Regulation of constitutive neutrophil apoptosis by the α,β-unsaturated aldehydes acrolein and 4-hydroxynonenal

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Finkelstein, Erik I., Jurjen Ruben, C. Wendy Koot, Milena Hristova, and Albert van der Vliet. Regulation of constitutive neutrophil apoptosis by the α,β-unsaturated aldehydes acrolein and 4-hydroxynonenal. Am J Physiol Lung Cell Mol Physiol 289:L1019–L1028, 2005. First published July 22, 2005; doi:10.1152/ajplung.00227.2005.—Reactive α,β-unsaturated aldehydes are major components of common environmental pollutants and are products of lipid oxidation. Although these aldehydes have been demonstrated to induce apoptotic cell death in various cell types, we recently observed that the α,β-unsaturated aldehyde acrolein (ACR) can inhibit constitutive apoptosis of polymorphonuclear neutrophils and thus potentially contribute to chronic inflammation. The present study was designed to investigate the biochemical mechanisms by which two representative α,β-unsaturated aldehydes, ACR and 4-hydroxynonenal (HNE), regulate neutrophil apoptosis. Whereas low concentrations of either aldehyde (<10 µM) mildly promoted apoptosis in neutrophils (reflected by increased phosphatidylserine exposure, caspase-3 activation, and mitochondrial cytochrome c release), higher concentrations prevented critical features of apoptosis (caspase-3 activation, phosphatidylserine exposure) and caused delayed neutrophil cell death with characteristics of necrosis/onsiosis. Inhibition of caspase-3 activation by either aldehyde occurred despite increases in mitochondrial cytochrome c release and occurred in close association with depletion of cellular GSH and with cysteine modifications within caspase-3. However, caspase-3 processing was also prevented, because of inhibited activation of caspases-9 and -8 under similar conditions, suggesting that ACR (and to a lesser extent HNE) can inhibit both intrinsic (mitochondrial dependent) and extrinsic mechanisms of neutrophil apoptosis at initial stages. Collectively, our results indicate that α,β-unsaturated aldehydes can inhibit constitutive neutrophil apoptosis by common mechanisms, involving changes in cellular GSH status resulting in reduced activation of initiator caspses as well as inactivation of caspase-3 by modification of its critical cysteine residue.

inflammation; reduced glutathione; caspase-3; adenosine 5’-triphosphate; cytochrome c

IT IS WELL APPRECIATED that cigarette smoking promotes airway inflammation and contributes to the etiology of common respiratory tract diseases such as asthma and chronic obstructive pulmonary disease (7, 48). Moreover, the adverse effects of cigarette smoking are associated with increased involvement of polymorphonuclear neutrophils, whose inappropriate activation and/or degranulation contributes to host tissues injury in chronic inflammatory diseases (45, 58). Although cigarette smoke contains thousands of chemical components, many of the acute cellular effects associated with cigarette smoke exposure have been attributed to volatile electrophilic compounds, of which α,β-unsaturated aldehydes such as acrolein (ACR) and crotonaldehyde form the major category (37, 40, 49). These and other α,β-unsaturated aldehydes, such as 4-hydroxynonenal (HNE), are also generated during active inflammation as a consequence of lipid peroxidation (11, 35, 53, 60). Hence, the respiratory tract is commonly exposed to a range of α,β-unsaturated aldehydes, from either environmental or endogenous sources. Recent analysis of aldehyde concentrations in saliva or airway secretions with the use of HPLC, capillary electrophoresis, or liquid chromatography-mass spectrometry, has demonstrated that ACR and other aldehydes are present in airway secretions in low micromolar concentrations and are elevated up to 10-fold in heavy smokers, respectively (1, 2). At these concentrations, these aldehydes have been shown to be capable of affecting a variety of biochemical processes, including transcription factor activation and gene expression, production of inflammatory cytokines, respiratory burst activation, and cell death (15, 30, 35, 43).

Although a number of studies have demonstrated that ACR and HNE induce apoptotic cell death in various cell types (6, 29, 36, 42), we recently observed that ACR markedly inhibits constitutive apoptosis in human neutrophils (15). Granulocyte apoptosis is a critical event in the downregulation and resolution of inflammation, because it limits their responsiveness to activating stimuli and promotes their phagocytic clearance by macrophages or other surrounding cells. As such, inadvertent release of potentially noxious granule enzymes is minimized (25, 50), and production of anti-inflammatory cytokines, such as TGF-β, is promoted to actively downregulate the inflammatory process (13, 27). Constitutive apoptosis of granulocytes is mediated by both intrinsic and extrinsic pathways. The first is caused by alterations in the proapoptotic Bcl protein family member Bax, which initiates release of mitochondrial cytochrome c and activation of caspase-9 and -3 (9, 38, 46). The extrinsic pathway involves activation of death receptors such as Fas and TNF receptors, presumably involving reactive oxygen species, and operates through activation of caspase-8 and -3 (31, 51, 59). Dysregulation of granulocyte apoptosis and their phagocytic clearance might present an additional mechanism by which environmental factors such as ACR in tobacco smoke contribute to development of chronic airway inflammation (20, 56).

Although the mechanisms by which α,β-unsaturated aldehydes induce apoptosis have been relatively well studied (6, 29, 36), it is less clear how ACR interferes with apoptosis (15, 32) and whether these inhibitory effects of ACR are representative of other α,β-unsaturated aldehydes. One proposed mech-
anism by which aldehydes, as well as other stress stimuli, block apoptosis and promote necrosis is by depleting cellular ATP that is required for caspase activation (32, 34). Alternatively, because of their high reactivity toward nucleophilic cellular targets such as GSH and protein cysteine residues (11), α,β-unsaturated aldehydes may cause interference with redox regulation of caspase activation (12, 26). The present studies were performed to further investigate the molecular mechanisms by which two representative α,β-unsaturated aldehydes, ACR and HNE, affect constitutive apoptosis of primary human neutrophils. Our results demonstrate that both ACR and HNE affect neutrophil apoptosis in a dual fashion, with mild proapoptotic effects at low micromolar concentrations and dramatic antiapoptotic effects at higher concentrations, which are associated with delayed cell death by nonapoptotic cytolysis. Inhibition of apoptosis was largely independent of effects on cellular ATP and closely associated with changes in cellular GSH status, which affect activation of initiator caspases as well as caspase-3.

MATERIALS AND METHODS

Neutrophil isolation and treatment. Human neutrophils were isolated from freshly drawn heparinized venous blood from healthy nonsmoking volunteers, as described previously (15), and according to procedures that were approved by our institutional review board. After isolation, neutrophils were resuspended in Krebs-Ringer buffer containing glucose (137 mM NaCl, 4.9 mM KCl, 5.7 mM Na2HPO4, and 5.5 mM d-glucose, pH 7.4) and aliquoted into 1.5-mL microcentrifuge tubes at a density of 2 × 106 cells/mL. Suspended neutrophils were briefly preincubated at 37°C and then treated with either ACR (Aldrich, Milwaukee, WI) or HNE (Calbiochem, La Jolla, CA) for 30 min, after which neutrophils were centrifuged (500 g, 10 min) and resuspended in serum-free RPMI medium (Life Technologies, Gaithersburg, MD) for up to 24 h. Stock solutions of ACR or HNE were made in sterile PBS immediately before use. Because of their high reactivity, most or all of the added ACR or HNE would have reacted with cell components within 30 min. In some experiments, neutrophils were incubated in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF; 10 ng/mL). After treatments for various time periods, cells were collected by centrifugation for the various assays described below.

Analysis of apoptosis by flow cytometry. To quantitate relative numbers of apoptotic or nonapoptotic cells, we labeled neutrophils with annexin V and propidium iodide (PI) using an annexin V-FITC apoptosis detection kit (Pharmingen, San Diego, CA) and performed flow cytometry analysis (Becton Dickinson, San Jose, CA). Briefly, cells were collected by centrifugation (10 min, 250 g), washed in cold PBS, and resuspended in assay buffer to which annexin V-FITC and PI were added. After incubation for 15 min in the dark, samples were analyzed by flow cytometry using a Becton Dickinson FACSCalibur (Becton Dickinson, San Jose, CA). PI-positive cells represent either late-stage apoptotic cells or necrotic cells. PI-negative and annexin V-negative cells were classified as nonapoptotic, whereas annexin V-positive/PI-negative cells were classified as apoptotic. PI-positive cells were added. After incubation for 15 min, cells were collected by centrifugation (12,000 g, 10 min). Collected supernatants were mixed with annexin V and propidium iodide (PI) and diluted in assay buffer and analyzed by flow cytometry. Cells that were PI-negative and annexin V-negative were considered viable, whereas annexin V-positive/PI-negative cells were classified as apoptotic. PI-positive cells represent either late-stage apoptotic cells or cytolytic cells and were classified as necrotic cells. Neutrophil cell death was also monitored by analysis of LDH release using the CyTox96 nonradioactive cytotoxicity assay (Promega).

Analysis of cytochrome c release. The release of cytochrome c from mitochondria into the cytoplasm was measured by digitonin permeabilization of neutrophils and Western blot analysis of cytosolic and mitochondrial extracts, essentially as described by Gao et al. (19). Cells were collected and resuspended in permeabilization buffer containing 20 mM HEPES (pH 7.4), 210 mM sucrose, 70 mM mannitol, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1% mammalian protease inhibitor cocktail (Sigma), and 100 μM digitonin. After incubation at 37°C for 5 min, cell suspensions were centrifuged for 10 min at 10,000 g, and supernatants (containing cytosolic proteins) were mixed with 6× Laemmli buffer (125 mM Tris-HCl, pH 6.7, 6% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.1% bromophenol blue) for analysis by SDS-PAGE. The remaining cell pellet (containing mitochondria and other cell organelles) was suspended in 100 μL of solubilization buffer (250 mM NaCl, 1.5 mM MgCl2, 50 mM HEPES, 1 mM EGTA, 1 mM PMSF, 2 mM Na3VO4, 10% glycerol, 1% Triton X-100, and 10 μg/mL aprotinin and leupeptin) and mixed with 6× Laemmli buffer for similar SDS-PAGE analysis. Samples were separated on 15% SDS-PAGE gels, transferred to polyvinylidene difluoride (PVDF) membranes, and immunoblotted with a monoclonal antibody against cytochrome c (Pharmingen), which was detected with a horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma) and enhanced chemiluminescence (ECL; Pierce, Rockford, IL). Band intensity was quantitated using NIH ImageJ software.

Caspase activity assays. After treatments, cells (2 × 106) were lysed in 75 μL of solubilization buffer, and caspase-3 activity was measured using the caspase-3 cellular activity assay kit (Calbiochem, La Jolla, CA). To this end, we mixed cell lysates with DTT-containing assay buffer and the colorimetric caspase-3 substrate Ac-DEVD-pNA in a 96-well plate, monitored the increase in absorbance over 2 h, and compared the results with standard incubations with recombinant caspase-3 (Calbiochem). Similarly, the activity of caspase-8 and -9 was assessed using a fluorometric assay (BioSource, Camarillo, CA), according to the manufacturer’s instructions.

Western blot analysis of procaspase processing. Proteolytic cleavage of procaspase-3, -8, and -9 was determined by separating cell lysates on 10 or 15% SDS-PAGE gels, transferring to PVDF membranes, and immunoblotting with a polyclonal antibody against procaspase-3 (Pharmingen), a monoclonal antibody against procaspase-8 (Cell Signaling, Beverly, MA), or a polyclonal antibody against procaspase-9 (Cell Signaling). Similarly, proteolytic cleavage of the X-linked inhibitor of apoptosis protein (XIAP) was analyzed using a polyclonal antibody (Cell Signaling). Antibodies were detected using HRP-conjugated secondary antibodies and ECL.

Analysis of cellular ATP. ATP was extracted from cells and analyzed by HPLC, according to the method of Sellevold et al. (52). Neutrophils (2 × 106) were centrifuged, washed with cold PBS, and extracted with 200 μL of 0.42 M perchloric acid by continuous vortexing for 2 min. Samples were neutralized by adding 80 μL of 1 M KOH, and insoluble material was removed by centrifugation (14,000 g, 15 min). Collected supernatants were buffered by adding 200 μL of 1 M KH2PO4 (pH 6.5) and were centrifuged again to remove traces of insoluble material before injection onto a 250 × 4.6-mm Spherisorb ODS-2 column (Waters, Milford, MA). ATP was eluted with 0.1 M KH2PO4 (pH 6.0) containing 8 mM tetrabutylammonium hydrogen sulfate-methanol (75:25 vol/vol) at a flow rate of 1 mL/min. ATP eluted at about 6 min, was detected by UV absorbance at 254 nm, and was quantitated by comparison with external standards that were subjected to the same extraction procedure.

Analysis of cellular GSH. Cellular GSH was analyzed by reaction of cell lysates with an equal volume of 4 mM monobromobimane (Calbiochem) in 50 mM N-ethylmorpholine (pH 8.0), which was allowed to proceed for 10 min in the dark. After protein precipitation (5% TCA final concentration) samples were analyzed by HPLC with fluorescence detection as described previously (55).

Determination of cysteine modification in procaspase-3. Recombinant procaspase-3 (Calbiochem) (25 μg/mL) was treated with ACR or HNE for 20 min and subsequently incubated in the presence of 1 mM of the sulfhydryl-reactive biotinylating agent 1-biotinamido-4-[4′-(maleimidomethyl)cyclohexanecarboxamido]butane (biotin-BMCC; Pierce) for 30 min at room temperature. Samples were analyzed by SDS-PAGE, and the extent of procaspase biotinylation was detected using streptavidin-peroxidase (Sigma) and ECL (Pierce). In similar studies, neutrophils (2 × 109/ml) were treated with ACR or HNE for...
30 min, lysed in solubilization buffer including 1 mM biotin-BMCC, and incubated for 60 min at 4°C. Caspase-3 was immunoprecipitated from neutrophil lysates from 10^7 neutrophils by using 2 μg of a monoclonal antibody (clone 19; Transduction Laboratories) and 4 mg of protein A-Sepharose (Sigma). After the Sepharose beads were washed three times with high-salt buffer (50 mM Tris, 500 mM NaCl, 1% NP-40, and 100 μM EDTA, pH 8.0), biotinylation of caspase-3 was determined by SDS-PAGE and detection with streptavidin-peroxidase. Caspase-3 was detected using a monoclonal antibody (Santa Cruz) to verify equal gel loading.

Determination of protein-aldehyde conjugates. Caspase-3 that was immunoprecipitated from aldehyde-treated neutrophils or aldehyde-treated recombinant procaspase-3 (Calbiochem) was derivatized with 2,4-dinitrophenylhydrazine (DNPH) for analysis of protein carbonyl adducts by SDS-PAGE and Western blotting (OxyBlot protein oxidation detection kit; Intergen, Purchase, NY). In control experiments, similar analysis of protein carbonyl adducts was performed by DNPH derivatization of whole neutrophil cell lysates (OxyBlot) according to the manufacturer’s instructions.

Statistical analysis. Data are expressed as means ± SE, and statistical comparisons were made using the two-tailed Student’s t-test, with P = 0.05 as the limit of significance.

RESULTS

Unsaturated aldehydes affect constitutive neutrophil apoptosis in a dual fashion. Freshly isolated neutrophils were allowed to age for up to 20 h at 37°C, and constitutive apoptosis was assessed using flow cytometry after dual cell labeling with annexin V/PI. Recovery of neutrophils for such analysis was 25–35% and was not significantly altered after
ACR or HNE treatment. As shown in Fig. 1, the percentage of apoptotic neutrophils appeared to increase slightly after treatment at the lowest ACR concentration tested (3 μM, equivalent to 1.5 mmol/10⁶ cells), but apoptosis was markedly inhibited at higher concentrations of ACR, as illustrated by reduced annexin V labeling, consistent with earlier observations (15). Under these conditions, enhanced numbers of viable cells were observed, and significant increases in necrotic cells (as illustrated by increased PI-positive cells) were observed only at the highest ACR concentration tested (30 μM). A similar concentration-dependent profile was observed with HNE. In this case the proapoptotic effect at low doses (up to 10 μM) was more pronounced and statistically significant, and inhibition of apoptosis by higher HNE concentrations (>30 μM) was associated with increased percentages of apparently viable (PI-negative) cells (Fig. 1). Similar flow cytometry analysis of dose-dependent effects of aldehydes on neutrophil apoptosis after 6 h of incubation yielded qualitative comparable results, except that markedly more viable neutrophils were recovered and the percentages of apoptotic cells were lower (results not shown). The effects of aldehydes on neutrophil apoptosis were also investigated in the presence of 10 ng/ml GM-CSF, a proinflammatory cytokine that delays constitutive neutrophil apoptosis (5). In this case, a similar dose-dependent effect was observed for HNE, and only antiapoptotic effects were observed with ACR at doses >3 μM (data not shown).

To explore whether the observed increase in neutrophil necrosis by the highest concentrations of ACR or HNE were caused by an alternative mode of cell death or accelerated apoptosis, we analyzed annexin V and PI labeling of aldehyde-treated and untreated cells. As shown in Fig. 1C, constitutive apoptosis of untreated neutrophils was associated with gradual decreases in viable cells and early increases in annexin V-positive/PI-negative cells (6 and 12 h) followed by markedly increased numbers of dual-positive cells at later time points (24 h), reflecting apoptosis followed by secondary necrosis. In contrast, the time-dependent increase in numbers of unstained (viable) cells was delayed after treatment with ACR, and time-dependent increases in dual-stained (necrotic) cells were not accompanied by early increases in annexin V-positive cells. Instead, increased numbers of PI-positive/annexin V-negative cells were observed at earlier time points (12 h), suggestive of necrotic/oncotic cell death in which neutrophils become initially permeable to PI and eventually to annexin V. Qualitatively similar results were obtained with 60 μM HNE, although the percentage of PI-positive (necrotic/oncotic) cells was generally lower (data not shown).

Collectively, these results indicate that inhibition of neutrophil apoptosis by ACR or HNE is associated with delayed cell death and with a switched mode of cell death to necrosis/oncrosis. Analysis of cell death by monitoring the release of LDH indicated slightly delayed cell death at earlier time points (6–12 h) after treatment with 10 μM ACR but no significant differences at later time points (20 h). Higher concentrations of ACR tended to increase LDH release at all time points, consistent with earlier findings (15). No significant differences in LDH release were observed after neutrophil treatment with HNE (not shown). Because measurement of LDH release does not distinguish between apoptotic or necrotic cell death, these findings indicate that the observed inhibition of apoptosis by ACR or HNE is primarily associated with a switch to necrotic cell death. Morphological analysis of neutrophils after ACR or HNE exposure showed consistent results, with a relative absence of typical apoptotic features (e.g., nuclear condensation) and increased cell swelling, indicative of necrosis (not shown).

Effects on caspase-3 activation. We next assessed the effects of ACR and HNE on caspase-3 activation, a key event in the execution of constitutive neutrophil apoptosis (5, 46). As shown in Fig. 2A, ACR and HNE dose dependently affected activation of caspase-3 during neutrophil aging, which closely mirrored their effects on apoptosis as assessed by annexin V labeling (Fig. 1). Lower concentrations of either ACR or HNE appeared to mildly enhance caspase-3 activity (in the case of HNE, this was statistically significant), whereas higher concentrations of either aldehyde dramatically inhibited caspase-3 activation, which was nearly completely prevented by 30 μM ACR or 60 μM HNE (Fig. 2A). Activation of caspase-3 in association with constitutive neutrophil apoptosis was reduced by ~50% in the presence of 10 ng/ml GM-CSF, consistent with its ability to delay constitutive neutrophil apoptosis (9). Nevertheless, ACR or HNE similarly prevented caspase-3 activation in neutrophils in the presence of GM-CSF (data not shown), suggesting that these aldehydes regulate apoptosis by a mechanism independent of GM-CSF-mediated pathways.

Alteration of cellular redox status by aldehydes: direct cysteine modification of caspase-3. On the basis of the known reactivity of α,β-unsaturated aldehydes with cellular nucleophiles such as GSH and protein cysteine residues, we hypothesized that caspase-3 inactivation by these aldehydes may have occurred through direct or indirect modification of its redox-sensitive cysteine residue (12, 39). Consistent with such a mechanism, both ACR and HNE caused a rapid (within 30 min) and dose-dependent depletion of cellular GSH (Fig. 2B), which is most likely due to direct or enzyme-catalyzed GSH conjugation with these aldehydes (49). ACR was about five times more potent than HNE in its ability to deplete GSH, possibly because of its higher hydrophilicity and higher reactivity with thiols (11). A similar difference in potency was observed with regard to their inhibitory effects on caspase-3 activation (Fig. 2A). Indeed, the effects of either aldehyde on cellular GSH were found to correspond closely with their inhibitory effects on caspase-3 activation (Fig. 2C), suggesting that the inhibition of caspase activation by these aldehydes is directly or indirectly related to depletion of cellular GSH. Although GSH depletion has been previously observed in association with apoptosis (51), kinetic analysis shows that this occurs much more slowly compared with the rapid depletion of cellular GSH by ACR (Fig. 2D) or HNE (not shown), which is complete within 30 min.

To explore the ability of aldehyde to modify reactive cysteine residues within caspase-3, we incubated recombinant pro-caspase-3 with ACR or HNE and analyzed changes in its cysteine content by selective cysteine labeling with the biotinylating agent biotin-BMCC. As shown in Fig. 3A, treatment of procaspase-3 with either ACR or HNE resulted in a dose-dependent decrease in biotin-BMCC labeling, indicating direct modification of one or more cysteine residues by these aldehydes. A similar approach was used to determine changes in cysteine content of endogenous procaspase-3 in neutrophils 30 min after treatment with ACR, by neutrophil lysis in the presence of biotin-BMCC and immunoprecipitation of procaspase-3. As shown, ACR dose dependently decreased the
cysteine content of procaspase-3 in neutrophils (Fig. 3A, bottom) under conditions that inhibit its activation (Fig. 2A).

We next attempted to determine whether ACR causes direct alkylation of caspase-3 by Michael addition, by derivatizing lysates of ACR-treated neutrophils with DNPH. Although a range of DNP-reactive proteins could be detected in whole lysates of ACR-treated neutrophils (43), we could not detect increased DNP reactivity in caspase-3 that was immunoprecipitated from ACR-treated neutrophils or in ACR-treated recombinant procaspase-3 (data not shown). Thus our findings are inconclusive regarding the precise mechanism of cysteine modification within caspase-3 in ACR-exposed neutrophils, which may be caused by irreversible oxidation as a result of GSH depletion or alternative modifications by ACR or HNE that are undetectable with DNPH.

**Effects of ACR and HNE on procaspase-3 processing.** The activation of procaspase-3 requires its proteolytic processing by upstream caspases such as caspase-8 or -9. As shown in Fig. 3, B and C, Western blot analysis of procaspase-3 processing revealed substantial procaspase-3 cleavage in neutrophils after incubation for 6 h, indicated by reduced levels of procaspase-3 (32 kDa) and increased amounts of cleaved caspase-3 (±20 and 17 kDa). Neutrophil exposure to ≥10 μM ACR markedly prevented procaspase-3 cleavage (Fig. 3B), consistent with the dose-dependent inhibition by ACR of caspase-3 activity (Fig. 2A). Qualitatively different results were obtained with HNE, for which case procaspase-3 processing was enhanced at low doses (up to 30 μM), whereas higher concentrations reduced detectable levels of active (p17/20) caspase-3 but did not result in corresponding increases in procaspase-3 (Fig. 3C). Thus, in addition to causing direct modification of its cysteine residues, ACR and HNE also prevent caspase-3 activation by interference with procaspase-3 processing.

**Analysis of metabolic stress by unsaturated aldehydes: mitochondrial cytochrome c release and ATP depletion.** The intrinsic pathway of constitutive neutrophil apoptosis, which depends on mitochondrial depolarization and release of cytochrome c into the cytoplasm (9), can be stimulated by stress-activated pathways, and we therefore explored whether ACR and HNE affect mitochondrial cytochrome c release. As shown in Fig. 4, cytoplasmic extracts of neutrophils that were incubated for 4 h contained detectable amounts of cytochrome c, and cytoplasmic cytochrome c levels were dose dependently increased after neutrophil exposure to either ACR and HNE, corresponding with decreases in mitochondrial levels of cytochrome c. The mild proapoptotic effects of these aldehydes are most likely related to their ability to increase cytochrome c release. However, the fact that higher aldehyde concentrations inhibit neutrophil caspase-3 activation and apoptosis despite more dramatic cytochrome c release suggests that these aldehydes interfere with processes either downstream or independent of mitochondrial dysfunction and cytochrome c release.

Another feature of metabolic stress that may interfere with apoptotic pathways is depletion of cellular ATP in response to cell molecular damage (34). We determined ATP levels in extracts of ACR- or HNE-treated neutrophils and observed
Fig. 3. ACR and HNE cause direct modification of procaspase-3 and affect caspase activation. A: recombinant caspase-3 (25 μg/ml in PBS) was treated with ACR or HNE for 30 min, and reduced cysteine content was analyzed after labeling with biotin-BMCC (top). Similar cysteine labeling was also performed in caspase-3 from neutrophils after ACR treatment for 30 min. Cells were lysed in the presence of 1 mM biotin-BMCC, and procaspase-3 was immunoprecipitated and analyzed by Western blotting (bottom). Representative blots of 2–3 experiments are shown. Rec., recombinant; IB, immunoblot; IP, immunoprecipitation. B: ACR inhibits procaspase-3 processing and activation. Neutrophils were treated with ACR and lysed after 6 h for Western blot analysis of procaspase-3 (p32) and activated caspase-3 (p17/20). Freshly isolated neutrophils were analyzed directly (lane C) for comparison. A representative blot (top) and densitometric analysis from 3–5 experiments (means ± SE; bottom) are shown. C: effects of HNE on procaspase-3 processing. Similar Western blot analysis and densitometry were performed after neutrophil treatment with various concentrations of HNE.

The activation of procaspase-3 is also regulated by XIAP, degradation of which promotes procaspase-3 activation (17, 33). However, in accordance with some earlier observations (38), we observed no evidence of XIAP degradation during neutrophils under our conditions, and ACR treatment did not significantly affect XIAP levels (Fig. 5C). Hence, aldehyde-induced effects on caspase-3 activation are not due to changes in XIAP.

DISCUSSION

The present studies show that ACR and HNE, two representative members of a family of α,β-unsaturated aldehydes that are generated during lipid peroxidation (11) or are present in environmental pollutants such as cigarette smoke (37), can markedly affect neutrophil apoptosis at concentrations that may be present in smokers’ lungs (1, 2). Although these aldehydes have been demonstrated to be capable of inducing apoptosis in several cell systems (29, 36, 42), their ability to suppress apoptosis may be highly relevant to inflammatory cell types that can undergo rapid expansion and/or turnover and for which constitutive or death receptor-mediated apoptosis are critical in regulating and maintaining overall cell number. The conclusion that aldehydes can inhibit apoptosis is based largely on the observed reduced cell labeling with annexin V, which may have been complicated by potential interference of reaction products of these aldehydes with components on the cell surface (32). However, the close association between decreases in annexin V labeling with inhibitory effects on caspase-3 activation, as well as the ability of ACR to prevent other apoptotic features such as nuclear condensation and DNA laddering (15), strongly suggests that the decreased annexin V...
binding in aldehyde-exposed cells is the result of a specific cellular response and reflects decreased apoptosis. The changes in annexin V labeling of phosphatidylserine externalization imply that phagocytic clearance of apoptotic neutrophils that is mediated by this apoptotic signal would also be prevented (14).

Furthermore, the apparent delay in neutrophil death and the switch to necrotic/oncotic cell death due to the presence of these aldehydes may cause increased proinflammatory cytokine production and release of noxious granule constituents due to cytolysis and thus promote chronic inflammation (15).

The ability of ACR and HNE to induce apoptosis has been related to the activation of mitogen-activated protein kinase pathways such as JNK and p38 (15, 35, 44), pathways that also have been implicated in the intrinsic (stress induced) apoptotic pathway in neutrophils (3, 16). Conversely, the molecular mechanisms by which these aldehydes can inhibit apoptosis are less well characterized (15, 32) and, hence, were the primary focus of the present study. The inhibitory effects of ACR and HNE on apoptosis appear to be independent of cytokine-mediated regulation of cell survival or apoptosis, because these aldehydes were similarly effective in the presence of GM-CSF, a proinflammatory cytokine that delays constitutive neutrophil apoptosis (9). Furthermore, the observed inhibition of caspase activation despite increased mitochondrial cytochrome c release (Figs. 3 and 4) implies that these aldehydes act on cellular pathways independent from or downstream of mitochondrial cytochrome c release.

Perhaps the strongest indication regarding the mechanisms by which ACR and HNE inhibit neutrophil apoptosis is the close association between the inhibition of caspase-3 activation and depletion of cellular GSH (Fig. 2). ACR and HNE share similar chemical reactivity, in both cases related to the strong

![Fig. 4. Metabolic changes in ACR- and HNE-treated neutrophils. A: ACR and HNE promote mitochondrial cytochrome c release. After aldehyde treatment, neutrophils were incubated for 4 h before preparation of cytosolic and mitochondrial extracts. Cytochrome c was analyzed in either fraction by Western blotting with a polyclonal antibody (Pharmingen). B: densitometric analysis of cytosolic cytochrome c. Data represent means ± SE from 3 separate experiments. C: effects of ACR or HNE on neutrophil ATP levels. Aldehyde-treated neutrophils were incubated for 6 h and extracted with perchloric acid for ATP analysis by HPLC. Data represent means ± SE from 3 separate experiments.](http://ajplung.physiology.org/)

![Fig. 5. Effects of ACR or HNE on activation of caspases-9 and -8. Neutrophils were treated with ACR or HNE and lysed after 6-h incubation for analysis of caspase-9 (A) or caspase-8 (B) activity, using fluorogenic substrates. Data represent means ± SE from 3–4 experiments. *P < 0.05 compared with untreated controls. C: Western blot analysis of neutrophil lysates for procaspase-8, procaspase-9, or the X-linked inhibitor of apoptosis protein (XIAP). Lane C represents freshly isolated, untreated neutrophils.](http://ajplung.physiology.org/)
electrophilic character of the α,β-double bond adjacent to the aldehyde group, which readily undergoes Michael addition to cellular nucleophiles such as thiols (11). Indeed, exposure of neutrophils to either ACR or HNE caused a rapid decrease in cellular GSH, presumably due to glutathione S-transferase-mediated conjugation to GSH (4, 49). Depletion of cellular GSH may sensitize caspase-3 to oxidative inactivation by facilitating oxidative modification of its critical cysteine residue (12, 39) or may allow direct alkylation of this cysteine residue by aldehydes, analogous to the previously proposed mode of inactivation of interleukin-1β-converting enzyme by HNE (8). Indirect evidence for cysteine modification within procaspase-3 was obtained using thiol labeling with biotin-BMCC, which revealed reduced cysteine content of recombinant procaspase-3 as well as neutrophil-derived procaspase-3 after exposure to ACR or HNE (Fig. 3). Attempts made using DNPH derivatization to verify whether cysteine residues were alkylated by these aldehydes were unsuccessful. This could imply that aldehyde-induced cysteine modification within procaspase-3 may have occurred indirectly or that generated products were undetectable by DNPH because of potential secondary reactions of initially formed Michael adducts (11, 24). Alternatively, cysteine modifications might also have occurred indirectly by reaction with Michael adducts of ACR with histidine and/or lysine residues (18, 28, 44, 53). More directed future studies are needed to determine the exact modifications within caspase-3 under these conditions. Nevertheless, our results demonstrate that ACR and other unsaturated aldehydes can block caspase activation, and this is closely related to GSH depletion and cellular redox changes and is accompanied by modification of cysteine residues within caspase-3.

Although the enzymatic activity of activated caspase-3 is sensitive to modification of its critical cysteine residue (12, 23, 39), it is unclear whether such modification also prevents procaspase-3 processing by upstream caspases. In this regard, the observation that neutrophil exposure to ACR markedly prevents procaspase-3 processing implies interference with upstream events, rather than direct modification of procaspase-3 itself. Inhibition of procaspase-3 processing by ACR was associated with increased recovery of procaspase-3, but this was not apparent for HNE, revealing perhaps a different mechanism by which this aldehyde affects caspase-3 activity. Alternatively, modification of procaspase-3 by high doses of HNE may have prevented its detection by Western blot analysis.

One proposed factor that prevents caspase-3 activation is stress-induced depletion of cellular ATP, for instance, as a consequence of activation of poly(ADP-ribose) polymerase (PARP) (32, 34, 57), because ATP is required in the intrinsic apoptotic pathway for proteolytic activation of caspase-9 within the apoptosome (32, 34). Although neutrophil exposure to ACR or HNE modestly reduced cellular ATP levels, this is unlikely to account for the more dramatic effect on caspase activation. For example, exposure of neutrophils to 10 μM ACR resulted in marked inhibition of apoptosis and caspase-3 activation and caused dramatic GSH depletion as well as procaspase-3 cysteine modification, but it did not significantly alter cellular ATP levels. These findings clearly imply that the inhibition of caspase-3 activation by ACR is primarily due to changes in cellular redox status, rather than metabolic stress and depletion of cellular ATP. The dramatically increased cytochrome c release by ACR or HNE may be another feature of metabolic stress but also may have occurred as a consequence of GSH depletion and may not necessarily result in cell death (21).

In accordance with the dose-dependent effects of ACR or HNE on caspase-3 activation, their effects on activation of caspase-9 and -8 in aging neutrophils were strikingly similar (Figs. 2 and 5). The modest activation of caspase-9 by low doses of these aldehydes most likely reflects activation of stress-mediated apoptosis initiated by mitochondrial cytochrome c release. The inhibitory effects of high doses of ACR and HNE on caspase-9 may be related to cellular redox changes due to GSH depletion (26) but also could be partly due to ATP depletion. Constitutive neutrophil apoptosis has recently been demonstrated to involve death receptor (CD95)-mediated pathways and activation of caspase-8 (51), and activation of this caspase was also dose dependently inhibited by ACR and HNE (Fig. 5) in strikingly close parallel to their inhibitory effects on caspase-3 (Fig. 2). The efficacy of either ACR or HNE in inhibiting caspase-8 or -3 activation was also remarkably similar to their ability to deplete cellular GSH, strongly suggesting that activation of caspase-8 and -3 are dependent on cellular redox status. This notion appears to conflict with the proposed role of NADPH oxidase-derived oxidants in constitutive and CD95-mediated neutrophil apoptosis (31), which are thought to initiate apoptosis by depleting GSH and altering cellular redox status (51). However, depletion of cellular GSH during apoptosis has been shown to be due to GSH extrusion, rather than its oxidation, and appears to occur downstream and perhaps as a result of caspase activation (22, 54). Accordingly, we observed gradual depletion of cellular GSH during constitutive neutrophil apoptosis (Fig. 2D), and this was largely recovered in the extracellular medium as a mixed disulfide with cysteine, attributable to its reaction with cystine (47) (results not shown). In contrast, the rapid depletion of cellular GSH by alkylating agents such as ACR and HNE (26, 49) is more likely to affect initial events in apoptosis, such as the activation of initiator caspases, which appear to be tightly controlled by redox regulation (26, 41).

Overall, our results indicate that the α,β-unsaturated aldehydes ACR and HNE are capable of both stimulating and preventing constitutive apoptosis by dual effects on the activation of caspase cascades. The ability to inhibit caspase activation was most pronounced for ACR but was also observed with related α,β-unsaturated aldehydes such as HNE. Importantly, although both aldehydes were capable of inducing a number of cellular changes, including mitochondrial cytochrome c release and ATP utilization, the inhibitory effects were closely associated with depletion of cellular GSH, which led to decreased activation of initiator caspases and allowed direct cysteine modification within caspase-3. Both events most likely contributed to the overall inhibition of caspase-3 activity and execution of apoptosis, causing delayed necrotic cell death. The inhibitory effects of α,β-unsaturated aldehydes on neutrophil apoptosis may be most relevant in conditions of elevated production or exposure to these agents (1, 2), such as in the airways of smokers, and may contribute to the development or exacerbation of chronic inflammatory diseases such as asthma and chronic obstructive pulmonary disease.
REFERENCES


