Glutathione prevents inhibition of fibroblast-mediated collagen gel contraction by cigarette smoke

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Cigarette smoking is the major etiological factor in pulmonary emphysema (4). Cigarette smoke contains 1018 free radicals/gram (2, 9, 26). A delicate balance between the toxicity of oxidants and the protective effects of the antioxidant defense system is critically important for the maintenance of normal pulmonary functions (32). An imbalance between oxidants and antioxidants has been proposed in the pathogenesis of chronic obstructive pulmonary disease (COPD), particularly emphysema (29). The ability of individuals to regulate antioxidant defense mechanisms, such as the level of glutathione (GSH) in response to cigarette smoke, may in part relate to why only 15–20% of smokers develop COPD (33).

GSH is involved in one of the fundamental antioxidant defense mechanisms protecting against oxidant-induced lung injury in inflammation (20, 27). GSH is a strong nucleophile and often inactivates electrophilic reactive compounds by either direct nonenzymatic conjugation or enzyme catalysis. GSH has been implicated in various cellular events, such as inflammatory response, modulation of redox-regulated signal transduction, regulation of cell proliferation, remodeling of extracellular matrix, maintenance of surfactant and antiprotease screen, apoptosis, immune modulation, and mitochondrial respiration (28). GSH synthesis is regulated by oxidants, antioxidants, growth factors, inflammatory, and anti-inflammatory agents (27, 28). N-acetyl-L-cysteine (NAC), a cysteine-donating and antioxidant thiol compound, is converted in the body into N-acetyl-L-cysteine (NAC), which serves as a substrate for glutathione (GSH) production, and buthionine sulfoximine (BSO), which inhibits GSH production, were incubated in the presence and absence of cigarette smoke extract (CSE) with fibroblasts in three-dimensional collagen gels. Neither agent alone altered gel contraction. CSE inhibition of gel contraction, however, was mitigated by NAC and potentiated by BSO. Parallel effects were observed on cigarette smoke inhibition of fibronectin production and mRNA expression as well as by changes in intracellular GSH content. Pretreatment of fibroblasts with NAC or BSO resulted in similar effects, suggesting that neither agent was acting directly on smoke but, rather, was altering cellular response to smoke. In conclusion, smoke inhibition of fibroblast repair, as reflected by collagen gel contraction and fibronectin production, may be modulated by intracellular GSH levels.

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Emphysema is a destructive process involving the lung parenchyma. Previous studies have suggested that cigarette smoke may impair the wound healing process by inhibiting fibroblast recruitment, proliferation, and contraction of extracellular matrix (24). Inhibition of matrix contraction by smoke, an effect that depends on smoke-mediated inhibition of fibronectin production, could contribute to the enlarged air spaces that develop in the injuries associated with pulmonary emphysema (7).

In the current study, therefore, we hypothesized that the effect of cigarette smoke on fibroblast contraction of collagen gels might result from an imbalance between free radicals derived from the smoke and the antioxidant defense of the cells. The effect of cigarette smoke could be modulated by agents that alter intracellular GSH synthesis. This study, therefore, supports the concept that alterations in thiol antioxidant in defense mechanisms, by altering repair responses, could account for individual susceptibility to the development of emphysema and could represent a therapeutic target to prevent the development of this disorder.

MATERIALS AND METHODS

Cell culture. Human fetal lung fibroblasts (HFL-1; lung, diploid, human) were obtained from American Type Culture Collection (Rockville, MD). The cells were cultured in 100-mm tissue culture dishes (Falcon; Becton-Dickinson Labware, Lincoln Park, NJ) in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Grand Island, NY) supplemented with 10% fetal calf serum (FCS; Biofluids, Rockville, MD), 50 μg/ml penicillin G sodium, 50 μg/ml streptomycin sulfate (penicillin-streptomycin, Invitrogen), and 1 μg/ml amphotericin B (Parma-Tek, Huntington, NY). Fibroblasts were fed three times weekly, and cells between passages 15 and 20 were used. Confluent fibroblasts were detached by 0.25% trypsin–EDTA and resuspended in 0.5% mM EDTA and resuspended in DMEM without serum.

Materials. Type I collagen [rat tail tendon collagen (RTTC)] was extracted from rat tail tendons by a previously published method (21). Briefly, tendons were excised from rat tails and washed several times with Tris-buffered saline (0.9% NaCl and 10 mM Tris, pH 7.5) and 95% ethanol. The collagen was then extracted under sterile conditions with 6 mM hydrochloric acid. Protein concentration was determined using the bicinchoninic acid method (21). Protein concentrations of 1 mg/ml have been found to inhibit fibroblast contraction by HFL-1 cells, whereas 1–2% of CSE had a subthreshold effect on gel contraction. In the current study, CSE concentrations of 1% and 5% were selected to determine whether the effect of smoke on collagen gel contraction might be modulated by either inhibitors of GSH synthesis or antioxidants.

Measurement of intracellular GSH. Determination of intracellular GSH in fibroblasts was performed as previously described by Tietze (35), using 5,5′-dithiobis(2-nitrobenzoic acid)-GSSG/GSH reductase recycling. Briefly, after drying the collagen gels containing fibroblasts with Kimwipes (Kimberly-Clark, Roswell, GA), gels were transferred to Eppendorf tubes. After we briefly sonicated the gels, they were immediately put on ice. Gels were then spun at 4°C at 2,000 rpm for 2 min. Supernatants were transferred to each well of a 96-well flat-bottom plate where GSH was quantitated at a 405-nm wavelength. The actual total concentration of GSH in the samples was determined using linear regression to calculate the values obtained from a standard curve. The absolute value for GSH concentrations in cells under control conditions was 272.2 ± 31.9 μmol/10^6 cells.

Measurement of cell injury. To evaluate cell viability, ethidium homodimer-1 and calcein AM were added to wells to a final concentration of 0.23 and 0.12 μM, respectively (Live/Dead kit; Molecular Probes, Eugene, OR). Ethidium homodimer-1 (excitation/emission: ~495 nm/~635 nm), excluded by the intact plasma membrane of live cells, enters cells with damaged membranes and undergoes a fluorescence enhancement on binding to nucleic acids. Calcein AM (excitation/emission: ~495 nm/~515 nm) is well retained within living cells. Microscopic visualization was carried out 30 min after addition of the dyes. Wells were examined with an inverted fluorescent microscope (IMT-2, Olympus) at ×400 magnification. Five images from each well were subsequently analyzed by counting stained cells, and the percentage of dead cells was determined.
RNA isolation and complementary DNA synthesis. From the cell pellets prepared as described above, total RNA was extracted with acid guanidine monothiocyanate, precipitated with isopropyl alcohol, and dissolved in Tris EDTA buffer. The amount of total RNA was quantified with a spectrophotometer (Pharmacia, Peapack, NJ). To get rid of possible contamination of genomic DNA, 1 μg of total RNA was treated with DNase I (GIBCO) for 15 min at room temperature, the reaction was stopped with 25 mM EDTA, and samples were heated at 65°C for 10 min followed by 95°C for 5 min. For cDNA synthesis, ~400 ng of total RNA was transcribed with cDNA transcription reagents (Perkin-Elmer) using random hexamers. The cDNA was used for quantitative real-time PCR.

Quantitative real-time PCR. Gene expression was measured with the use of an ABI Prism 7700 Sequence Detection System (Perkin-Elmer) as described previously (5). Primers and TaqMan probes were designed using Primer Express TM 1.0 software (Perkin-Elmer) to amplify fewer than 150 base pairs. Probes were labeled at the 5’ end with the reporter dye molecule FAM (6-carboxy-fluorescein; emission λmax = 518 nm) and at the 3’ end with the quencher dye molecule TAMRA (6-carboxytetramethyl-rhodamine; emission λmax = 518 nm). On amplification, probes annealed to the template are cleaved by the 5’ nuclease activity of Taq polymerase reaction. This process separates the fluorescent label from the quencher and allows release of 1 unit of fluorescence for each unit of amplification. By determining the fluorescence with each cycle, one can determine the number of cycles necessary to reach a certain amount of fluorescence in a test sample. Target genes expressed relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Real-time PCRs of DNA specimens were conducted in a total volume of 50 μl with 1× TaqMan Master Mix (Perkin-Elmer) and primers at 300 nM and probes at 200 nM. Primer sequences were as follows: fibronectin EIIIA (forward): 5′-ATG TCG ATT CCA TCA AAA TTG CT-3′; fibronectin EIIIA (backward): 5′-CTG CAG TGT CTT CTT CAC CAT CA-3′; fibronectin EIIIA (probe): 6FAM-CCT ACT CGA GCC CTG AGG ATG GAA TCC-TAMRA; fibronectin EIIIB (forward): 5′-GAG GTG GAC CCC GCT AAA CT-3′; fibronectin EIIIB (backward): 5′-TAC CCT CTC CGG CAA CT-3′; fibronectin EIIIB (probe): 6FAM-TCC ACC ATT AGT GAC TGG TAG CGC ATC ACA -TAMRA; GAPDH (forward): 5′-CAG AAA ATG GAG CTT GAG AAA GT-3′; GAPDH (reverse): 5′-CCC ACT CCT CCA CCT TTG AC-3′; GAPDH (probe): 6FAM-CGT TGA GGG CAA TGC CAG CCC-TAMRA.

Thermal cycles parameters included 2 min at 50°C, 10 min at 95°C, and 40 cycles involving denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min.

Statistical analysis. All data were expressed as means ± SE. Statistical analyses were performed using Student’s t-test and by analyzing the variance using the Bonferroni procedure for multiple comparisons (Stat View 4.5 package; Abacus Concepts, Berkeley, CA). P << 0.05 was considered to be significant for t-test.

Results

Effect of NAC on 5% CSE-induced fibroblast-mediated gel contraction. Control gels contracted rapidly during the first day of incubation, reaching 50.2 ± 2.8% of the initial area, and contracted further during the next 3 days (Fig. 1). However, 5% CSE exposure significantly inhibited the fibroblasts’ ability to contract gels (94.4 ± 1.2% of initial area) over 3 days. Addition of NAC (10 mM) to the media of control gels had no effect on gel contraction. But added to media containing 5% CSE, NAC largely blocked the smoke-induced inhibition of fibroblast-mediated collagen gel contraction (46.9 ± 1.4% of initial area).

To evaluate the concentration dependence of NAC on gel contraction of 5% CSE-exposed fibroblasts, different concentrations of NAC were added to media of CSE-exposed collagen gels. Smoke-inhibited contraction was blocked in a concentration-dependent manner up to 1 mM of NAC (data not shown).

Effect of BSO on 1% CSE-exposed fibroblast-mediated gel contraction. BSO is a specific inhibitor of γ-glutamylcysteine synthetase, the key enzyme in intracellular GSH synthesis. To confirm the role of GSH on CSE’s effect on fibroblast-mediated gel contraction, BSO (1 mM) was added to the media of control gels and 1% CSE-exposed gels. Control gels and gels treated with BSO contracted rapidly within the first 24 h and reached a final area of 30.5 ± 1.5% and 34.00 ± 1.7% at 3 days, respectively (Fig. 2). In contrast to the inhibition observed with 5% CSE, no significant difference between 1% CSE-exposed gels and control was observed. However, BSO added to media containing 1% CSE resulted in inhibition of contraction to 82.1 ± 1.0% of the initial area (Fig. 2).

Effect of NAC on fibronectin release by 5% CSE-exposed fibroblasts in gel culture. Since we have previously observed that cigarette smoke inhibition of fibroblast-mediated collagen gel contraction may depend on inhibition of fibronectin release (7), the ability of NAC to modulate fibronectin release was assessed. Fibroblasts in gels conditioned with 1 or 10 mM of NAC released the same amount of fibronectin as control.
fibroblasts (Fig. 3). A significantly reduced amount of fibronectin was released in culture with 5% CSE. The addition of NAC (1 or 10 mM) to the medium containing 5% CSE significantly restored the fibronectin release. NAC added to 5% CSE cultures restored the fibronectin release to control levels, and, in lower concentrations, 0.1 or 0.01 mM NAC restored release partially, but significantly, compared with 5% CSE-exposed cultures.

**Effect of 1% CSE with BSO on fibronectin release by fibroblasts in collagen gels.** BSO (1 mM) added alone to fibroblasts had no effect on fibronectin release, whereas 1% CSE slightly but not significantly increased fibronectin release (Fig. 4). However, BSO added to media containing 1% CSE resulted in significant inhibition of fibronectin release (Fig. 4).

**Effect of pretreatment with NAC or BSO on CSE-exposed, fibroblast-mediated gel contraction.** To further evaluate the effect of NAC and BSO, fibroblasts contained in collagen gels were pretreated with NAC (10 mM) or BSO (1 mM) in serum-free DMEM for 1 day, rinsed, and then fed with 1% FCS-DMEM for 3 days. Fibroblasts pretreated with NAC (10 mM) significantly contracted collagen gels when exposed to 5% CSE, compared with smoke-exposed gels without NAC pre-
treatment (62.7 ± 1.1 vs. 91.1 ± 3.6% on day 1, \( P < 0.005, \) Fig. 5A). Fibroblasts in control, BSO-pretreated, or gels exposed to 1% CSE contracted similarly over 3 days of culture. But fibroblasts pretreated with BSO and exposed to 1% CSE resulted in significant inhibition of contraction compared with 1% CSE exposure alone (91.0 ± 2.6 vs. 57.1 ± 1.5%, \( P < 0.001, \) Fig. 5B).

**Effect of NAC and BSO pretreatment on fibroblast growth.** Fibroblasts pretreated with NAC (10 mM) and exposed to 5% CSE significantly released more fibronectin than fibroblasts exposed to 5% CSE alone (251.2 ± 26.1 vs. 25.2 ± 1.9 ng·ml\(^{-1}·\mu g\(^{-1}\) DNA, \( P < 0.001, \) Fig. 6A). BSO-pretreated fibroblasts added with 1% CSE significantly inhibited fibronectin production compared with 1% CSE exposure only (28.9 ± 3.7 vs. 419.9 ± 38.5 ng·ml\(^{-1}·\mu g\(^{-1}\) DNA, \( P < 0.001, \) Fig. 6B). No difference in fibronectin production was observed among control cells and NAC- or BSO-pretreated cells without CSE exposure.

**Effect of CSE on intracellular GSH in fibroblasts.** Intracellular GSH was measured after 24-h treatment with NAC or BSO on CSE-exposed cells. NAC-treated cells not exposed to CSE produced higher GSH levels than control fibroblasts (459.3 ± 39.6 vs. 100%, \( P < 0.005)\). In contrast, GSH was significantly decreased when exposed to 5% CSE (60.3 ± 13.8 vs. 100%, \( P < 0.05)\). However, in the presence of NAC and when fibroblasts were exposed to 5% CSE, the GSH level was increased significantly (867.7 ± 159.3% vs. 100% of control, \( P < 0.001, \) Fig. 7A). When fibroblasts were
exposed to 1% CSE for 24 h, GSH content was significantly increased (315.6 ± 69.4% vs. 100% of control, *P < 0.05). When smoke was added with BSO, this increase in GSH was completely inhibited (Fig. 7B).

To determine whether the effects of NAC and BSO were due to direct effects of cigarette smoke components or were mediated by effects on the fibroblasts, fibroblasts were pretreated with NAC or BSO for 24 h. Control cells were pretreated with serum-free medium. Pretreatment with NAC also both increased control fibroblast GSH (119.4 ± 6.8% vs. 100% of control, *P < 0.05, Fig. 7C). Pretreatment of fibroblasts for 24 h with BSO resulted in a 34% decrease in GSH levels (*P < 0.001, Fig. 7D).

**Effect of CSE and NAC on fibronectin mRNA.** The effect of NAC on fibronectin mRNA expression was also evaluated. Specifically, fibronectin mRNAs containing the EIIIA and EIIIB splicing regions were both assessed because fibronectin mRNA splice variants containing these regions are upregulated in some repair processes and fibrosis (13, 37). As expected, more EIIIA fibronectin mRNA than EIIIB mRNA was observed in all conditions (Fig. 8), as measured by real-time PCR. NAC-treated fibroblasts expressed more EIIIA and EIIIB fibronectin mRNA compared with fibroblasts exposed to 5% CSE alone. Fibroblasts cultured with NAC alone expressed more EIIIA and EIIIB fibronectin mRNA than other groups studied.

**DISCUSSION**

The current study demonstrates that the inhibitory effects of cigarette smoke on fibroblast fibronectin production and contraction of collagen gels can be modulated by agents that alter intracellular GSH. NAC, an agent that increases GSH production, attenuates the effect of cigarette smoke. Conversely, BSO, an agent that decreases intracellular GSH, increases the sensitivity of fibroblasts to smoke in a concentration-dependent manner. The changes in fibronectin production...
were paralleled by alterations in fibronectin mRNA levels, and both the EIIIA and EIIIB fibronectin splice variances were affected. Together, these data support the concept that cigarette smoke can inhibit the ability of fibroblasts to participate in repair responses and suggest that the sensitivity of fibroblasts to these effects of cigarette smoke may be modulated by GSH levels.

The ability of cigarette smoke to inhibit fibroblast-mediated collagen contraction has been demonstrated by Carnevali and colleagues (7). The effect appears to be mediated by an inhibition of fibronectin production and is not due to general toxicity because addition of exogenous fibronectin can restore contractility to smoke-exposed fibroblasts. Mechanisms other than modulation of fibronectin production are also possible for smoke-mediated alterations in fibroblast function. In this regard, fibroblast contraction of collagen gels requires interaction of the cells with collagen fibers through \( \alpha_2 \beta_1 \)-integrins (15). The contractile process, moreover, appears to be regulated by prostaglandin \( \text{E}_2 \) (PGE\(_2\)), which is produced by the fibroblasts and functions as a paracrine regulator (19, 25). In the Carnevali et al. studies, no effect of smoke either on the expression of \( \alpha_2 \beta_1 \)-integrins or on PGE\(_2\) production was noted (7). The current study confirms and extends these observations.

Carnevali and colleagues (7) undertook partial characterization of the toxic moieties present in cigarette smoke responsible for inhibition of collagen gel contraction. Activity was lost with volatilization or storage, suggesting that either volatile or labile compounds were responsible. Acrolein and acetaldehyde, reactive aldehydes present in high concentrations in cigarette smoke, were tested individually. Each compound inhibited collagen gel contraction at concentrations comparable with that present in cigarette smoke extract. Interestingly, neither compound inhibited as much as smoke, even at the maximal noncytotoxic concentrations. Acetaldehyde and acrolein together, however, were more potent than either compound alone (7).

The present study suggests that GSH can block the effect of cigarette smoke. GSH is a major intracellular antioxidant and, in some body compartments, such as the epithelial lining fluid of the lung, is also present in high concentrations (6, 10, 23, 38). The reactive sulfhydryl in GSH is capable of interacting not only with oxidants and free radicals but also with reactive aldehydes such as acrolein and acetaldehyde (12). The current study, therefore, is consistent with the potential toxic effects of acrolein and acetaldehyde on fibroblast-mediated collagen gel contraction. The data are also consistent with a potential role for smoke-derived oxidants. In this context, among the 4,700 compounds included in cigarette smoke, many function as oxidants. It is estimated that each puff of cigarette smoke contains \(~10^{14}\) radicals in a variety of chemical species (26). In addition, many compounds contained in cigarette smoke are capable of inducing cellular generation of oxidants. An antioxidant effect of GSH, therefore, could be either direct on smoke-contained oxidants or indirect on smoke-induced, cellular-derived oxidants (3, 36).

That intracellular GSH modulates the effects of cigarette smoke is supported by several lines of evidence in the current study. First, NAC, an agent that increases intracellular GSH by virtue of its conversion to cysteine and use as substrate, attenuated the effect of 5% cigarette smoke, a concentration that significantly inhibited collagen gel contraction by HFL-1 cells. Conversely, BSO, an inhibitor of GSH synthase, depleted intracellular GSH levels. Similarly, BSO increased the sensitivity of fibroblasts to the effects of 1% cigarette smoke. NAC, however, can itself function as an antioxidant. It is unlikely, however, that the effects of either NAC or BSO were mediated directly by an effect of either agent on cigarette smoke in the extracellular space. This was demonstrated by pretreating the cells with either agent to modulate intracellular GSH levels and then exposing the cells to smoke only after changing the medium. In addition, the effect of NAC and BSO on cigarette smoke inhibition of fibroblast gel contraction and fibronectin production was paralleled by the expected changes in intracellular GSH assessed by direct measurement. In this regard, not only did NAC stimulate and BSO inhibit GSH production but smoke was also found to alter GSH levels.

It has previously been reported that cigarette smoke acutely depletes GSH but results in a stimulation of GSH synthesis (17, 18). As a result, cigarette smoke-exposed fibroblasts rapidly recover GSH levels that may actually exceed control levels. The current study is also consistent with these observations in that 1% CSE...
or NAC-treated fibroblasts had modest increases in GSH levels, whereas NAC added to smoke-treated fibroblasts had marked increases in intracellular GSH levels. This resembles the reported observation that smokers have increased levels of GSH in alveolar epithelial fluid as assessed by bronchoalveolar lavage (6, 23).

The in vitro experiments performed in the current study used an aqueous extract of cigarette smoke prepared by a method originally developed by Carp and Janoff (8). Although this method has been widely used, the exposure of cells to this extract does not strictly reproduce in vivo experience after inhalation of cigarette smoke. The nature of the exposure in the in vitro model differs from in vivo exposure to smoke. The concentration of acetaldehyde in cigarette smoke extract prepared as used in the current study has been quantified (7, 39), and these concentrations are thought to resemble the amounts of acetaldehyde to which a smoker is exposed. Cigarette smoke contains an estimated 6,000 compounds, many of which can have complex toxicities. It is likely that some of these components of smoke will have very different in vivo exposures than those used in the current experimental model system. Thus although the current study suggests that cigarette smoke may modulate repair responses through mechanisms sensitive to endogenous GSH levels, in vivo studies will be required as a further test of this concept.

The current study demonstrates that cigarette smoke inhibits fibroblast production, at least in part, by decreasing steady-state mRNA levels. Both the EIIIA and EIIIB fibroblast splice variances are affected, although the ratio between them appears to be differentially affected. Because the splice variances EIIIA and EIIIB have slightly different spectrums of biological activities (31), this raises the possibility that some may alter not only the amount of fibronectin present but also its biological activity.

GSH is only one among many potential antioxidant defenses. In vivo, therefore, it is likely that the effect of smoke may be mitigated by a variety of mechanisms. The current study, however, suggests that GSH may be particularly important, at least with regard to smoke-induced alteration of fibroblast-mediated repair responses. Because GSH levels may vary due to either exogenous or endogenous factors, this may account for varying individual susceptibility to smoke-induced diseases related to altered repair. Such mechanisms may, moreover, offer potential therapeutic opportunities to intervene and potentially modify the adverse effects of cigarette smoke.

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