per nucleus, reveals the frequency of DSBs. Using multiparameter cytometric analysis of γH2AX-IF, we previously observed that DSBs are induced in normal human bronchial epithelial (NHBE) and A549 pulmonary adenocarcinoma cells following exposure to cigarette smoke (CS) or smoke condensate. In the present study, we show that N-acetyl L-cysteine (NAC) and glutathione, both effective scavengers of free radicals, prevented induction of DSBs by CS in these cells. In contrast, the glutathione synthesis inhibitor, DL-Buthionine-[S,R]-sulfoximine (BSO), enhanced the induction of DSBs by CS. The observed reduction of DSBs by NAC correlated with protection of the reproductive capability (clonogenicity) of A549 cells treated with CS. The data implicate formation of free radicals by CS as factors generating DSBs and affecting cell survival. Interestingly, at the conditions of exposure to CS when clonogenicity was only moderately affected, S-phase cells showed significantly higher sensitivity in terms of induction of DSBs compared with G_1 or G_2M cells. In light of the evidence that CS increases oxidative stress and induces cell proliferation in the lungs of smokers, the high propensity of S-phase cells to develop DSBs upon exposure to CS has to be considered as a potentially pathogenic event in smoke-induced tumor development. This is the first report to reveal cell cycle-phase specificity in both the induction of DSBs by CS and their prevention by free radical scavengers. The detection of γH2AX to assess the induction of CS-induced DSBs and their relationship to cell cycle phase provides a convenient tool to explore approaches to protect cells from this type of genotoxic damage.

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Introduction

The leading preventable cause of death and disability in the United States is the chronic use of tobacco products, in particular, cigarettes. In addition to lung cancer, tobacco use plays important direct and indirect roles in the etiology of a wide range of other cancers, including those of the upper aerodigestive tract (i.e., oral cavity, pharynx, larynx, and esophagus), bladder, stomach, kidney, pancreas, uterine cervix, and blood (1-3). While numerous studies have elucidated some of the biological effects of cigarette smoke that result in its ability to induce this diverse range of malignancies in smokers, little is known about the precise nature of genetic events that drive specific stages in the multi-step processes that result in clinically evident disease (2,4,5). This is due to the fact that CS is a complex chemical mixture of gases and suspended particulate matter that contains a wide range of carcinogens, mutagens, and free radicals that can induce an array of DNA lesions (5) that have been strongly implicated in the development and progression of human cancers by epidemiological studies (6,7).

DSBs are a major type of DNA damage that can lead to translocations and chromosomal instability, two important mechanisms in the generation of malignant tumors (8-10). Consequently, detection of DSBs may provide a more precise measurement of the potential cancer risk in individuals exposed to CS. A sensitive means to detect formation of DSBs is to assess histone H2AX phosphorylation. Histone H2AX, a variant of a family of at least eight protein species of the nucleosome core histone H2A (11,12), becomes phosphorylated shortly upon induction of DSBs (13,14). The phosphorylation of H2AX on Ser-139 at sites flanking the DSBs is carried out by ATM (13-15), ATR (16), and/or DNA-dependent protein kinases (DNA-PKs) (17). The phosphorylated form of H2AX is denoted γH2AX. The availability of antibodies to γH2AX allows for immunocytochemical detection of DSBs (18). After

induction of DSBs, the appearance of γH2AX in chromatin manifests as discrete foci (14,18), each focus considered to represent a single DSB (14). The intensity of γH2AX immunofluorescence (IF) measured by cytometry was reported to strongly correlate with the dose of ionizing radiation and, thus, with the number of induced DSBs (19). In fact, γH2AX IF has been proposed as a surrogate for cell killing in viability assays of irradiated cells (20).

In previous studies, we expanded the utility of γH2AX antibody in conjunction with multiparameter flow- and laser scanning cytometry as an assay of DSBs, to detect and measure their induction in individual, live cancer cells exposed to antitumor drugs *in vitro* (21). Within certain limits, the intensity of γH2AX IF correlated well with the concentration of several antitumor drugs and duration of cell exposure to the drug, indicating a relationship between the incidence of DSBs induced by these drugs and γH2AX IF intensity. Likewise, γH2AX IF correlated strongly with the dose of UV-B irradiation of HL-60 or HeLa cells (22). Multiparameter analysis of γH2AX IF and cellular DNA content made it possible to relate the abundance of DSBs (extent of DNA damage) to the position of the cell in the cycle (21,23).

We recently used this assay to demonstrate for the first time that DSBs are induced in both NHBE and A549 human pulmonary adenocarcinoma cells exposed either to CS or smoke condensate. However, the specific components of cigarette smoke that caused these DSBs were not investigated. In the present report, we show that the thiol *N*-acetyl-L-cysteine (NAC), a potent free radical scavenging compound with antioxidant activity, can prevent formation of most of the DSBs induced by CS. Our data also show that at longer CS-exposure times, although cells at the G_1 phase of the cell cycle exhibited the greatest DNA damage compared to S-phase cells, the latter were more sensitive to DNA damage if either the time of exposure or the concentration of CS was diminished. Furthermore, the protective effect of NAC was less pronounced in the case of S- than G_1-phase cells. Further evidence that CS induces DSBs via reactive intermediates was that the addition of the cellular antioxidant, glutathione (GSH), during CS exposure reduced, while depletion of glutathione by DL-Buthionine-[S,R]-sulfoximine (BSO) increased, the sensitivity of A549 cells to the DSB-inducing effect of CS. Moreover, with both BSO and GSH treatments, S-phase cells demonstrated increased sensitivity compared to G_1- and G_2M-phase cells. These observations suggest that S-phase cells require more effective oxidant suppression to ensure adequate protection from CS-induced DSBs. Since it is known that CS depresses antioxidant potential, increases oxidative stress, and induces lung cell proliferation in smokers (24-27), the increased sensitivity of actively dividing S-phase cells to CS-induced DSBs may play a pivotal role in the generation of genotoxic events such as translocations that contribute to pulmonary carcinogenesis.

Materials and methods

Cell culture and smoke treatment. A549 cells were purchased from American Type Culture Collection (ATCC no. CCL-185, Manassas, VA) and were cultured in Ham's F12K medium with 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate (ATCC) and supplemented with 10% fetal bovine serum (ATCC). NHBE cells were purchased from Cambrex BioScience, Walkersville, MD and were cultured in complete bronchial epithelial cell growth medium (BEGM), prepared by supplementing bronchial epithelial basal medium (BEBM) with retinoic acid, human epidermal growth factor, epinephrine, transferrin, triiodothyronine, insulin, hydrocortisone, bovine pituitary extract and gentamicin by addition of SingleQuots™ . BEBM and SingleQuots were purchased from Cambrex BioScience. All incubations were at 37˚C in a humidified atmosphere of 5% CO_2 in air.

Smoke treatment was performed in the same manner for both A549 and NHBE cells. Dual-chambered slides (Nunc Lab-Tek II, VWR International, West Chester, PA) were seeded with 1 ml of $5x10^4$ cells/ml cell suspension per chamber 48 h before exposure and were typically at 70% confluency at the time of smoke treatment. The cell culture medium was replaced with 37˚C Dulbecco's PBS (D-PBS) containing calcium and magnesium (BioSource, Rockville, MD) for the smoke exposure. Slide chamber covers were removed and the slides were placed in a smoke exposure chamber (20.6 cm x 6.7 cm x 6.3 cm - LxWxH). Smoke was generated either from IM16 cigarettes (Industry Monitor #16, Philip-Morris, Richmond VA, containing 15.7 mg 'tar' and 1.01 mg nicotine per cigarette) or commercially available packs of the leading U.S. brand of full-flavored cigarettes (containing 14.8 mg 'tar' and 1.16 mg nicotine per cigarette) under Federal Trade Commission (FTC) (28) smoking conditions (35±0.3 cc puff, one puff every 60 sec, 2-second puff duration with none of the ventilation holes blocked) using a KC 5 Port Smoker (KC Automation, Richmond, VA). Cigarettes were smoked to within 3 mm of the filter tip. All cigarettes had been equilibrated at 23.9±1.1˚C and 60±2% relative humidity for a minimum of 24 h and a maximum of 14 days. The smoke exposure chamber was designed to deliver smoke uniformly diluted with 5% CO_2 in air and passed through the cell exposure chamber at a constant flow rate of 500 cc/min. Briefly, each 35-cc puff was first drawn into a 250-cc round chamber containing 5% CO_2 in air and mixed via a stir bar. The standard smoke dilution used in most of our experiments was 35 cc delivered over 1 min in a 500-cc volume, and the intensity of exposure was varied by varying the length of time the cells spent in the exposure chamber. In one set of experiments, the exposure time was kept constant but the intensity of exposure was varied by diluting the smoke with 5% CO_2 in air such that only a portion of the 35-cc puff was delivered while maintaining a constant flow rate of 500 cc/min. The time and distance that the smoke traveled from the end of the cigarette to the exposure chamber was minimized by using the shortest lengths of tubing possible between the parts of the apparatus.

Mock-exposed cells were treated under the same conditions as the exposed cells except for the absence of a cigarette in the smoking port. Following treatment or mock treatment, the D-PBS covering the cells was aspirated and replaced with 1 ml per chamber of fresh culture medium at 37˚C. The cells were placed in the 37˚C, 5% CO_2 incubator and incubated for 1 h. For H2AX measurements, the medium was aspirated and the cells fixed with 1% paraformaldehyde by gently rocking the slides at room temperature for 15 min. Following aspiration of the fixative, the chamber slides were disassembled and the

slides submerged in 50-ml conical tubes filled with 70% ethanol for storage prior to analysis.

In some experiments cells were exposed to the vapor phase of CS. This was accomplished by placing a 44-mm Cambridge glass fiber filter pad (Performance Systematix Inc., Caledonia, MI) just after the cigarette port such that the smoke flowed through it before reaching the cell exposure chamber. This technique removes over 99% of all particles 0.3 micron or larger from whole smoke (29).

N-acetyl-L-cysteine (NAC) treatment. Cells were treated with up to 25 mM NAC (Sigma, St. Louis, MO) during and/or following exposure to cigarette smoke. NAC was dissolved in culture medium for the post-exposure treatment in order to minimize shock to the cells and was prepared in D-PBS for the concurrent treatment; the pH of the NAC solution was equilibrated to 7.2 before being added to the cells. The NAC was rinsed from the cells using 37˚C D-PBS after the designated time period in order to remove any carryover.

Treatment of cells with reduced L-glutathione (GSH). Cells were treated with up to 25 mM GSH during exposure to cigarette smoke. GSH was prepared in D-PBS for concurrent treatment; the pH of the GSH solution was equilibrated to 7.2 before being added to the cells. The GSH was rinsed from the cells using 37˚C D-PBS after the designated time period in order to remove any carryover.

DL-Buthionine-[S,R]-sulfoximine (BSO) treatment. Cells were treated with 5 μM BSO (Sigma) for 18 h prior to exposure to cigarette smoke. BSO was prepared in culture medium for the pre-treatment; the pH of the BSO solution was equilibrated to 7.2 before being added to the cells. The BSO was rinsed from the cells using 37˚C D-PBS after the designated time period in order to remove any carryover.

Dimethyl-thiourea (DMTU) treatment. Cells were treated with either 10 or 20 mM DMTU (Sigma) for 18 h prior to exposure to cigarette smoke. DMTU was prepared in culture medium for the pre-treatment; the pH of the DMTU solution was equilibrated to 7.2 before being added to the cells. The DMTU was rinsed from the cells using 37˚C D-PBS after the designated time period in order to remove any carryover.

Clonogenic cell assays. A short-term clonogenic cell assay was used to assess the ability of CS to suppress colony formation in A549 cells. Briefly, following smoke exposure, A549 cells were returned to the 37˚C, 5% CO_2 incubator for 2-3 h. The cells were then harvested, along with their supernatant, by trypsinization with trypsin-EDTA (0.25% trypsin-0.38 mg/ml EDTA, Invitrogen Corporation, Carlsbad, CA). Cells were centrifuged at 260 x g for 8 min and pellets were resuspended in 1 ml of Ham's F-12K medium, 10% FBS (complete medium) per pellet and counted. Cells were serially diluted in complete medium so that the mock treated had ~65 cells/well and smoke treated had ~300 cells/well when seeded onto 96-well flat bottom tissue culture plates; one plate per condition. The plates were incubated for 5 days in a 37˚C, 5% CO_2 humidified incubator. After 5 days, the medium was removed and the colonies were washed and fixed with 5% formaldehyde/PBS, and then stained with 0.8% crystal violet solution for visualization. The colonies were counted with the aid of a macroscopic dissecting microscope. Stained colonies containing more than 10 cells were scored and counted. The percentage of colony formation was normalized to colonies formed following mock treatments which served as negative controls for CS exposure.

Immunocytochemical detection of phosphorylated histone H2AX and caspase-3 activation. Cells were treated with CS and fixed as described above, then rinsed twice in PBS and immersed in 0.2% Triton X-100 (Sigma) in a solution of 1% (w/v) bovine serum albumin (BSA; Sigma) in PBS for 30 min to suppress non-specific antibody binding. The cells were then incubated in a 100-μl volume of 1% BSA containing a 1:200 dilution of anti-phosphorylated (*Ser*-139) histone H2AX (γH2AX) mouse monoclonal antibody (Ab) (Upstate, Charlottesville, VA). After overnight incubation at 4˚C, the slides were washed twice with PBS and then incubated in 100 μl of a 1:200 dilution of Alexa Fluor 488 goat anti-mouse IgG (H+L) (Molecular Probes, Eugene, OR) for 45 min at room temperature in the dark. Parallel samples were incubated with 1:100 diluted anti-cleaved (activated) caspase-3 rabbit polyclonal Ab (Cell Signaling Technology, Beverly, MA) overnight at 4˚C, washed twice with PBS and incubated with 1:30 diluted FITC-conjugated F(ab')2 fragment of swine anti-rabbit immunoglobulin (Dako, Carpinteria, CA) for 30 min at room temperature in the dark. The cells were then counter-stained with 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI, Molecular Probes) in PBS for 5 min. Each experiment was performed with an IgG control in which cells were labeled only with secondary antibody, Alexa Fluor 488 goat anti-rabbit IgG (H+L) or FITC-conjugated F(ab')2 fragment of goat anti-mouse immunoglobulins, without primary antibody incubation to estimate the extent of non-specific binding of the secondary antibody to the cells.

Measurement of cell fluorescence by Laser Scanning Cytometry. Cellular green (phosphorylated histone H2AX or activated caspase 3), and blue (DAPI) fluorescence emission was measured using a Laser Scanning Cytometer (LSC; iCys; CompuCyte, Cambridge, MA), utilizing standard filter settings; fluorescence was excited with 488-nm argon ion and violet diode lasers, respectively. The intensities of maximal pixel and integrated fluorescence were measured and recorded for each cell. At least 3,000 cells were measured per sample.

Statistical analysis. To compare the changes in γH2AX immunofluorescence intensity (γH2AX IF), the mean fluorescence intensity (integral values of individual cells) was calculated for cells in each phase of the cycle by gating G_1, S and G_2M cells based on differences in DNA content. The means of the fluorescence value for G_1, S and G_2M populations of cells in the IgG control groups were then subtracted from the respective means of the condensate or smoke-treated cells. After this subtraction, the means of γH2AX IF of mock-treated cells were subtracted from the γH2AX IF means of the CS-treated cells to obtain the CS-induced change in γH2AX IF (Δ γH2AX IF). All experiments were run under identical instrument settings. Data is presented as mean γH2AX

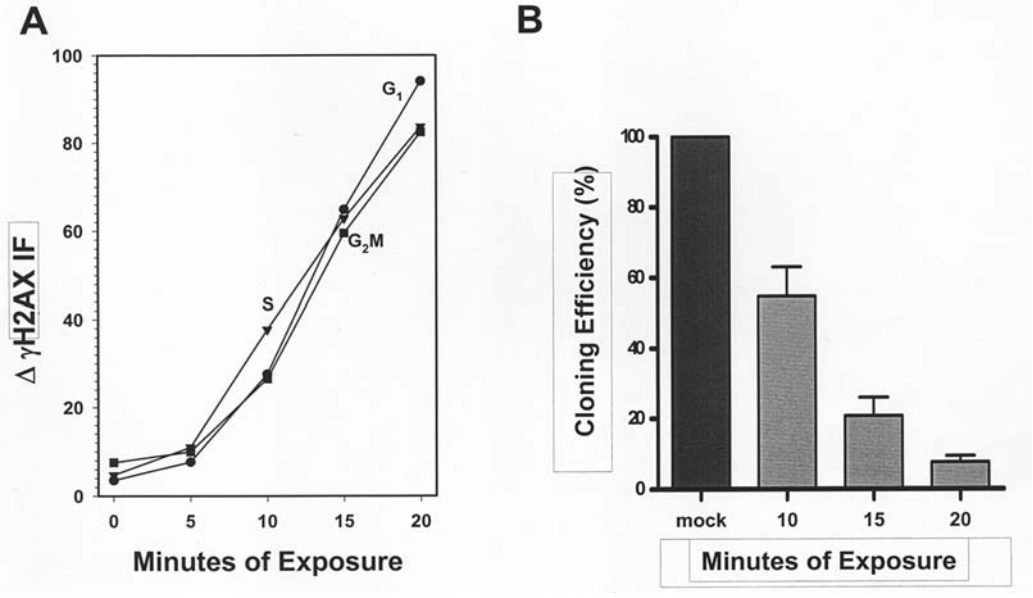

Figure 1. Histone H2AX phosphorylation and cloning efficiency of A549 cells exposed to CS from IM16 for various lengths of time. (A) The increase (△) in mean γH2AX IF (per unit of DNA) of A549 cells exposed to CS for different time intervals was calculated for cells in G_1, S and G_2M phase as described in Materials and methods. (B) Colony formation (cloning efficiency) of A549 cells exposed to CS for the indicated lengths of time is expressed as percent of that of mock-treated cells. The cloning efficiency of mock-treated cells was 23.6%.

fluorescence of each cell cycle compartment or, where not indicated, of the entire population (G_1, S and G_2M). All experiments were performed at least twice.

Results

Induction of histone H2AX phosphorylation following exposure to CS. A549 cells were exposed to CS from IM16 cigarettes for increasing periods of time, then purged of smoke constituents by PBS rinses, and cultured for an additional hour before being harvested and subjected to analysis of H2AX phosphorylation. This timing is based on our repeated observations of the kinetics of increase in γH2AX IF following exposure of A549 cells to DNA damaging agents, which approaches a plateau at that time (22,23).

The expression of γH2AX was estimated for populations of cells in G_1, S and G_2M by gating analysis using cellular DNA content to define the respective cell cycle phases in the course of a multivariate analysis. Histone and DNA content double in content as cells proceed from G_1 to G_2 phase. Thus, in order to express the increase in H2AX phosphorylation per unit of DNA or histone (i.e. to represent the degree of histone H2AX phosphorylation), the mean values of γH2AX IF for S and G_2M phase populations were divided by 1.5 and 2.0, respectively. In order to express the increase (Δ) in H2AX phosphorylation as a function of cell treatment with CS under different conditions, in some of the data presented, the mean γH2AX IF values of G_1, S and G_2M cell populations of mock-treated cells were subtracted from the respective means of the CS-treated cells (Δ γH2AX). In all cases, the values presented represent the means of γH2AX IF for populations consisting of 3-5x10^3 cells.

As illustrated in Fig. 1A, there was little or no change in γH2AX IF when exposure of A549 cells to CS was limited to 5 min. However, as the exposure exceeded 5 min there was essentially a linear time-related increase in expression of γH2AX. Following 10 min of exposure to smoke, S-phase cells were most sensitive to DNA damage expressing approximately 37% higher levels of γH2AX than G_1 phase cells. When the length of exposure to CS was increased to 20 min (which reduced cell viability by >90%; see below), G_1 phase cells expressed 10-20% higher levels of γH2AX-associated IF.

Parallel to analysis of DNA damage, we quantified the effects of CS exposure on inhibition of the reproductive capability of A549 cells with a 5-day clonogenicity assay. This assay integrates multiple types of cell and genomic damage that ultimately leads to reproductive death. As shown in Fig. 1B, increasing exposure of A549 cells to CS to 20 min suppressed colony formation by up to 90-95% whereas >50% of the cells survived when exposure was limited to 10 min. Thus, Fig. 1 reveals a strong correlation between increasing intensity of γH2AX IF and decreasing cell reproductive capability after CS exposure.

As most, if not all, American brands of cigarettes appear to confer similar risks to the chronic smoker (30), we were interested in determining the effects of a popular cigarette sold in the United States which has a similar tar and nicotine value as IM16. Fig. 2A shows that the extent of H2AX phosphorylation observed in A549 cells after exposure to the CS from a leading commercial cigarette brand (FTC values of 14.8 mg tar and 1.16 mg nicotine) was essentially equivalent to that seen with IM16 (FTC values of 15.7 mg tar and 1.01 mg nicotine), and Fig. 2B shows that CS from both IM16 and the commercial cigarette reduced clonogenic capability to a similar degree.

A complementary way of investigating the effects of exposure to IM16 CS on A549 cells was to maintain a constant length of exposure but dilute the CS with a greater proportion

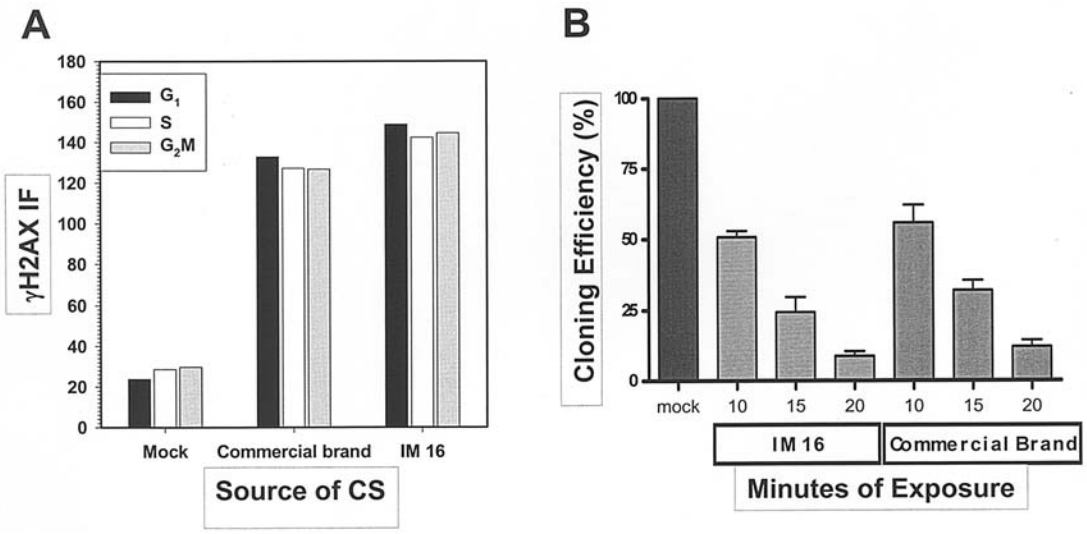

Figure 2. Comparison of the extent of γH2AX IF and cloning efficiency of A549 cells exposed to CS from either IM16 or a commercial brand of cigarette. (A) A549 cells exposed for 20 min to either air (mock) or to CS from IM16 or a commercial brand of cigarette were washed in fresh medium, returned to culture for 1 h and then fixed for analysis of γH2AX expression. The mean γH2AX IF of cells in G_1, S or G_2M phase was determined by gating analysis based on DNA content. (B) Cloning efficiency of A549 cells exposed to CS from an IM16 or a commercial brand of cigarette for different lengths of time was expressed as percent of that of cells exposed for 20 min to air (mock).

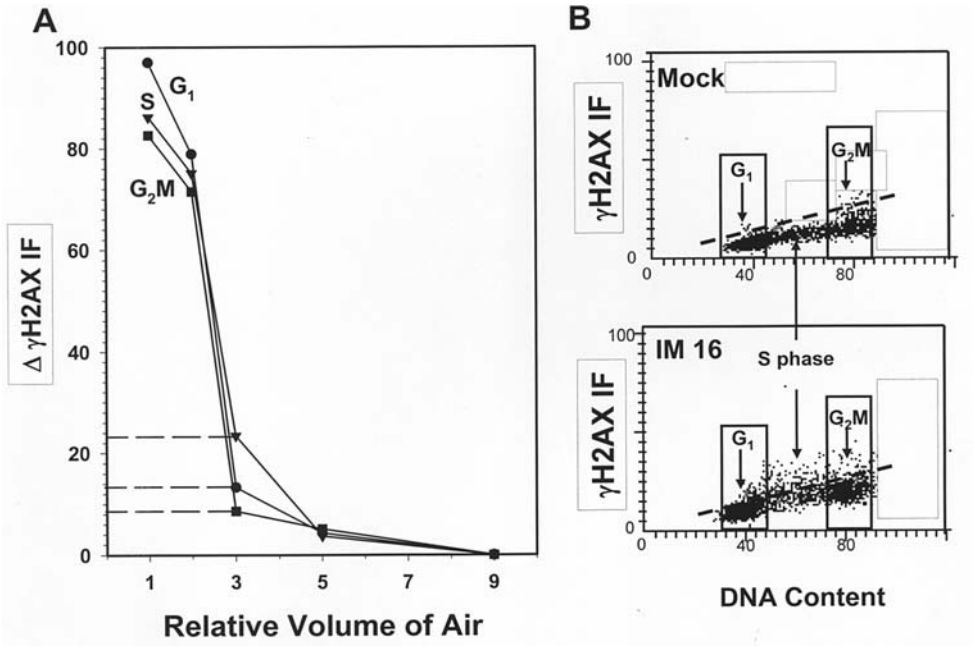

Figure 3. Mean γH2AX IF of A549 cells exposed to various concentrations of CS from IM16 for 20 min; the change in CS concentration was obtained by combining CS with 95% air and 5% CO_2 at proportions of 1:1, 1:2, 1:3, 1:5 and 1:9, respectively. (A) Cellular γH2AX IF and DNA content were measured by iCys and, through gating analysis, the mean intensity of γH2AX IF was estimated for cells in G_1, S and G_2M phases. At a 1:3 dilution of CS, S-phase cells continued to exhibit 76% and 172% higher γH2AX IF than G_1 or G_2M cells, respectively. (B) Bivariate (γH2AX IF vs DNA content) distributions of control (mock) cells and cells exposed to a 1:3 dilution of CS from IM16. The dashed line indicates the level of γH2AX IF below which 98% of S-phase cells in mock-treated cultures express γH2AX. Note that many more cells in the S phase as compared to cells in the G_1 or G_2M phase have elevated levels of γH2AX IF (above the dashed line) in cultures exposed to CS.

of 5% CO_2 in air. Under our standard conditions (e.g., 20-min exposure to CS at one 35-cc puff/min followed by 1-h incubation in fresh medium) cells sustained a substantial degree of damage in the form of DSBs (Fig. 1). However, dilution of CS resulted in a decidedly non-linear decrease in γH2AX IF. Thus, dilution of CS by 50% had a minimal effect on DSB formation for cells in all cell cycle phases. However, when the CS was diluted to approximately 33% of that used in our standard conditions (Fig. 1), γH2AX IF of G_1- and G_2M-phase cells decreased by ~60% while S-phase cells were much less effected (Fig. 3A). At the same CS dilution, S-phase cells showed a 76% higher level of γH2AX IF than G_1 cells, and

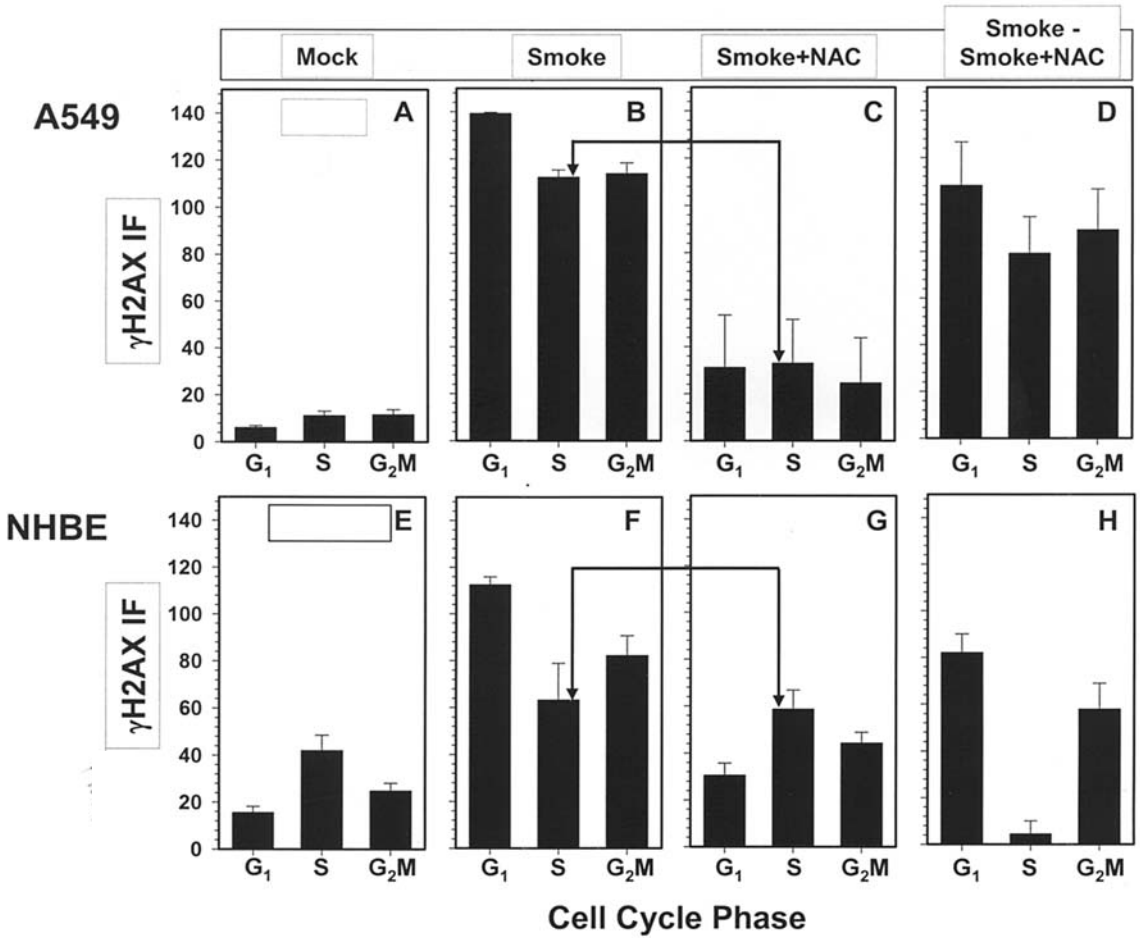

Figure 4. Protective effect of NAC on the increase in γH2AX IF in A549 (top panels) or NHBE cells (bottom panels) exposed to CS from IM16. Following exposure to CS in the absence or presence of 25 mM NAC, cells were transferred to culture for 1 h, then fixed and stained for DNA content and γH2AX IF. The mean γH2AX IF was estimated for G_1, S and G_2M cells, respectively. (A and E), γH2AX IF of cells from mock-treated cultures. (B and F), γH2AX IF after exposure to CS from IM16. (C and G), γH2AX IF of cells exposed to CS in the presence of NAC. (D and H), The difference between γH2AX IF of cells exposed to CS in the absence and presence of NAC. Arrows highlight the apparent difference in NAC-induced protection of S-phase cells for A549 and NHBE cells.

172% higher level of γH2AX IF than G_2M cells. These differences are illustrated further in Fig. 3B where the raw data in the form of bivariate (i.e., DNA content vs γH2AX IF) distributions of mock-treated and CS-treated cells are presented. It is quite evident that compared with G_1 or G_2M, many more S-phase cells expressed increased levels of γH2AX IF following treatment with CS. When the smoke was further diluted, all cell cycle-phase specific differences disappeared at 20% of the original CS concentration and, at 11%, the level of γH2AX IF was indistinguishable from that observed in mock-treated cells (Fig. 3A). In each instance, the data points represent the mean γH2AX IF of thousands of cells gated by DNA content for cell cycle position minus the mean of the same cell cycle populations of mock-treated cells.

Apoptosis is not the cause of DSBs. Due to extensive DNA fragmentation, apoptotic cells are characterized by dramatically increased levels of γH2AX IF compared to relatively modest increases of γH2AX IF in response to primary DSBs induced by genotoxic agents (23). In our previous studies, we observed that exposure to CS for longer than 20 min leads to a significant increase in apoptotic cells in the population when measured 24 h after CS exposure (31). Although, in the present studies, induction of γH2AX was assayed at a much shorter interval of post-CS exposure (1 h), we wanted to determine whether apoptosis could still contribute to the observed increase in γH2AX IF following CS exposure. Therefore, activation (cleavage) of caspase-3, considered to be a reporter of induction of apoptosis, was measured immunocytochemically in samples parallel to those that were subjected to analysis of H2AX phosphorylation. Exposure of A549 cells to smoke for up to 40 min followed by their fixation at 15 min or up to 20 min exposure followed by fixation at 4 h had little or no effect on caspase-3 activation: less than 0.5% of the cells demonstrated the presence of activated caspase-3 in either mock-exposed or smoke-treated cultures (data not shown). However, if A549 cells exposed to smoke for 20 min were allowed to grow in culture for an extended period of time (24 h), virtually half the cells were positive for activated caspase-3 (31). Therefore, in the absence of apoptosis, the formation of γH2AX in A549 cells observed after 20 min of exposure to CS and fixed at 1 h post-exposure would appear to be entirely the result of CS-induced primary DSBs and not due to CS-induced apoptosis-associated DNA fragmentation. However, as noted in Fig. 1B,

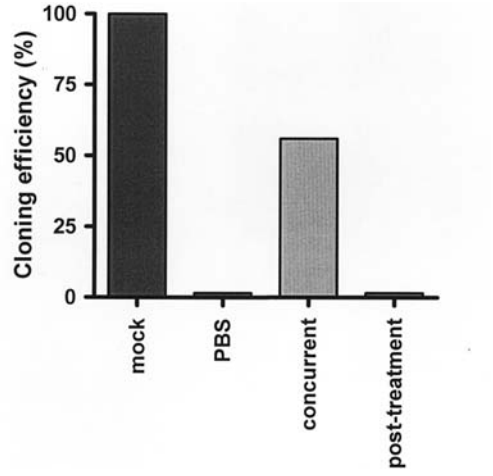

Figure 5. Cloning efficiency of A549 cells exposed to CS from IM16 for 20 min in the absence of 25 mM NAC (PBS) or when NAC was present during (concurrent) or after (post-treatment) exposure to CS. While NAC present for 1 h subsequent to exposure to CS had little effect on rescuing A549 cells from reproductive death induced by CS, when added concurrently with CS it increased the survival of A549 cells to greater than 55%.

<10% of A549 cells survived to form colonies following 20-min exposure to CS in contrast to a >55% survival rate when exposure was limited to 10 min.

DSBs and cell death induced by CS can be suppressed by exogenous antioxidants. CS contains a large number of highly reactive DNA damaging organic and inorganic substances that either are free radicals or can generate free radicals. In order to determine whether these reactive species contribute to the observed induction of H2AX phosphorylation by CS, A549 and NHBE were exposed to IM16 CS in the absence and presence of extracellular NAC, a known radical scavenger and nucleophilic antioxidant. First, multiple CS exposure experiments were performed in the presence of increasing concentrations of NAC from 100 μM to 25 mM (data not shown). Maximum γH2AX protection was observed with an NAC concentration of 25 mM. We therefore chose to use 25 mM NAC for all future comparative experiments. The protective effect of NAC, in terms of preventing H2AX phosphorylation, was then measured as a function of cell cycle phase as follows. CS-treated cells were incubated with NAC at a concentration of 25 mM under several conditions: a) concurrent with the 20-min exposure to CS; b) only during the 1-h incubation that followed the CS exposure; and c) during both the 20 min CS exposure period and the post-CS exposure 1-h incubation time. Parallel cultures were exposed to CS but not treated with NAC. In A549 cells (Fig. 4, top panel), IM16 CS, in the absence of NAC, caused a dramatic increase in H2AX phosphorylation compared to that observed in mock-treated cultures (Fig. 4A and B). Adding NAC to the media only during the 1-h post-CS exposure incubation did not have any appreciable affect on reducing the levels of γH2AX IF, which remained high (not shown). However, when NAC was present, concurrent with CS exposure, γH2AX IF intensity was reduced by >80% for the entire cell population, i.e. cells in all phases of the cell cycle (Fig. 4C). When estimated for cells in a particular phase of the cell cycle, the reduction in H2AX phosphorylation was most prominent for G_1 cells and least pronounced for S-phase cells; the G_2M cells showed an intermediate level of suppression (Fig. 4D). The presence of NAC during both the 20-min CS exposure and the 1-h post-exposure period provided somewhat more protection, in terms of reducing γH2AX IF, than when NAC was present only during the CS exposure period (90% versus 80% protection for the entire cell population; not shown).

Similar to A549 cells, induction of γH2AX in NHBE cells exposed to IM16 CS was the most pronounced for the cells in G_1 phase (compare Fig. 4E and F). Also similar was the overall pattern of protection by NAC for both NHBE and A549 cells. For example, protection was evident in NHBE cells only when NAC was present during the 20-min exposure to CS and not when it was present during the post-exposure incubation time (not shown). Likewise, in NHBE cells, we observed somewhat higher protection when NAC was present during both the 20-min exposure to CS and the 1-h post-exposure period. Interestingly, however, in contrast to A549 cells, the reduction in the rise of CS-induced γH2AX expression by NAC was much less apparent for S-phase than for G_1- or G_2M-phase NHBE cells (Fig. 4G). Thus, as shown in Fig. 4H, NAC present during the 20-min exposure period diminished the CS-induced rise in γH2AX IF of G_1 cells by approximately 79% and of G_2M cells by 65% but S-phase cells were provided almost no protection (<5% reduction).

More importantly, when NAC was present during CS exposure, we observed a direct correlation between diminishing levels of DNA damage in A549 cells, as evidenced by an 80% decrease in γH2AX IF intensity (Fig. 4D), and an increase in cell viability, as reflected by the clonogenicity assay (Fig. 5). However, Fig. 5 also shows that when NAC was only present after CS exposure, no protection was provided and cell viability was decreased by >90%. As NAC functions primarily as a free radical scavenger and antioxidant when present extracellularly for a short period of time (as in the current experiments), these data indicate in all probability that CS-generated free radicals and/or membrane permeable components in CS that generate free radicals within the cell contribute significantly to DNA damage and a loss of cell viability as reflected by a rise in phosphorylated H2AX levels and in cell death.

We next sought to determine whether GSH was also effective at suppressing γH2AX IF and cell death. Although both NAC and GSH function as free radical scavengers and nucleophiles, there are two significant differences that distinguish these compounds. First, in terms of rates of reactivity with highly reactive free radical species (e.g., hydroxyl, alkyl, nitrogen dioxide etc.), both GSH and NAC will scavenge highly reactive free radicals at near diffusion-controlled rates and display, therefore, similar radical scavenging ability. Moreover, NAC and GSH can also act as a nucleophile to scavenge and/or sequester reactive species that exist in CS (e.g. acrolein, HCN, reactive oxygen species, reactive nitrogen species etc.) which are known to cause oxidative stress. However, GSH is generally considered to be more efficient than NAC in this process since it contains multiple nucleophilic groups while NAC contains only one. This difference in antioxidant potency between NAC and GSH is expected to be reflected in a difference in the respective

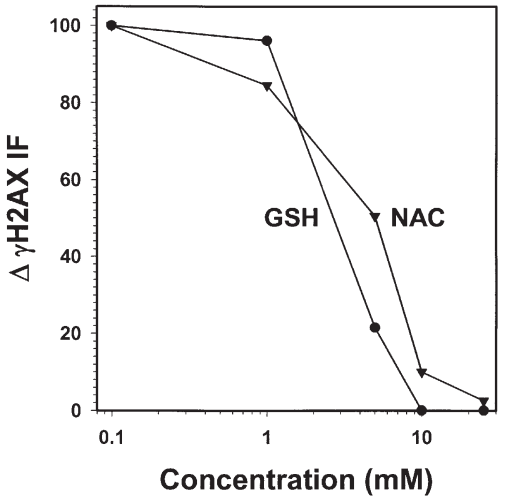

Figure 6. The effect of varying concentrations of GSH or NAC added to A549 cells during exposure to CS. A549 cells were exposed to CS from IM16 for 20 min in the presence of varying (100 μM-25 mM) concentrations of GSH or NAC, washed, incubated for 1 h in fresh medium and fixed for analysis. Mean γH2AX IF was measured for all cells (G_1, S and G_2M phase) in each population and the values normalized to that of samples receiving CS but not GSH or NAC. Prior to normalization, the mean γH2AX values of cells exposed to CS and GSH or NAC were subtracted from that of mock-treated cells.

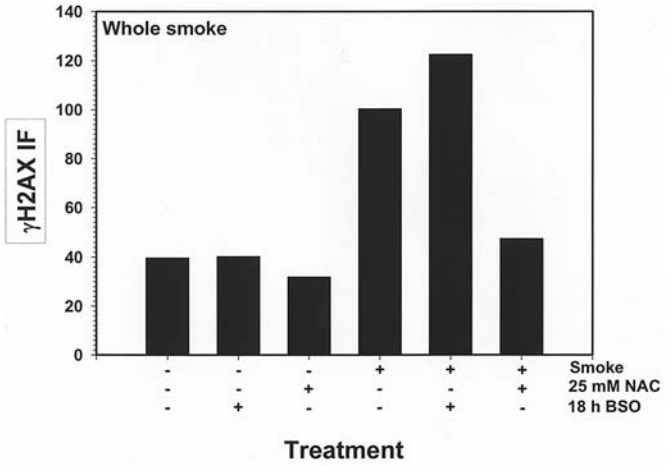

Figure 7. The effect of pretreatment of A549 cells with BSO prior to exposure to CS either in the absence or presence of 25 mM NAC. Cells were pretreated for 18 h with either medium or medium containing 5 μM BSO. Prior to exposure to CS, cells were washed and either PBS or PBS containing 25 mM NAC was added. The cells were either mock-exposed or exposed to CS from IM16 for 20 min. Following a 1-h post-exposure incubation in fresh medium, cells were harvested and the entire population (G_1, S and G_2M phase cells) analyzed for the extent of H2AX phosphorylation.

concentrations that provide equi-effective protection. To test this idea, we used the same experimental design as described for NAC to test the impact on γH2AX induction of exposing cells to CS in the presence of increasing concentrations of GSH ranging from 100 μM to 25 mM. As shown in Fig. 6, exogenously added GSH at a concentration of 10 mM suppressed the formation of DSBs as effectively as 25 mM NAC, supporting the idea that GSH is more efficient at preventing γH2AX induction than NAC.

The second major difference is that NAC, unlike GSH, can readily cross the lipid membrane of the cell and undergo deacetylation to yield cysteine, a necessary precursor for the intracellular production of GSH. To test the hypothesis that the protective effects of NAC were primarily due to its ability to act as an exogenous antioxidant and not due to its capacity to increase the intracellular concentration of GSH, we performed the following experiments with BSO, an irreversible inhibiter of γ-glutamyl cysteine synthetase, the rate-limiting enzyme in GSH synthesis. Specifically, A549 cells were pre-treated with 50 μM BSO for 18 h, rinsed with PBS solution and then exposed to CS for 20 min in the presence or absence of 25 mM NAC. If NAC inhibits the induction of γH2AX IF simply by increasing intracellular GSH concentrations, then the presence of BSO would prevent GSH from being formed from NAC and there should be potent induction of γH2AX IF. However, the results shown in Fig. 7 indicate that BSO-treated cells in the presence of 25 mM NAC prevented γH2AX IF induction almost to the levels observed in control cells. These data indicate that, under the experimental conditions used, NAC protects cells from CS-induced DNA damage by acting primarily as an exogenous antioxidant and not by increasing GSH levels. Further support for this conclusion comes from the fact that, when cells were treated with BSO only (which reduced endogenous glutathione levels by >90%; data not shown) and exposed to CS for 20 min, there was only a modest (~20%) increase in γH2AX IF compared to control cells exposed only to smoke (Fig. 7).

DSBs and cell death caused by CS can be mediated with DMTU. The results discussed above indicated that CS-induction of γH2AX could be inhibited by broad-spectrum nucleophilic antioxidants such as GSH and NAC. In order to address the specific issue of whether the prime species inhibited by NAC and GSH are highly reactive free radicals or radical generators/oxidants in CS (e.g. acrolein, epoxides, reactive nitrogen species, etc.) that can enter the cell and lead to the intracellular production of free radicals, we assessed the impact of the hydroxyl radical scavenging, non-nucleophilic compound, dimethyl-thiourea (DMTU). A549 cells were pretreated with either 10 or 20 mM DMTU for 18 h, rinsed with PBS solution and exposed to CS for 20 min. Fig. 8A shows that DTMU provides a substantial degree of protection (44% decrease in γH2AX IF) against CS-induced DSB formation. These data are interesting given the highly reactive nature and short lifetimes of free radical species that can result in DSBs. Collectively, these experimental results suggest that the intracellular generation of DNA damaging free radicals plays an important role in DSB formation. Moreover, these data also strongly suggest that the primary species in CS that ultimately lead to γH2AX induction are not reactive free radicals themselves but rather free radical generators that can ultimately lead to the formation of free radicals (e.g., $^{\bullet}OH$, $^{\bullet}O_2^-$, or H_2O_2) within the cell and/or nucleus. Equally interesting is the fact that, as shown in Fig. 8B, DMTU is only minimally effective at preventing loss of reproductive capacity in CS-treated cells as measured by the clonogenic assay. This observation supports the conjecture that CS contains, in addition to free radical generators that enter the cell and mediate the formation of DNA-damaging free radicals, other compounds that directly damage one or more cellular

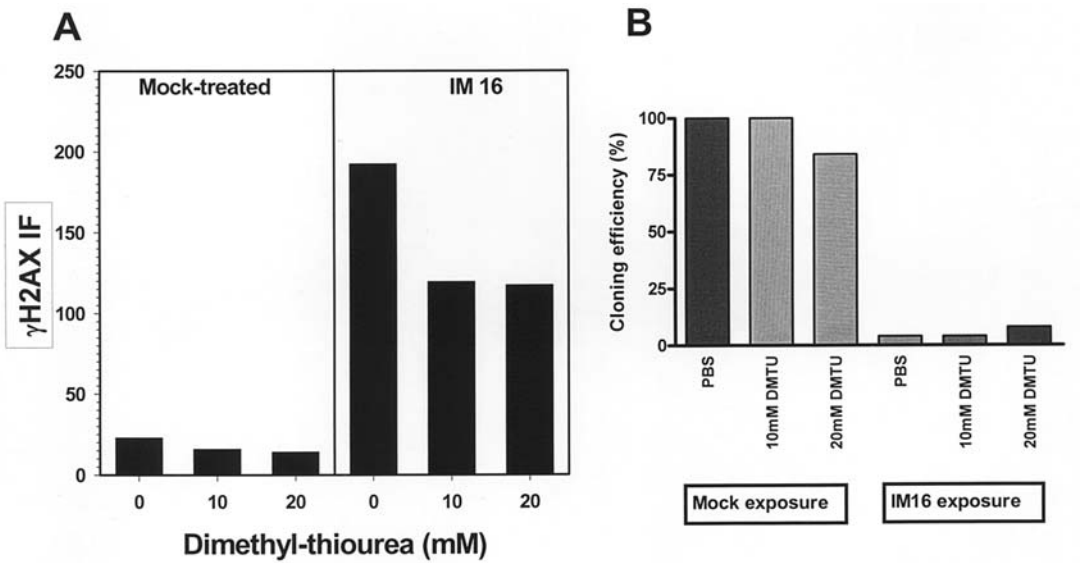

Figure 8. The effect of DMTU pre-treatment on the response of A549 cells to CS. Cells were incubated with 0, 10 or 20 mM DMTU for 18 h prior to exposure to CS from IM16. Cells were rinsed prior to 20-min CS exposure followed by a 1-h post-exposure incubation in fresh medium. The extent of H2AX phosphorylation was calculated for the entire (G_1, S and G_2M) population of cells. Pre-treatment with both 10 and 20 mM DMTU lowered γH2AX IF by ~45% relative to that of control cells exposed to CS.

components (e.g., lipids, proteins, organelles) or negatively impact a critical molecular function resulting in cell death above that caused solely by DSB formation.

Protective effect of NAC is cell cycle-phase specific. The data presented above indicated that the pattern of induction of H2AX phosphorylation by CS across the cell cycle was quite different for NHBE than for A549 cells. In order to determine in more detail the variability of cells to the protective effect of NAC in different phases of the cycle, the induction of γH2AX in A549 cells was studied at different concentrations of NAC. For comparison, the data were expressed as a fraction of response of G_1 phase cells which, under the present conditions (e.g., 20-min exposure to CS from IM16), exhibited the greatest amount of DNA damage as indicated by the increase in γH2AX IF. As can be seen in Fig. 9, the induction of γH2AX by CS in S-phase A549 cells was unaffected by the presence of NAC up to a concentration of 5 mM. In contrast, reduction in the levels of γH2AX induced in both G_1- and G_2M-phase cells was already evident at 1 mM NAC. However, at 10 mM, the differences in protection of cells in different cell cycle phases by NAC began to narrow. When the concentration of NAC reached 25 mM, the S-phase cells were protected to almost the same degree as G_1- and G_2M-phase cells. The concentration of NAC that reduced DNA damage by 50% for each cell cycle phase can be determined from the plot in Fig. 9. For G_1-, S- and G_2M-phase A549 cells, the IC_{50} values were approximately 4.5, 7.5 and 2.6 mM NAC. These data indicate that G_2M cells were the most protected by NAC, whereas S-phase cells were 3-fold more resistant and G_1 cells showed an intermediate degree of protection by NAC.

S-phase cells are most sensitive to non-toxic levels of CS. When cells were exposed to CS for less than 20 min (Fig. 1)

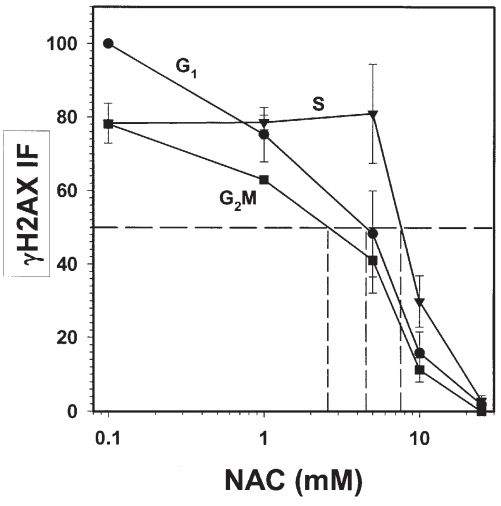

Figure 9. The effect of various concentrations of NAC on induction of γH2AX IF in A549 cells exposed to CS. Following 20-min exposure to CS in the absence and presence of varying concentrations of NAC, the cells were washed and returned to culture for 1 h, then fixed and stained for DNA content and γH2AX IF; gating analysis was used to calculate the mean γH2AX IF for cells in G_1, S and G_2M phase. Note that S-phase cells were the most resistant to the protective effect of NAC, such that no protection was observed at 1 and 5 mM NAC. The NAC concentrations at which γH2AX IF was reduced by 50% were 2.6, 4.5 and 7.5 mM NAC for cells in the G_2M-, G_1- and S-phase, respectively (vertical dashed lines).

or to diluted levels of CS (Fig. 3), or when the ROS-scavenger, NAC, was present during exposure (Fig. 9), S-phase cells tended to express the greatest levels of γH2AX IF. This increased sensitivity of S-phase cells is summarized in Fig. 10. Following 8-min exposure to CS, S-phase cells expressed 35-36% higher levels of γH2AX IF than G_1- or G_2M-phase cells (Fig. 10A). If 5 mM NAC was present during CS exposure, cells in the S phase expressed higher levels of γH2AX IF;

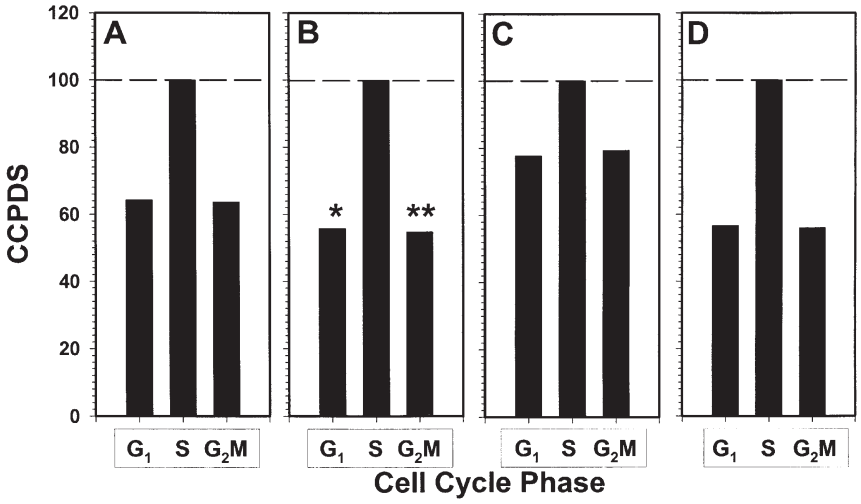

Figure 10. Cell cycle-phase differential sensitivity (CCPDS) of A549 cells under various conditions of exposure to CS. (A) Cells were exposed to CS for 8 min and the γH2AX IF determined for G_1, S, and G_2M cells based on intensity of DAPI (DNA) fluorescence. The values of γH2AX IF of G_1- and G_2M-phase cells was then normalized to that of S-phase cells and multiplied by 100. (B) Calculated as in A, data for A549 cells exposed to CS in the presence of 5 mM NAC as in Fig. 9; asterisk and double asterisk indicate statistically significant differences between the values for S and G_1 and S and G_2M cells ($p<0.05$ and $p<0.01$), respectively. (C) Cells exposed to CS diluted 3:1 with air (as in Fig. 3) calculated as described in A. (D) Cells exposed to CS in the presence of 5 mM GSH; relative expression of γH2AX IF for the various cell cycle phases were calculated as in A.

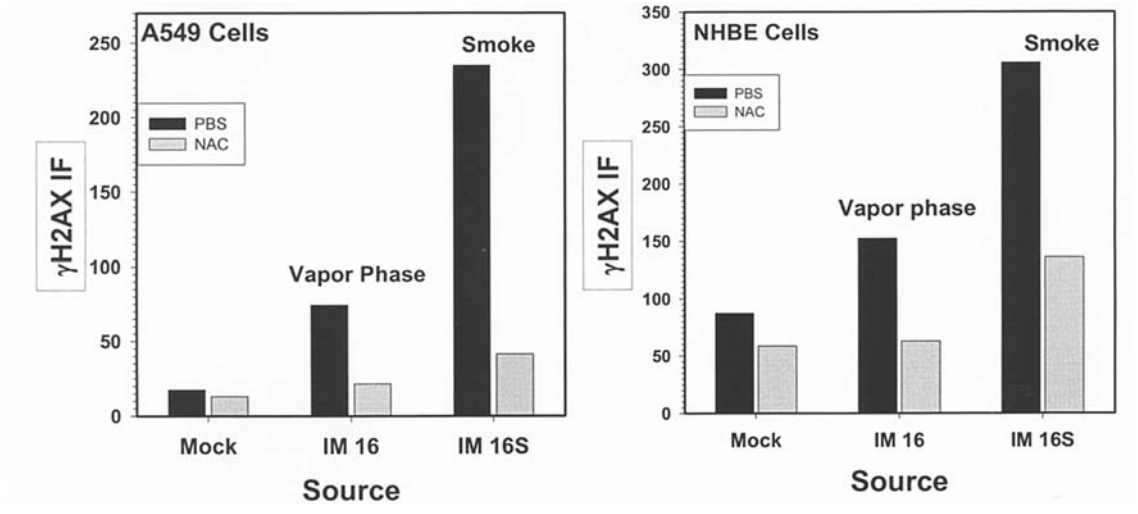

Figure 11. A comparison of the effect of CS and the vapor phase of smoke in the presence or absence of NAC on the induction of γH2AX IF. Cells were either grown for 20 min in the absence of smoke (mock) or in the presence of the vapor phase or CS. Parallel cultures were exposed to the vapor phase or CS in the presence of 25 mM NAC. While NAC significantly lowered the levels of γH2AX IF when present during exposure to CS, it had a much more pronounced protective effect on induction of γH2AX IF when present during exposure of both A549 and NHBE cells to the vapor phase of smoke.

44% higher than cells in G_1 ($p<0.05$) and 45% higher than cells in G_2M ($p<0.01$) (Fig. 10B). Dilution of CS with air to a level approximately 1/3 that of the typical concentrations used in most experiments resulted in S-phase cells having 21-23% higher levels of γH2AX IF compared to G_1- or G_2M-phase cells (Fig. 10C). If cells were exposed to 5 mM GSH during exposure to CS, S-phase cells again expressed 43-44% higher levels of γH2AX IF than G_1 or G_2M phase cells (Fig. 10D). It should be noted that in several of these instances, the conditions in which S-phase cells appeared to be most sensitive to the effects of CS coincided with the conditions that resulted in only modest decreases in cell survivability (Figs. 1B and 5).

Vapor phase of smoke also induces H2AX phosphorylation which is abrogated by NAC. Whole cigarette smoke is an aerosol that contains both gaseous (i.e. vapor phase) and suspended particulate material (i.e. particulate phase). The vapor phase is primarily a mixture of gases (i.e. nitrogen, oxygen, and carbon dioxide) and multiple volatile and semi-volatile organic species, while the particulate phase consists of a wide variety of condensed organic compounds, a number of which are carcinogens (2,32). In addition, both the vapor and particulate phases of CS contain large amounts of free radicals which are presumed to cause both direct and indirect stress to the cell (33,34). As we have previously shown that the particulate phase of CS can trigger H2AX phosphorylation

(31), in the present study we examined whether the vapor phase component can also induce DSBs as detected by an increase in H2AX phosphorylation. Fig. 11 illustrates the ability of the vapor phase from IM16 CS to induce γH2AX in A549 cells as compared to whole CS from IM16. The data show that under standard conditions of exposure, the vapor phase caused an increase in expression of γH2AX that was 31.5% of that observed after exposure to whole CS from the same cigarettes.

NAC present during A549 cell exposure to the vapor phase of IM16 CS reduced γH2AX induction by 93% compared to cells similarly exposed but in the absence of NAC. The same experiment performed on NHBE cells demonstrated comparable results, i.e. IM16 vapor phase caused an increase in expression of γH2AX that was 29.8% of that observed after exposure to whole CS. However, the presence of NAC virtually eliminated any increase in γH2AX IF in these cells as well (Fig. 1). Collectively, these results indicate that the vapor phase of CS contributes significantly to the induction of DSBs in both normal and malignant lung cells, and this DNA damage appears to be due predominately to free radicals, either directly delivered from or induced by components of CS.

Discussion

It is well documented that increased oxidative stress is a major mechanism by which CS causes airway damage that can lead to a host of pathogenic conditions, including asthma, pulmonary fibrosis, chronic obstructive pulmonary disease, and lung cancer (35-37). However, a detailed understanding of the specific molecular mechanisms that link oxidative stress with CS-induced pathologies is still lacking. Increased knowledge in this area may be useful in developing new approaches to mitigating or reversing the damage caused by CS either by chemopreventive strategies and/or reducing the toxicity of cigarettes.

It is assumed that despite efficient antioxidant defense mechanisms in the respiratory tract, the large amounts (on the order of >1014/puff) of short-lived free radicals (such as $^{\bullet}O_2^-$, $^{\bullet}NO$, $^{\bullet}OH$), and more stable organic reactive species/ oxidants (such as acrolein, epoxides) that exist in both the gas and particulate phases of CS can transiently and, perhaps, chronically overwhelm the cell's steady-state antioxidant capacity (38,39). In fact, there is strong evidence that active smoking causes a marked imbalance in an individual's redox state and an overall increase in oxidative stress, especially in the respiratory tract (26,40,41). One possible result of this disruption to the lung's redox status is the induction of oxidatively derived DNA damage which can manifest as missing bases, altered or mismatched bases, deletions and insertions, strand breaks, intra- and inter-strand cross-links, structural or numerical chromosomal aberrations, abnormal sister chromatid exchanges, and the formation of micronuclei. Each of these defects can not only be genotoxic and cytotoxic but can also play a fundamental role in tumorigenesis (42,43). In particular, the ability of an agent to induce cytogenetic damage, such as structural aberrations in chromosomes and chromatids, correlates well with enhanced cancer risk in exposed individuals (7,44). Among the most critical DNA lesions that can lead to these types of genetic abnormalities are DSBs (10). Chronic induction of DSBs can, therefore, be an important factor in the ultimate generation of genetic instability and multiple types of malignancies, especially if effective repair of these lesions is compromised (9,45,46).

A wealth of published evidence supports the conclusion that H2AX is specifically phosphorylated in response to the occurrence of DSBs caused by a variety of genotoxic agents (13,20,47,48). Moreover, at least with ionizing radiation, there appears to be a direct linear correlation between the number of γH2AX IF foci and the number of DSBs, which suggests that each γH2AX IF focus may represent an individual break (14,49). In addition, a strong correlation has been observed between increasing γH2AX IF foci and decreasing cell survival (50), leading to the conjecture that a single DSB can be lethal to a cell (51). However, when a DSB remains unrepaired or is improperly repaired and the cell survives, preserving its proliferative potential, it may potentially give rise to a carcinogenic lesion. These observations led us to previously determine that short-term exposure of A549 and NHBE cells to CS or cigarette smoke condensate induces γH2AX phosphorylation in a dose-dependent manner, and to conclude that the rise in expression of γH2AX following CS exposure reflects the induction of DSBs (31). Thus, the H2AX phosphorylation assay is an important addition to the battery of available DNA-damage tests that are being used to assess the genotoxic properties of tobacco products (31).

Until now, while there was clear evidence that CS and specific key tobacco smoke constituents, such as free radicals, can cause DNA nicks and single-strand breaks (52-54), there was no convincing data that CS-generated free radicals cause DSBs as well. Therefore, in light of the implicated but unproven role of CS-generated free radicals in the induction of DSBs, the present experiments explored whether NAC, a synthetic acetylated form of the amino acid, L-cysteine, and a thiol-containing antioxidant that has been shown to inhibit DNA damage and carcinogenesis (55), could suppress the induction of DSBs by CS. The results presented here clearly demonstrate that the induction of H2AX phosphorylation was significantly reduced and, in some instances, nearly prevented when cells were exposed to whole CS or to vapor phase smoke in the presence of NAC. The observed reduction of CS-induced H2AX phosphorylation by NAC can be explained either by a suppressive effect of NAC on protein kinases that phosphorylate H2AX (e.g. ATM, ATR and/or DNA-PK) or the prevention of induced DSB formation due to inhibition of one or more radical species that derive from CS. The latter explanation appears more tenable as there is no published evidence to suggest that NAC is an inhibitor of any of the protein kinases that are involved in H2AX phosphorylation (55). Moreover, we have observed that when these protein kinases are inhibited (e.g. by caffeine), the reduction in H2AX phosphorylation is paralleled by a significant decrease in cell survival (induction of apoptosis) (22) whereas, in contrast, the present study shows that the reduction in H2AX phosphorylation by NAC is paralleled by an increase in cell survival, as measured by a clonogenicity assay.

Combined results from the NAC, GSH, BSO, and DMTU experiments indicate the possibility that membrane permeable components in CS, which can generate free radicals within

the cell, contribute significantly to a loss of cell viability and mediate the induction of a predominant fraction of DSBs in exposed cells. These CS components can be intercepted by using nucleophilic antioxidants exogenously but, once inside the cell, can overwhelm the cellular defense mechanisms and generate reactive free radicals through multiple mechanisms. While it remains our current focus to determine the specific CS generated free radical(s) and free radical generating compound(s) causing DSBs and cell death that are attenuated by NAC, it is known that NAC can impact a broad range of compounds, many of which cause some form of oxidative stress (55,56). For example, NAC can scavenge several reactive oxygen species (ROS) generated in CS such as $^{\bullet}O_2^-$, H_2O_2, and $^{\bullet}OH$ (56-58), as well as directly bind and attenuate various reactive CS compounds that can generate free radicals intracellularly (e.g. aldehydes, epoxides, quinones, etc.).

Interestingly, differences were observed between A549 and NHBE cells in terms of the degree of H2AX phosphorylation across the cell cycle prior to CS exposure (i.e. 'programmed' expression of γH2AX). The variability in 'programmed' expression of γH2AX is in concordance with observations of Banath *et al* (59) and Huang *et al*, (60) who reported a similar degree of variability between different cell lines. Perhaps more interesting were the cell cycle-phase differences in the protective effect of NAC on CS-induced γH2AX phosphorylation in A549 and NHBE cells. We observed that S-phase NHBE cells could not be significantly protected from CS-induction of DSBs, even at a 25-mM concentration of NAC (Fig. 4, bottom). This is in contrast to A549 cells in which 25 mM NAC could provide nearly full protection regardless of the cell cycle phase (Fig. 4, top). However, at lower concentrations of NAC, cell cycle phase differences were observed in A549 cells. Specifically, G_2M A549 cells were protected from CS-induced DSB formation at an NAC concentration that was 3-fold lower than that required to protect the more resistant A549 S-phase cells to a similar degree (Fig. 9). What is the possible significance of these data? One obvious conclusion is that S-phase cells appear to require a higher level of protection against free radicals than do other cell cycle phases, possibly indicating an increased vulnerability to oxidant damage during DNA replication. This conclusion is further supported by the smoke dilution experiment which showed that, at a level of CS exposure in which there is little increase in DNA damage in either G_1- or G_2M-phase cells, H2AX IF of the order of 75-175% greater than that of either G_1- or G_2M-phase cells, respectively, is still observed in S-phase cells. The observed cell cycle phase specificity for DSB accumulation may also be explained by the possibility that successful repair of DSBs depends on cell cycle position (61). Consequently, if proliferating cells exposed to CS experience similar levels of DSBs during each phase of the cell cycle but dissimilar repair rates, they may be particularly susceptible to accumulating deleterious DNA defects during that specific phase.

Why S-phase NHBE cells are less protected by NAC than S-phase A549 cells is unknown as yet. One possibility is that there are intrinsic differences between NHBE and A549 lung adenocarcinoma cells as a result of the malignant status of the latter that affect one or more mechanisms responsible for attenuating oxidative stress. This possibility is supported by a wealth of data showing that a wide range of genetic and biochemical alterations related to redox imbalance distinguish malignant from normal cells (62-64). Another possibility is that A549 cells may have increased levels of the main endogenous free radical scavenger, glutathione, for which NAC can act as a precursor (55). Higher intrinsic glutathione levels in A549 cells than in NHBE cells may provide more effective scavenging of free radicals (e.g. in G_2M cells) and, therefore, lower exogenous amounts of NAC are needed for protection against induction of DSBs. It is also possible that the overall levels of other free radical scavenging thiol-containing proteins vary between normal and malignant lung cells. Levels of these proteins may also vary across the cell cycle. Precedent for this possibility comes from the fact that glutathione levels can modulate during the cell cycle (65,66). In addition, the present data do not exclude the possibility that a small but significant fraction of CS-induced DSBs is generated by mechanisms other than reactive radicals. For example, the induction of DNA adducts may play a more significant role in the development of DSBs in S-phase NHBE cells than in A549 cells due to differential metabolism of CS constituents between the two cell types. Moreover, both the vapor and particulate components of tobacco smoke contain numerous substances whose toxicity is activated only after biotransformation by cellular enzymes into reactive nucleophiles which can attack DNA, another process that may distinguish normal and malignant lung cells (5).

Regardless of the mechanism, the observation that S-phase lung cells require higher levels of antioxidant protection to avoid DNA damage (such as DSBs) may have long-term etiological implications for the chronic cigarette smoker who is at significant risk of various pulmonary diseases. Specifically, it is known that a) CS contains a number of substances that cause oxidative stress, partly by depleting glutathione stores in cells (67-69); b) the lungs of persistent smokers undergo a significant increase in the number of proliferating cells due to smoke-induced damage (70,71); and c) in general, cells actively dividing at the time of carcinogen exposure are at particular risk for transformation-related events (44). Thus, if it proves to be a general phenomenon that S-phase lung cells need an augmented oxidative stress defense mechanism, then the ability of CS to cause DSBs while simultaneously depleting intracellular GSH stores and inducing cell proliferation could prove particularly problematic for pulmonary cells, especially since the difficulty in faithfully repairing DSBs can result in genetic instability and chromosomal rearrangements, two major mechanisms involved in the etiology of multiple types of malignancies, including lung cancer (9,10,45,46,72,73). Furthermore, if an individual smoker has specific polymorphisms in relevant DNA-repair genes that reduce their effectiveness, the induction of DSBs by CS could further exacerbate the risks associated with smoking (74-76). There is clear evidence that genetic factors render certain smokers more susceptible to cancer, in part by altering the functions of critical pathways, including those involved in DNA repair (77-79). Finally, a rapid induction of high levels of DSBs by CS, such as observed in this study, may present an independent hazard to the smoker since one recent study indicates that the probability for a DSB to be

inaccurately rejoined is relatively low when DSBs are spatially separated but increases considerably when multiple breaks coincide (61), while another study shows that heavily clustered DSBs can lead to complex genetic changes similar to those seen in human cancers (80).

While DSBs have been found in lung cancer (9), it remains to be determined to what extent DSBs exist in premalignant lung lesions and in the normal, but stressed, bronchial epithelium of chronic smokers. However, it has recently been shown that an activated DNA damage response, which includes the expression of phosphorylated ATM and H2AX, occurs in early precursor lung lesions and it is hypothesized that this response may be a dominant anti-cancer barrier preventing the cell from undergoing genetic instability and malignant conversion (81,82). Mutations in the genes within this pathway (e.g. ATM, Chk2, p53, etc.) (82), and/or a chronic assault on this pathway such as occurs with CS and oxidative stress (this report), may allow the cell to prematurely breach this DNA damage barrier and progress (82). At least two observations provide support for this possibility: i) one of the most common mutations in lung cancer is mutation of the p53 tumor suppressor gene (83,84); and ii) Chk2 kinase expression is down-regulated in non-small cell lung cancer due to promoter methylation (85). Moreover, it is interesting to note that cells from patients with ataxia telangiectasia show genetic instability and a continuous state of oxidative stress (86). These studies suggest that genetic instability may be a consequence of oxidative stress in combination with defects in the ATM pathway (87). In conclusion, the data presented here support the possibility that one of the earliest and most chronic genomic defects caused by CS is the induction of a large number of DSBs, which are potentially tumorigenic lesions.

Acknowledgements

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