Cigarette smoke extract-induced suppression of caspase-3-like activity impairs human neutrophil phagocytosis

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Stringer KA, Tobias M, O’Neil HC, Franklin CC. Cigarette smoke extract-induced suppression of caspase-3-like activity impairs human neutrophil phagocytosis. Am J Physiol Lung Cell Mol Physiol 292: L1572–L1579, 2007. First published March 9, 2007; doi:10.1152/ajplung.00325.2006.—Neutrophils are the primary inflammatory cell in smokers’ lungs, but little is known about the ability of cigarette smoke to modulate neutrophil function. Neutrophils undergo caspase-3-dependent spontaneous, as well as phagocytosis-induced, apoptosis. This study investigated the ability of cigarette smoke extract (CSE) to alter neutrophil caspase-3 activity, apoptosis, and phagocytosis. CSE treatment resulted in a dramatic suppression of neutrophil caspase-3-like activity, which correlated with reduced cleavage of glutamate-cysteine ligase catalytic subunit, a known target of active caspase-3. CSE did not affect procaspase-3 processing to its active fragment, suggesting a direct effect of CSE on active caspase-3. Consistent with this, CSE inhibited active recombinant caspase-3 activity, which was abolished by dithiothreitol, suggesting a redox-sensitive mechanism. CSE-induced suppression of caspase-3 activity did not alter spontaneous apoptosis but did impair phagocytic activity. Since CSE treatment resulted in profound suppression of caspase-3 activity but did not alter apoptosis, the possibility of a threshold level of caspase-3 activity was investigated. CSE reduced caspase-3 activity in a concentration-dependent manner. Despite near complete suppression of caspase-3 activity, spontaneous apoptosis was not altered. Conversely, treatment with the pan-caspase inhibitor, Z-Val-Ala-Asp-fluoromethylketone, reduced spontaneous apoptosis. These data demonstrate that CSE does not suppress caspase-3 activity below a threshold level to prevent spontaneous apoptosis, but the level of inhibition is sufficient to impair neutrophil phagocytic activity. These divergent functions of caspase-3 may contribute to the persistence of neutrophils in the lungs of smokers, as well as a factor in their higher incidence of community-acquired pneumonia.

neutrophils; caspase-3; apoptosis; chronic obstructive pulmonary disease

EXPOSURE OF THE LUNGS TO CIGARETTE SMOKE (CS) leads to an influx of inflammatory cells, particularly neutrophils, which participate in the pathogenesis of chronic obstructive pulmonary disease (COPD) and lung cancer (4, 5, 19, 23, 33). In addition, this exposure may contribute to neutrophil dysfunction that results in a compromised host defense (26, 29). Although it is known that proteases and inflammatory cytokines are involved, the pathophysiology of COPD is complex, and the ability of CS to modulate neutrophil function has not been well characterized (4, 5).

A number of studies have demonstrated that CS modulates caspase-3 activity in a variety of cell types (2, 20, 22, 27, 35–38). It is generally accepted that caspase-3 activation is required for the successful execution of spontaneous neutrophil apoptosis (18, 28). Spontaneous neutrophil apoptosis is an essential mechanism for the maintenance of neutrophil homeostasis, so disruption of this process could have significant physiological consequences (e.g., persistent inflammation) (40). In addition, caspase-3 activation has been shown to participate in phagocytosis-induced neutrophil apoptosis (39). However, it is not known whether CS alters neutrophil caspase-3 activation that results in modulation of these essential neutrophil functions. In this regard, it is likely that any CS-induced modification of caspase-3 activity would have a dramatic effect on these processes. Improving our understanding by which CS influences neutrophil function is important, since alterations may be involved in inflammatory processes that participate in the pathogenesis of COPD and contribute to diminished host defense in cigarette smokers. Therefore, since caspase-3 is the only effector caspase in neutrophils, this study sought to determine whether CS exposure alters caspase-3 activity, spontaneous apoptosis, and phagocytosis in normal peripheral human neutrophils.

MATERIALS AND METHODS

Isolation of human neutrophils. This study was approved by the Colorado Multi-Institutional Review Board, and written, informed consent was obtained from all study participants. Neutrophils were isolated from human whole blood as previously described (11). Briefly, neutrophils were obtained by direct venipuncture of healthy, nonsmoking, medication-free volunteers using 3.8% sodium citrate as anticoagulant. The polymorphonuclear layer obtained from a sodium diatrizole-Dextran 300 gradient (Polymorphprep, Greiner Bio-One, Longwood, FL) was resuspended in RPMI 1640 (37°C), and the red cells were lysed with hypotonic saline. The neutrophils were washed with KRPD (Krebs-Ringer-phosphate-dextrose, pH 7.4) buffer and then resuspended (5 × 10⁶ cells/ml) in RPMI 1640 with 1-glutamine and 1% FBS. Neutrophil purity was verified as >95% with overall cell viability >98% by Trypan blue exclusion.

Preparation of CS extract. Immediately before experimentation, CS extract (CSE) was freshly generated from a single, lit, unfiltered Camel cigarette (R. J. Reynolds, Winston-Salem, NC; 26 mg tar/cigarette) by bubbling the smoke into PBS (10 ml, 37°C) using a mechanical peristaltic pump (125 ml/min; Masterflex, Cole-Parmer Instrument, Chicago, IL), as previously described (11). The CSE was subsequently sterile filtered (0.22 μm), cooled on ice, and used within 30 min. This was considered to be a 100% solution of CSE. This method of CSE acquisition and the final CSE concentration (% vol/vol) used in experimentation are consistent with that of others who...
utilize in vitro models for studying CS-induced changes in cellular function (2, 11, 27, 34).

**Treatment protocol.** Neutrophils were untreated, treated with CSE (4% vol/vol), or treated with the broad-spectrum caspase inhibitor Z-Val-Ala-Asp-fluoromethylketone (Z-VAD-FMK; 100 μM) (Biomol, Plymouth Meeting, PA) and incubated at 37°C (5% CO2).

**Determination of caspase-3-like activity.** Caspase-3-like activity was determined using the fluorogenic substrate N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC; Biomol), as previously described (16). Briefly, cells were harvested by centrifugation (10,000 g, 5 min, 4°C), and the pellet was resuspended in lysis buffer (10 mM Tris, 5 mM EDTA, 5 mM NaF, 1 mM Na3VO4, 20 mg/ml aprotinin, 10 μg/ml leupeptin, and 0.1 mM PMSF) and subjected to one freeze-thaw cycle. Protein levels were determined using a modified Lowry method (BioRad, Hercules, CA) (31). To perform the assay, 50 μg of cell extract protein were brought to a final volume of 50 μl with 2× caspase buffer (40 μM PIPES, pH 7.2, 200 mM NaCl, 20% sucrose, 0.2% CHAPS, 20 mM DTT) containing 40 μM Ac-DEVD-AMC in a sterile, opaque 96-well microtiter plate (Dynex Technologies, Chantilly, VA). The plate was incubated for 30 min (37°C) and then read at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Data were obtained using Softmax (La Jolla, CA) on a Spectra Max Gemini EM fluorescence microplate reader (Molecular Devices, Sunnyvale, CA), and specific activities were calculated against a standard curve of AMC (Sigma).

**Analysis of procaspase-3, caspase-3, and glutamate-L-cysteine ligase catalytic subunit proteins.** Following incubation, cells (5 × 106) were harvested by centrifugation (10,000 g, 5 min, 4°C), and the pellet was resuspended in 150-μl lysis buffer and sonicated on ice. The protein concentration of each lysate was determined as described above.

Cleavage of procaspase-3 (32 kDa) to active caspase (17 kDa) was determined by immunoblotting. Protein (60 μg) was separated by 10–15% gradient SDS-PAGE and transferred to nitrocellulose. The membrane was blocked in 5% milk Tris-buffered saline-Tween (1× PBS, 0.1% Tween) for 1 h at room temperature and then was cut into two sections, according to the molecular weights of procaspase-3 and active caspase-3. Each respective blot was probed for procaspase-3 (1:500 dilution in 5% milk; mouse monoclonal anti-procaspase-3 antibody; BD Pharmigen, San Jose, CA) or active caspase-3 (1:500 dilution; rabbit anti-active-caspase-3 antibody; Cell Signaling, Danvers, MA) overnight (4°C). After being washed, the membranes were incubated with secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature and washed again. Signal detection was accomplished using enhanced chemiluminescence (ECL Plus Reagent kit; Amersham Biosciences, Piscataway, NJ) followed by exposure to radiographic film (Amersham). To measure protein loading, the procaspase-3 blot was reprobed with an anti-actin antibody (1:1,000 dilution, rabbit anti-actin, Sigma).

Our laboratory has recently demonstrated that the catalytic subunit of glutamate-L-cysteine ligase (GCLC) is a direct target for caspase-mediated cleavage during apoptotic cell death (16). Cleavage of GCLC was measured by immunoblotting, as described above, with the exception that the membrane was blocked in 5% milk overnight (4°C) and probed for GCLC (73 kDa) and its cleavage product (60 kDa) (1:5,000 dilution in 0.5% milk; rabbit anti-GCLC antibody, kindly provided by Dr. Terrance Kavanagh, University of Washington) for 1 h at room temperature and then reprobed for actin to measure protein loading.

To directly assess the effects of CSE on caspase-3 activity, clarified extract from bacteria expressing active recombinant caspase-3 was incubated with CSE for 1 h, and caspase-3-like activity was measured as described above. BL21(DE3) cells expressing pGEX-KG-caspase-3 were induced, harvested, and lysed in PBS, and clarified extracts were used as a source of active caspase-3, as previously described (7, 16). Assessment of morphology and membrane integrity. Neutrophils were analyzed for apoptotic nuclear morphology using a modified Wright-Giensa stain (Protocol Hema 3, Fisher Scientific, Swedesboro, NJ). Cells were cytospun (Cytospin 3, Shandon, Pittsburgh, PA) onto microscope slides (25 × 75 × 1.0 mm). The cells were then fixed, stained, and destained in double-distilled H2O. Cells were examined using light microscopy (40×); 200 cells per slide utilizing a minimum of three separate fields were counted in a blinded fashion.

Leakage of intracellular lactate dehydrogenase (LDH) was used to assess neutrophil membrane integrity and thereby assess cytotoxicity (8). The LDH activity of the supernatant was collected after centrifugation (400 g), measured as the reduction of pyruvate to lactate coupled to the oxidation of NADH to NAD+, and read as the rate of decrease in absorbance at 340 nm. The supernatant from untreated cells was utilized for the negative control, and supernatant from baseline cells lysed with 1% Triton X-100 was utilized for the positive control for LDH activity. Supernatants were diluted 1:1 with Tris-NaCl buffer (81.3 mM Tris base, 203.3 mM NaCl, pH 7.2) and vortexed. An NADH solution (81.3 mM Tris, 203.3 mM NaCl, 0.244 mM NADH, pH 7.2) was added to sample/buffer combination and incubated with periodic shaking in a plate reader for 10 min (30°C). Following incubation, 20 μl pyruvate solution (81.3 mM Tris base, 203.3 mM NaCl, 9.76 mM pyruvate, pH 7.2) were added to each well, and the absorbance was analyzed using Softmax PRO 4.1 on a ThermoMax microplate reader (Molecular Devices). Data are presented as percent cytotoxicity using the following formula:

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\text{Mean experimental value} = \frac{\text{negative control} - \text{positive control}}{100} \times \text{negative control}
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**Detection of apoptosis by fluorescent-activated cell sorting.** Annexin V, a polypeptide that binds strongly and specifically to cell surface phosphatidylserine, is a sensitive marker of apoptosis (21). Following incubation, neutrophils (2.5 × 106 cells) were washed with KRPD and resuspended in 1× binding buffer (100 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 25 mM CaCl2) to which annexin V-FITC and propidium iodide (PI) were added. The cells were incubated at room temperature for 10 min, after which they were subjected to fluorescent-activated cell sorting (FACSCaliber cytometer, Becton-Dickinson, San Jose, CA), and fluorescence was analyzed by CellQuest software. After gating out debris, 10,000 events were collected for each analysis, and data were further analyzed using Summit (Cytomation, Ft. Collins, CO). Data are presented as the percentage of annexin V-FITC-positive and PI-negative cells, which represents cells that are apoptotic but not necrotic.

**Phagocytosis assay.** Neutrophils (1 × 106 cells/ml) were incubated in the absence or presence of CSE (4%) or Z-VAD-FMK (100 μM) for 2 h, or cytochalasin D (10 μM) for 10 min. Cells were then exposed to fluorescein-labeled heat-killed E. coli (Molecular Probes) that had been opsonized (37°C, 1 h; opsonizing reagent, Molecular Probes) for 30 min (37°C, 5% CO2) (39). Approximately 10 E. coli per neutrophil were used. After exposure to E. coli, cells were pelleted by centrifugation (10,000 g, 4°C, 5 min) and resuspended in ice-cold 0.2% Trypan blue in PBS (100 μl) to quench extracellular fluorescence. The samples were then transferred to an opaque 96-well microtiter plate, and phagocytosis was quantified by detection of fluorescence using an excitation wavelength of 485 nm and an emission wavelength of 530 nm (Spectra Max Gemini EM, Molecular Devices). Data are presented as percent reduction in phagocytosis relative to control (untreated cells) for each experiment. Cytochalasin D-treated cells represent maximal suppression of neutrophil phagocytosis.

**Data analysis.** Radiographic images were acquired and quantified using video densitometry (ImageQuant, Molecular Dynamics). The ratio density of the active caspase-3 and GCLC cleavage product protein bands to the corresponding actin protein band was determined for each sample. Data from all experiments were analyzed by
ANOVA using StatView (SAS, Cary, NC). Fisher protected least significant difference post hoc analysis was performed where appropriate. In all cases, a \( P \) value of \( \leq 0.05 \) was considered significant.

RESULTS

Neutrophils undergo spontaneous apoptosis that is dependent on the activation of caspase-3. To assess the effects of CSE on neutrophil apoptosis, we measured caspase-3-like activity using the fluorogenic substrate N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC). Data are mean \( \pm \) SE of five experiments. CSE suppressed caspase-3-like activity compared with untreated cells at all time periods analyzed: 2 h (\( *P = 0.0340 \)), 4 h (\( *P = 0.0003 \)), and 8 h (\( *P = 0.0083 \)).

Fig. 1. Cigarette smoke extract (CSE) suppresses caspase-3-like activity during spontaneous neutrophil apoptosis. Neutrophils were incubated in the absence or presence of CSE (4%) for the time periods indicated and harvested, and caspase-3-like activity was measured utilizing the fluorogenic substrate N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC). Data are mean \( \pm \) SE of five experiments. CSE suppressed caspase-3-like activity compared with untreated cells at all time periods analyzed: 2 h (\( *P = 0.0340 \)), 4 h (\( *P = 0.0003 \)), and 8 h (\( *P = 0.0083 \)).

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RESULTS

Neutrophils undergo spontaneous apoptosis that is dependent on the activation of caspase-3. To assess the effects of CSE on neutrophil apoptosis, we measured caspase-3-like activity using the fluorogenic substrate DEVD-AMC as a biochemical marker of apoptotic cell death. Caspase-3-like activity was readily detected within 2 h in untreated neutrophils and progressively increased with time (Fig. 1). However, treatment with CSE (4%) resulted in a robust suppression of caspase-3-like activity at all time points analyzed. To determine whether CSE-mediated suppression of caspase-3-like activity was due to an effect on the processing of the inactive procaspase-3 zymogen, we examined procaspase-3 processing by immunoblot analysis. Spontaneous neutrophil apoptosis was accompanied by the time-dependent appearance of the active 17-kDa caspase-3 fragment (Fig. 2, lanes 1–4), which correlated temporally with the appearance of caspase-3-like activity (see Fig. 1). While treatment with the pan-caspase inhibitor Z-VAD-FMK abolished the processing of procaspase-3 to its active fragment (lanes 8–10), CSE treatment did not affect the time-dependent appearance of the active caspase-3 fragment (lanes 5–7). These findings suggest that CSE-induced suppression of caspase-3-like activity was due to a direct inhibitory effect of CSE on active caspase-3. To verify that CSE suppressed caspase-3 activity during spontaneous neutrophil apoptosis, we examined the effect of CSE on the cleavage of an established endogenous caspase-3 target protein. Our laboratory recently demonstrated that GCLC is a direct target for caspase-mediated cleavage during apoptotic cell death, and cleavage correlates temporally with, and is dependent on, caspase-3-like activity (16, 17). Immunoblot analysis confirmed that GCLC is cleaved during spontaneous neutrophil apoptosis and that cleavage is abolished by treatment with either CSE or Z-VAD-FMK (Fig. 3). Collectively, these data show that exposure of neutrophils to CSE (4%) resulted in the profound suppression of caspase-3 activity that is not attributed to an alteration in procaspase-3 processing, but

Fig. 2. CSE does not prevent the processing of procaspase-3 to active caspase-3 during spontaneous neutrophil apoptosis. A: neutrophils were incubated in the absence (Un) or presence of CSE (4%) or Z-Val-Ala-Asp-fluoromethylketone (Z-VAD-FMK; 100 \( \mu \)M) (Z-VAD) for the time periods indicated. Cell extracts were analyzed for procaspase-3, active caspase-3, and actin by immunoblotting. Cells were treated with Z-VAD-FMK to inhibit procaspase cleavage, and actin is shown as a control for protein loading. B: quantitation of procaspase-3 processing. Relative protein levels were assessed by densitometry, and the mean \( \pm \) SE density of active caspase-3 protein-to-actin ratios was calculated for untreated, CSE-treated, and Z-VAD-FMK-treated neutrophils at 2, 4, and 8 h. There was no difference in the amount of active caspase-3 in untreated compared with CSE-treated cells at any time point examined and no difference in the amount of active caspase-3 detected in Z-VAD-FMK-treated cells at any time point compared with baseline cells.
caspase-3 activity was examined. As shown in Fig. 4 was incubated with increasing concentrations of CSE, and caspase-3. To test this hypothesis, active recombinant caspase-3 processing, suggesting that CSE has a direct effect on active neutrophil caspase-3 activity without affecting procaspase-3 cleavage (14, 15, 38). Our results demonstrate that CSE inhibits caspase activation, the mechanism by which this occurs is not direct, irreversible, redox-sensitive mechanism that is independent of an effect on procaspase-3 processing.

While recent studies report that CS or its constituents inhibit caspase activation, the mechanism by which this occurs is not clear (14, 15, 38). Our results demonstrate that CSE inhibits neutrophil caspase-3 activity without affecting procaspase-3 processing, suggesting that CSE has a direct effect on active caspase-3. To test this hypothesis, active recombinant caspase-3 was incubated with increasing concentrations of CSE, and caspase-3 activity was examined. As shown in Fig. 4A, incubation with CSE resulted in a dose-dependent inhibition of caspase-3 activity. Interestingly, preincubation of caspase-3 with CSE before initiation of the caspase assay was required to detect this inhibitory effect. As the caspase activity assay employed is performed in the presence of high levels of reducing equivalents (10 mM DTT), we examined whether CSE-mediated inhibition of caspase activity was redox sensitive, thus obviating the need to preincubate caspase-3 with CSE in the absence of reducing equivalents. For these studies, caspase-3 was preincubated with CSE in the absence or presence of DTT (1 mM) before initiation of the caspase assay. Preincubation in the presence of DTT abolished the inhibitory effects of CSE on caspase-3 activation (Fig. 4B). The inability of DTT in the caspase assay to reverse the inhibitory effect of CSE on caspase-3 indicates that this is an irreversible inhibition of caspase-3 activity. In aggregate, these findings indicate that CSE-mediated inhibition of caspase-3 activity occurs by a direct, irreversible, redox-sensitive mechanism that is independent of an effect on procaspase-3 processing.

As spontaneous neutrophil apoptosis is dependent on caspase-3 activation, we examined whether CSE-induced suppression of caspase-3-like activity inhibited neutrophil apoptotic cell death. Externalization of phosphatidylserine, as detected by annexin V-FITC staining, is a sensitive, reliable, and quantitative measurement of apoptosis (13). Untreated neutrophils exhibited a time-dependent increase in annexin V staining (data not shown) that correlated temporally with the increase in caspase-3-like activity (Fig. 1). Surprisingly, treatment with CSE did not affect the percentage of annexin V-FITC-positive neutrophils or the percentage of cells with morphological evidence of apoptosis compared with untreated cells at any time point examined (4-h data are shown in Fig. 5, A and B, respectively). In contrast, spontaneous apoptosis was inhibited in neutrophils treated with the pan-caspase inhibitor Z-VAD-FMK (Fig. 5). There was also no difference in the percentage of PI-positive cells in untreated and CSE-treated cells at any time point (data not shown), demonstrating that CSE treatment did not compromise the cell membrane. This result was substantiated in that CSE treatment did not potentiate LDH release, which was used to assess cytotoxicity (data not shown). These results demonstrate that CSE treatment neither altered spontaneous neutrophil apoptosis nor caused acute cytotoxicity.

Caspase-3 is activated during phagocytosis-induced neutrophil cell death and is required for neutrophil apoptosis, but it is not known whether caspase-3 activity influences phagocytosis (39). To determine the putative involvement of caspase activation in neutrophil phagocytosis and whether CSE-induced suppression of caspase-3 activity may function to inhibit neutrophil phagocytosis, neutrophils were incubated in the presence or absence of CSE, Z-VAD-FMK, or cytochalasin D, and phagocytosis of heat-inactivated fluorescein-labeled E. coli was measured. As shown in Fig. 6, CSE treatment reduced neutrophil phagocytosis to a level comparable to that observed in cytochalasin D-treated cells, which represents the maximal...
obtainable suppression of neutrophil phagocytosis. The ability of Z-VAD-FMK to also inhibit neutrophil phagocytosis (Fig. 6) suggests that caspase activation is required for neutrophil phagocytosis and that the inhibitory effects of CSE are likely mediated by suppression of caspase-3 activity.

Collectively, these findings suggest that, while CSE does not suppress caspase-3 activity below a threshold level necessary to prevent spontaneous neutrophil apoptosis, the level of inhibition attained is sufficient to impair neutrophil phagocytic activity. To determine whether a threshold level of caspase-3 activity was required for neutrophil cell death, we examined whether increasing concentrations of CSE were capable of suppressing neutrophil caspase-3 activity to levels sufficient to inhibit spontaneous apoptosis, as assessed by annexin V-FITC/PI staining. As shown in Fig. 7, increasing concentrations of CSE resulted in a concentration-dependent suppression of caspase-3-like activity, with 16% CSE inhibiting caspase-3-like activity by ~90%. Surprisingly, CSE concentrations as high as 16% had no effect on neutrophil cell death, and only complete suppression of caspase-3 activity with Z-VAD-FMK treatment was found to reduce spontaneous neutrophil apoptosis (Fig. 8). These results support the concept that the relative level of caspase-3 activation during spontaneous neutrophil apoptosis greatly exceeds that required to efficiently activate the cell death program. Furthermore, this level is below that necessary for suppression of neutrophil phagocytic activity.

**Fig. 4.** CSE inhibits caspase-3 activity by a redox-sensitive mechanism in vitro. Bacterial extract containing active recombinant caspase-3 was preincubated at 37°C for 1 h in the absence or presence of increasing amounts of CSE (A) and DTT (1 mM) (B), as indicated. Caspase-3-like activity was measured utilizing the fluorogenic substrate DEVD-AMC. Data are the means ± SE of at least three experiments performed in duplicate.

**Fig. 5.** CSE does not alter spontaneous neutrophil apoptosis. Neutrophils were incubated in the absence or presence of CSE (4%) or Z-VAD-FMK (100 μM) for 4 h. Apoptotic cell death was assessed by fluorescent-activated cell sorting analysis of annexin V-FITC/propidium iodide (PI)-stained cells, whereby annexin V-positive/PI-negative were judged to be apoptotic (A), and the appearance of apoptotic nuclear morphology (condensed chromatin) of Wright-Giemsa stained cells (B). Data are the means ± SE from at least five separate experiments. There was no difference between untreated and CSE-treated cells in any of the measured end points. Treatment with Z-VAD-FMK resulted in less annexin +/PI− cells and morphological apoptosis compared with untreated (*P = 0.05) or CSE-treated (+P = 0.02) cells.

**Fig. 6.** CSE and Z-VAD-FMK suppress neutrophil phagocytosis. Neutrophils were incubated in the absence or presence of CSE (4%) or Z-VAD-FMK (100 μM) for 2 h or cytochalasin D (10 μM) for 10 min. Phagocytosis of opsonized heat-inactivated fluorescein-labeled E. coli was measured as described in the MATERIALS AND METHODS section. Treatment with cytochalasin D, CSE, or Z-VAD-FMK reduced phagocytic activity by 25.5, 33.4, and 28.0%, respectively, compared with untreated cells (P < 0.0001; *cytochalasin D, †CSE, and ††Z-VAD-FMK treatment compared with untreated cells). Data are the means ± SE of six experiments.
DISCUSSION

This study demonstrated that exposure of human neutrophils to CSE in vitro resulted in a dramatic suppression of caspase-3-like activity. The mechanism of CSE-induced suppression of caspase-3 activity was a direct redox-sensitive inhibitory effect of CSE on the active caspase-3 protein that was not associated with modulation of the processing of procaspase-3 to active caspase-3. Unexpectedly, CSE-induced suppression of caspase-3 activity did not inhibit spontaneous apoptosis, but rather impaired the phagocytic ability of neutrophils, suggesting that activation of caspase-3 serves a previously unrecognized nonapoptotic function in neutrophils.

Other studies have shown that CSE treatment alters caspase-3 activity in a number of different cell types with a range of cell viability outcomes (2, 20, 22, 27, 35–38). For example, CSE treatment of Jurkat T cells resulted in necrosis that was associated with inhibition of procaspase-3 processing (38). Treatment of macrophages with CSE induced apoptosis that was independent of caspase-3 activation (2). Although culture conditions and treatment intensities varied, these divergent results demonstrate that CSE is capable of influencing cellular fate and apoptotic signaling to different extents, depending on the cell type. Our studies in neutrophils demonstrate that CSE has the ability to directly suppress the catalytic activity of active caspase-3 protein while not affecting the processing of the inactive procaspase-3 zymogen to active caspase-3. Several lines of evidence support this conclusion. First, CSE treatment inhibited neutrophil caspase-3-like activity as assessed using the fluorogenic DEVD-AMC substrate. CSE-induced suppression of neutrophil caspase-3 activity was confirmed by the ability of CSE to prevent the cleavage of endogenous GCLC, which is a direct target for caspase-3-dependent cleavage during apoptotic cell death (16). Furthermore, the ability of CSE to inhibit active recombinant caspase-3 in vitro indicates that this is a direct effect on the active caspase-3 protein. This inhibitory effect was abolished in the presence of DTT, suggesting the involvement of a redox-sensitive modification of active caspase-3. Finally, CSE was found to have no effect on the time-dependent appearance of the active 17-kDa caspase-3 fragment during spontaneous neutrophil apoptosis, indicating that CSE did not prevent the faithful processing of procaspase-3 during neutrophil cell death. While caspase-3 is generally considered to be the critical executioner caspase in mediating neutrophil apoptosis, these results do not exclude additional potential effects of CSE on other upstream caspases, such as caspases-8 and/or -9.

Caspase-3 is required for spontaneous neutrophil apoptosis, but it is not known if there is a threshold level of caspase-3 activity that is necessary for the initiation of neutrophil apoptosis. To test whether a threshold level of caspase-3 activity exists, we exposed cells to a broad range of CSE concentrations. This resulted in a CSE concentration-dependent suppression of caspase-3-like activity. Surprisingly, while treatment with the highest concentration of CSE (16%) resulted in very inhibited neutrophil caspase-3-like activity as assessed using the fluorogenic DEVD-AMC substrate. CSE-induced suppression of neutrophil caspase-3 activity was confirmed by the ability of CSE to prevent the cleavage of endogenous GCLC, which is a direct target for caspase-3-dependent cleavage during apoptotic cell death (16). Furthermore, the ability of CSE to inhibit active recombinant caspase-3 in vitro indicates that this is a direct effect on the active caspase-3 protein. This inhibitory effect was abolished in the presence of DTT, suggesting the involvement of a redox-sensitive modification of active caspase-3. Finally, CSE was found to have no effect on the time-dependent appearance of the active 17-kDa caspase-3 fragment during spontaneous neutrophil apoptosis, indicating that CSE did not prevent the faithful processing of procaspase-3 during neutrophil cell death. While caspase-3 is generally considered to be the critical executioner caspase in mediating neutrophil apoptosis, these results do not exclude additional potential effects of CSE on other upstream caspases, such as caspases-8 and/or -9.

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In conclusion, we have shown that human neutrophils exposed to whole CSE resulted in an irreversible, redox-sensitive, marked suppression of caspase-3 activity that was associated with the direct inhibition of the proteolytic function of active caspase-3 protein. While this did not alter spontaneous neutrophil apoptosis, an apparent physiological consequence of CSE exposure is a reduction in neutrophil phagocytic ability. Collectively, these data suggest that caspase-3 serves additional physiological functions in neutrophils that extend beyond the execution of apoptosis. Given the importance of neutrophils in inflammatory processes, these results have significant application in furthering our understanding of the cellular mechanisms that may participate in the pathogenesis of COPD and provide insight into a reason why smokers are more susceptible to infection.

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