Captopril inhibits apoptosis in human lung epithelial cells: a potential antifibrotic mechanism

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Captopril inhibits apoptosis in human lung epithelial cells: a potential antifibrotic mechanism. Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 19): L1013–L1017, 1998.—The angiotensin-converting enzyme inhibitor captopril has been shown to inhibit fibrogenesis in the lung, but the mechanisms underlying this action are unclear. Apoptosis of lung epithelial cells is believed to be involved in the pathogenesis of pulmonary fibrosis. For these reasons, we studied the effect of captopril on Fas-induced apoptosis in a human lung epithelial cell line. Monoclonal antibodies that activate the Fas receptor induced epithelial cell apoptosis as detected by chromatid condensation, nuclear fragmentation, DNA fragmentation, and increased activities of caspase-1 and -3. Apoptosis was not induced by isotype-matched nonimmune mouse immunoglobulins or nonactivating anti-Fas monoclonal antibodies. When applied simultaneously with anti-Fas antibodies, 50 ng/ml of captopril completely abrogated apoptotic indexes based on morphology, DNA fragmentation, and inducible caspase-1 activity and significantly decreased the inducible activity of caspase-3. Inhibition of apoptosis by captopril was concentration dependent, with an IC50 of 70 pg/ml. These data suggest that the inhibitory actions of captopril on pulmonary fibrosis may be related to prevention of lung epithelial cell apoptosis.

Apoptosis has been shown to be an important component of the elimination of excess mesenchymal cells from diseased human lung (18) and also is thought to be involved in the removal of excess epithelial cells in remodeling hyperplastic human lung (2). On the other hand, increased expression of Fas (APO1, CD95) and apoptosis was observed in both bronchial and alveolar epithelial cells during the pathogenesis of bleomycin-induced pulmonary fibrosis in mice (11). These observations suggested that epithelial cell apoptosis might be involved in the pathogenesis of lung fibrosis as well as in the resolution of hypercellularity during healing of the lung. A critical role for lung epithelial cell apoptosis as a pathogenic mechanism is supported by the recent finding that ligation of the Fas receptor in vivo by intratracheal administration of anti-Fas antibodies induced epithelial cell apoptosis and pulmonary fibrosis in mice (10).

The angiotensin-converting enzyme (ACE) inhibitor captopril ameliorated pulmonary fibrosis induced in rats by the plant alkaloid monocrotaline (15). Captopril also inhibited the accumulation of collagen and mast cells in irradiated rat lung (25). These observations suggested a novel role for ACE inhibitors as modifiers of the response to pneumotoxicity, but little is known about the cellular and molecular mechanisms underlying these actions. Given the importance of epithelial integrity in the pathogenesis of lung fibrosis, we hypothesized that captopril might ameliorate lung fibrosis through direct inhibition of apoptosis in lung epithelial cells. We report here that Fas-induced apoptosis of human lung epithelial cells in culture is potently inhibited by captopril at concentrations readily attained in vivo.

METHODS

Reagents and materials. Monoclonal activating antibodies to human Fas (clone CH-11, purified mouse IgM) were obtained from Upstate Biotechnology (Saranac Lake, NY). Purified nonimmune mouse IgM was obtained from Sigma (St. Louis, MO). Monoclonal nonactivating antibodies to human Fas (clones SF-7 and SF-9) were obtained from Kamiya Biomedical (Seattle, WA). All other materials were from sources described earlier (23) and were of reagent grade.
Cell culture. The human lung adenocarcinoma cell line A549 was obtained from the American Type Culture Collection and cultured in Ham's F-12 medium supplemented with 10% fetal bovine serum. Cells were seeded on 12-mm sterile glass coverslips in 24-well chambers at a density of 20,000 cells/well. All experiments were conducted at subconfluent densities of 80–90% in Ham's F-12 medium supplemented with 1% fetal bovine serum. Antibodies and captopril were diluted with Ham's F-12 medium and applied for 20 h at 37°C in a 5% CO₂ incubator.

Fluorescence detection of apoptosis. Detection of apoptotic cells with propidium iodide was conducted as described earlier (23) after digestion of ethanol-fixed cells with DNase-free RNase in PBS containing 5 µg/ml of propidium iodide. In these assays, detached cells were retained by centrifugation of the 24-well culture vessels during fixation with 70% ethanol. In situ end labeling (ISEL) of fragmented DNA was conducted as described by Gorczyca et al. (9). Briefly, ethanol was removed by rinsing coverslips in distilled water for at least 10 min. The coverslips were then placed in a saline-sodium citrate solution (0.3 M NaCl and 30 mM sodium citrate, pH 7.0) at 37°C for 20 min. After four rinses in PBS and four rinses in buffer A (50 mM Tris·HCl, 5 mM MgCl₂, 10 mM β-mercaptoethanol, and 0.005% BSA in water, pH 7.5), the coverslips were incubated at 18°C for 2 h with an ISEL solution (0.001 mM biotinylated dUTP, 20 U/ml of DNA polymerase I, and 0.01 mM each dATP, dCTP, and dGTP in buffer A). Afterward, the sections were rinsed thoroughly five times with buffer A and three additional times with 0.5 M PBS. Incorporated biotinylated dUTP was detected by incubation for 1 h at 37°C with avidin-rhodamine; the coverslips were then rinsed in distilled water three times and mounted under Fluoromount solution (Southern Biotechnology Associates, Birmingham, AL).

Caspase assays. The enzymatic activities of caspase-1/-interleukin-1β-converting enzyme (ICE) and caspase 3/caspase protease protein-32 (CPP32)/Yama were determined in intact A549 cell cultures preincubated under conditions identical to those used for morphological assays of apoptosis. After 20 h of incubation with antibodies and/or captopril, the cells were trypsinized from the culture vessels and resuspended in serum-free culture medium containing anti-Fas antibodies and/or captopril at the same concentrations as during the preincubation. After 1 h, fluorogenic peptide substrates specific to each enzyme were added separately to cuvettes containing the cell suspension for measurement of total activity in intact cells. For measurement of caspase-1/ICE activity, the peptide substrate N-acetyl-Tyr-Val-Ala-Asp-7-amino-4-methylcoumarin (Ac-YVAD-AMC, Pharmingen, San Diego, CA) was used at 50 µM final concentration. For caspase-3/CPP32/Yama, the peptide substrate N-acetyl-Acryl-Val-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC, Upstate Biotechnology) was added at 200 µM final concentration. Production of fluorescent product over time was monitored in a spectrofluorometer at 380-nm excitation and 570-nm emission. Both enzyme assays were linear with time and protein concentration (data not shown).

RESULTS

Spontaneous or stimulated apoptosis was quantitated as described earlier (23) in the human lung epithelial cell line A549 by fluorescence detection of chromatin condensation and nuclear fragmentation (Fig. 1). By this assay, apoptosis was induced in A549 cells by the monoclonal activating antibody CH-11 (Fig. 2) but not by isotype-matched nonimmune mouse IgM at an equivalent concentration. The monoconal nonactivating antibodies 5F-7 and 5F-9 also failed to induce apoptosis in A549 cells; the latter results are consistent with recent findings by others (26) using the same antibody preparations, which did not activate Fas in the absence of other inducers.

The more sensitive assay of ISEL of fragmented DNA (Figs. 3 and 4) revealed a potent inhibitory action of captopril on Fas-induced cell death in A549 cells. Although captopril alone had no significant effect on basal ISEL labeling, 50 ng/ml of captopril essentially abolished the generation of ISEL-positive cells in response to ligation of Fas antigen (Fig. 4). The same concentration of captopril also abrogated Fas-induced stimulation of caspase-1/ICE activity (Fig. 5A) and significantly inhibited Fas-induced activity of caspase-3/CPP32/Yama (Fig. 5B), both cysteine proteases believed to be critical in the execution of apoptosis (16).

Fig. 1. Fluorescence assay of apoptosis in human lung epithelial cells. Human lung epithelial cell line A549 was cultured on glass coverslips as described in METHODS. At end of test period, detached cells were retained by centrifuging culture vessels during fixation of cells in 70% ethanol. Fixed cells were incubated for 30 min with 5 µg/ml of propidium iodide in PBS containing DNase-free RNase under these conditions, red fluorescence (~570 nm) is specific for DNA. Field displayed is from Fas-activated cells (see Fig. 2). As in a recent work by Uhal et al. (23), apoptotic cells were identified by presence of discrete nuclear fragments containing condensed chromatin (arrow).

![Image](http://apjplung.physiology.org/)
The effect of captopril on apoptosis was concentration dependent (Fig. 6); inhibition of Fas-induced ISEL labeling was measurable at a concentration of 5 pg/ml of captopril and exhibited an IC50 of 70 pg/ml. Inhibition of apoptosis was maximal at 50 ng/ml of captopril, a concentration known to be physiologically attainable in humans and maximally inhibitory for ACE (7).

**DISCUSSION**

Captopril has been shown to ameliorate radiation-induced fibrosis of the lung (25) and kidney (5), as well as lung fibrosis induced by the plant alkaloid monocrotaline (15). Examinations of human lung fibroblasts in culture suggest that this action might be due to direct inhibitory effects on fibroblast proliferation, which were observed in the presence of the mesenchymal cell mitogen basic fibroblast growth factor (17). Captopril is also known to inhibit the proliferation of cultured human mammary duct carcinoma cells (21) and to slow the growth rate of experimental fibrosarcomas in rats (24). Direct inhibition of zinc-dependent 72- and 92-kDa metalloproteinases produced by endothelial cells has also been reported (24). Inhibition of mesenchymal cell proliferation and metalloproteinase activities are both potential antifibrotic mechanisms.

Our data suggest a complementary but entirely different mechanism of antifibrotic potential by captopril. Numerous works have suggested a relationship between incomplete epithelial repair and fibrogenesis within the underlying interstitium in both experimental animal models of lung injury (1, 12, 27) and biopsy specimens from patients with fibrotic lung disease (13). In addition to providing intact barrier functions, the alveolar epithelium (4) is believed to be an important

![Figure 3](image-url) In situ end labeling (ISEL) assay for DNA fragmentation. Ethanol-fixed A549 cells on glass coverslips were subjected to ISEL labeling of fragmented DNA as described in METHODS. DNA fragments labeled with biotinylated dUTP were detected with rhodamine-conjugated avidin. A, C, and E, phase-contrast images of the same field of cells photographed under fluorescence illumination (B, D, and F, respectively). A and B: CONT incubation without added antibody. C and D: 500 ng/ml of Fas MAb was applied under same conditions as in Fig. 1. E and F: Fas MAb was applied in presence of 50 ng/ml of captopril. See Fig. 4 for quantitation of ISEL-positive cells.

![Figure 4](image-url) Inhibition of Fas-induced DNA fragmentation by captopril. ISEL-positive A549 cells were quantitated in at least 4 separate culture vessels exposed with (+) and without (−) indicated test materials as described in Fig. 3. Concentrations were 500 ng/ml for Fas MAb and 50 ng/ml for captopril. Values are means ± SE of at least 4 determinations. *P < 0.001 compared with CONT by ANOVA and Dunnett's test.

![Figure 5](image-url) Captopril inhibits Fas-induced stimulation of caspase activities in human lung epithelial cells. A549 cells were exposed as described in Fig. 2 to Fas MAb alone or in presence of 50 ng/ml of captopril. Cells were then analyzed in suspension culture for activity of caspase-1/interleukin-1ß-converting enzyme ICE with peptide substrate Ac-YVAD-AMC (A) or caspase-3/caspase protease protein-32Yama with peptide substrate Ac-DEVD-AMC (B) as described in METHODS. Values are means ± SD of at least 4 separate determinations. Significant difference (P < 0.01) compared with: * CONT; ** Fas MAb (by ANOVA and Student-Newman-Keuls test).

![Figure 6](image-url) Concentration dependence of captopril inhibition of Fas-induced apoptosis. A549 cells were exposed to Fas MAb as described in Fig. 2 in presence of indicated concentrations of captopril. Inhibition of apoptosis was detected as decrease in ISEL-positive cells, scored as described in Fig. 4. IC50 = 0.07 ng/ml. Values are means ± SE of at least 6 determinations.
constitutive producer of prostaglandin E2, an inhibitor of fibroblast proliferation (3). Normal alveolar epithelial cells also produce urokinase-type plasminogen activator and plasminogen activator inhibitor-1 and exhibit a potent capacity for the degradation of fibrin clots in vitro (20). For these reasons, the loss of normal alveolar epithelium would be expected to decrease the capacity of the alveolus to clear intra-alveolar fibrin and would also deepen the commitment to fibrogenesis by eliminating a known source of inhibitors of lung fibroblast proliferation.

Apoptosis is now known to be an important regulator of alveolar epithelial cell number in vitro (22, 23) and is a prominent feature of the fibrotic lung in vivo (2, 11, 14). Expression of functional Fas has been demonstrated in alveolar epithelial cells of the mouse and rat lung in vitro (8) and in vivo (11). Furthermore, lung infiltration by lymphocytes expressing Fas ligand has been speculated to contribute to the induction of alveolar epithelial cell apoptosis observed after intratracheal instillation of bleomycin in mice (11). The demonstration that intratracheal administration of activating antibodies to Fas was capable of inducing pulmonary fibrosis in mice (10) supports the contention that Fas-induced apoptosis of the epithelium is a critical component of the pathogenesis of pulmonary fibrosis.

To our knowledge, this is the first demonstration of the inhibition of apoptosis by an ACE inhibitor in a cell type not of hematopoietic lineage. Deas et al. (6) recently demonstrated inhibition of Fas-induced apoptosis in activated human peripheral T cells by captopril and other thiol compounds but not by nontoxic antioxidants. Those results led the authors to speculate that the inhibition of T-cell apoptosis was the result of sulfhydryl redox regulation of critical molecules involved in the apoptotic signaling cascade. The caspases are cysteine proteases critical to the signaling of apoptosis and sensitive to sulfhydryl redox poise (16). Our results show that at least two of these enzyme activities, caspase-1/ICE and caspase-3/CPP32/Yama, are inhibited in situ by exposure of the intact cell to captopril. We are currently addressing the possibility that captopril and other thiol compounds inhibit lung epithelial cell apoptosis through direct inhibition of caspase-1 and -3 activities and/or other cysteine proteases required for the induction of apoptosis.

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