Captopril Inhibits Proliferation of Human Lung Fibroblasts in Culture: A Potential Antifibrotic Mechanism (43681)

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Abstract. **The angiotensin converting enzyme (ACE) inhibitor captopril, a free-thiol compound used widely as an antihypertensive agent, also inhibits radiation-induced pulmonary fibrosis in rats (Ward *et al.*, Int J Radiat Oncol Biol Phys 19:1405, 1990). In an attempt to clarify the antifibrotic mechanism of captopril in vivo, the present study examined the effect of the drug on proliferation of human lung fibroblasts in culture. Captopril produced a drug dose–dependent reduction in fibroblast proliferation and ^{3}H-thymidine incorporation during a 24–72-hr incubation. This cytostatic action of captopril was not the result of cytotoxicity as assessed by trypan blue exclusion, or by ^{51}Cr or lactate dehydrogenase (LDH) release. Fibroblasts stimulated to proliferate by basic FGF were more sensitive to the antimitotic effect of captopril than were unstimulated cells. The ability of captopril to inhibit ^{3}H-thymidine incorporation was not reversed by exogenous angiotensin 2, and was not mimicked by the nonthiol ACE inhibitor lisinopril. These data indicate that the cytostatic effect of captopril was not attributable to ACE inhibition. Penicillamine, a thiol compound with virtually no ACE inhibitory activity, also reduced fibroblast ^{3}H-thymidine incorporation, indicating that the antimitotic action of captopril may represent a nonspecific sulfhydryl effect. This study suggests that the antifibrotic activity of captopril in irradiated lung may result in part from a direct inhibition of fibroblast proliferation, particularly in fibroblasts responding to mitogenic stimuli.** [P.S.E.B.M. 1994, Vol 205]

Captopril (D-3-mercapto-2-methylpropanoyl-L-proline) is an orally active competitive inhibitor of angiotensin converting enzyme (ACE, EC 3.4.15.1), the enzyme that metabolizes angiotensin 1 to the vasoconstrictor angiotensin 2 and inactivates the bradykinins. Captopril is used by 5–10 million people worldwide in the management of hypertension and congestive heart failure (1, 2). Captopril also has been found to ameliorate radiation-induced pulmonary endothelial dysfunction in rats (3), and to spare pulmonary fibrosis induced by radiation (4) or by the pneumotoxic alkaloid monocrotaline (5) in that species. In irradiated rat lung, captopril not only reduces hydroxyproline content and concentration (per unit wet weight), but also decreases the number of interstitial mast cells (4). The mechanism of captopril's antifibrotic action is not clear. The ACE inhibitor may reduce radiation lung fibrosis indirectly, as a result of its sparing effect on either the pulmonary endothelium (3) or on mast cell accumulation (4). Conversely captopril may act directly to inhibit fibroblast proliferation in irradiated lung. Captopril exhibits antimitotic activity against human neuroblastoma cells (6), canine kidney epithelial cells (7), and hamster pancreatic carcinoma cells (8) *in vitro*. Furthermore, captopril lowers angiotensin 2 (A2) concentration *in situ*, and A2 is a reported mitogen for several cell types including 3T3 mouse fibroblasts (9–11). The present study determined whether captopril inhibits proliferation and ^{3}H-thymidine incorporation in human lung fibroblasts *in vitro*. This study was stimulated by observations that patients with idiopathic lung fibrosis exhibit low glutathione (GSH) concentration in the alveolar lavage

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fluid (12), and that GSH inhibits fibroblast proliferation *in vitro* (13). Like GSH, captopril possesses a free-thiol group. In the present study, lisinopril, a nonthiol ACE inhibitor, and penicillamine, a thiol compound with virtually no ACE inhibitory activity, were cotested with captopril as part of a structure-activity analysis. Penicillamine also inhibits radiation- (14) and monocrotaline-induced (5) pulmonary fibrosis in rats, as does captopril (4, 5), and is an antimitotic agent *in vitro* (15–18).

Materials and Methods

Cells and Culture Technique. Human lung fibroblasts (CCL-153; American Type Culture Collection, Rockville, MD), a diploid, nonimmortalized cell line, were used before the 20th passage in these studies. Cells were grown in Dulbecco's modified Eagle's medium (DME) containing 10% newborn calf serum and supplemented with 2 m*M* L-glutamine, penicillin G (100 U/ml), streptomycin (0.1 mg/ml) and amphotercin B (0.25 μg/ml). All culture reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Cells were incubated at 37.0 ± 0.1°C in humidified 5% CO_2–95% air.

Proliferation Assay. Approximately 10^4 cells were seeded in 35 mm plastic culture dishes, allowed to attach for 24 hr, then fresh medium containing one of the test compounds was added. Captopril, penicillamine, angiotensin 2, and basic fibroblast growth factor (bFGF) were purchased from Sigma Chemical Co., and were replaced in the culture medium every 24 hr when necessary. After incubation for 24, 48, or 72 hr, the dishes were washed twice with Hank's balanced salt solution without calcium or magnesium salts (Sigma Chemical Co.). The cells were then trypsinized and placed in a test tube containing 100 μl of 0.1% crystal violet (w/v) and 0.1% Triton X-100 (v/v) in 0.1 *M* citric acid. The tube was vortexed for 30 sec, the sample was loaded into a hemocytometer, and nuclei were counted in replicate at ×600 magnification.

^{3}H-Thymidine Incorporation. Approximately 5 × 10^3 fibroblasts were plated in 1.0 ml of complete DME into each well of a 24-well incubation plate. After a 24-hr attachment period, the medium was replaced with 1 ml of test medium containing 0.5 μCi of ^{3}H-thymidine (87 Ci/m*M*, Amersham, Arlington Heights, IL). Cells were incubated for an additional 24, 48, or 72 hr, with media replaced every 24 hr. At the end of the incubation, the radioactive medium was removed, and the cells were washed twice with Dulbecco's phosphate buffered saline (Gibco, Grand Island, NY). They were then rinsed three times with 10% trichloroacetic acid (TCA) at 4°C, with the final rinse lasting 10 min. The cells were air dried, then 350 μl of 1 *N* NaOH was added to each well. The samples were left at room temperature for 16–20 hr, then were neutralized with an equal volume of 1 *N* acetic acid, and were transferred quantitatively to scintillation vials containing 5 ml of liquid scintillation cocktail. The samples were counted for 5 min in a liquid scintillation counter, and data were expressed as counts per min (CPM) minus background.

Cytotoxicity Assays. ^{51}Cr Release: approximately 5 × 10^3 cells were seeded in each well of a 24-well culture plate, and allowed to attach for 24 hr in complete DME. Cells then were prelabeled for 16 hr with 1 μCi of ^{51}Cr (Na_2 ^{51}Cr O_4, ICN Biomedical, Costa Mesa, CA) per well. The labeled cells were washed twice with DME, then incubated for 24 hr in DME with and without captopril (1×10^{-6} to 5×10^{-3} *M*). ^{51}Cr radioactivity in the incubation medium and the cell lysate was determined in a Minigamma 1275 counter (Pharmacia, Gaithersburg, MD).

Lactate Dehydrogenase (LDH) Release: approximately 10^4 cells were seeded on 35 mm plastic culture dishes, allowed to attach for 24 hr, then incubated in complete DME or in DME containing 5 m*M* captopril for an additional 24 or 48 hr. The culture media then were collected and analyzed for LDH activity by an automated spectrophotometric technique on a Technicon RA-1000 System (Technicon Instruments, Tarrytown, NY).

Trypan Blue Exclusion: the cells remaining attached to the dish after removal of media for LDH analysis (above) were trypsinized and stained with 0.4% trypan blue solution for 10 min. The proportion of blue (nonviable) and clear (viable) cells was determined at ×600 magnification.

Statistical Analysis. The significance of differences between group means was determined by the Student's t-test (19). Values represent the mean ± one standard error of the mean (SEM).

Results

Effect of Captopril. Fibroblasts incubated in control DME increased in number by a factor of 3.5 during a 72-hr incubation, whereas cells exposed to 5 m*M* captopril exhibited less than a 2-fold increase during that time (Fig. 1). The incorporation of ^{3}H-thymidine into TCA-insoluble molecules generally paralleled cell (nuclear) number, and also demonstrated a significant inhibitory effect of 5 m*M* captopril (Fig. 1). Both endpoints suggested that the antimitotic effect of captopril increased with increasing time of exposure to the drug. Trypan blue exclusion tests at 48 hr revealed that more than 95% of both the control and the captopril-treated cells were viable. LDH activity in the incubation media of the two treatment groups also was similar (data not shown).

A captopril dose-response analysis at 24 hr demonstrated a drug dose-dependent decrease in ^{3}H-thymidine incorporation over the dose range of 5 ×

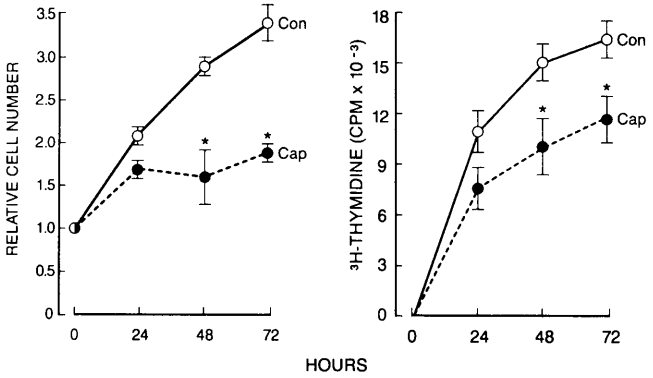

Figure 1. Captopril inhibits proliferation (left) and ^{3}H-thymidine incorporation (right) in cultured human lung fibroblasts. Cells were incubated for 24–72 hr in the presence (broken line) or absence (solid line) of 5 m*M* captopril. Mean ± SEM; *N* = 4–8 dishes per data point. Asterisks denote values significantly lower than control, $P < 0.05$.

10^{-5} to 5×10^{-3} *M*, a decline that was significant ($p < 0.05$) at doses of 5×10^{-4} *M* or higher. Maximum inhibition of approximately 30% occurred at a captopril concentration of 1 m*M* (Table I). The release of ^{51}Cr from prelabeled cells was not increased by 24-hr exposure to captopril doses of 1 m*M* or lower (data not shown).

Table I. ^{3}H-Thymidine Incorporation in Human Lung Fibroblasts *in Vitro*[a]

Treatment	^{3}H-Thymidine incorp. (% of control)
Captopril (*M*)	
0	100.0 ± 3.0[b]
5×10^{-7}	100.1 ± 2.0
5×10^{-6}	101.8 ± 3.8
5×10^{-5}	102.0 ± 3.0
1×10^{-4}	99.0 ± 1.0
2×10^{-4}	91.0 ± 1.0
5×10^{-4}	83.0 ± 3.1*
1×10^{-3}	73.0 ± 5.8*
5×10^{-3}	77.5 ± 2.5*
Fibroblast growth factor-basic (bFGF)	
bFGF (10 ng/ml)	
24 hr	111.8 ± 7.6
48 hr	108.1 ± 7.1
72 hr	123.7 ± 6.6*
Angiotensin 2 (A2)	
A2 (*M*)	
0	100.0 ± 2.9
10^{-8}	101.4 ± 1.3
10^{-7}	103.1 ± 1.6
10^{-6}	100.4 ± 1.6
10^{-5}	103.3 ± 2.6
Penicillamine	
Control	100.0 ± 2.3
Captopril (5 m*M*)	79.6 ± 3.2*
Penicillamine (5 m*M*)	75.8 ± 1.4*

[a] Cells were incubated for 24 hrs unless otherwise indicated.
[b] Mean ± SEM; n = 4–16 wells per group.
* Different from control, $P < 0.05$.

Effect of Captopril on bFGF-Stimulated Fibroblasts. Fibroblasts incubated in the presence of bFGF (10 ng/ml) exhibited a significant increase in ^{3}H-thymidine incorporation compared to cells grown in the absence of growth factor, even though the DME contained 10% serum. The ability of bFGF to stimulate ^{3}H-thymidine incorporation increased with time, from approximately 10% greater than control at 24 hr, to approximately 25% higher by 72 hr (Table I). Fibroblasts stimulated with bFGF were more sensitive to the antimitotic effect of captopril than were unstimulated cells. In the absence of captopril, bFGF-treated cells incorporated significantly more ^{3}H-thymidine during a 24-hr incubation than did nonstimulated cells. As captopril concentration increased from 1×10^{-4} to 5×10^{-3} *M*, however, ^{3}H-thymidine incorporation in the bFGF-stimulated cells declined steadily relative to unstimulated captopril-treated cells. At captopril concentrations of 5×10^{-4} *M* or higher, ^{3}H-thymidine incorporation in the bFGF-stimulated fibroblasts was significantly lower than that of unstimulated cells (Fig. 2).

Effect of Angiotensin 2. Angiotensin 2 (A2) alone in concentrations ranging from 10^{-8} to 10^{-5} *M* had no significant effect on ^{3}H-thymidine incorporation during a 24-hr incubation (Table I). Neither did 1 μ*M* A2 reverse the inhibition in ^{3}H-thymidine incorporation produced by 5 m*M* captopril after 24 hr (Fig. 3).

Effect of Lisinopril. Lisinopril in concentrations ranging from 1×10^{-6} to 5×10^{-3} *M* also had no significant effect on ^{3}H-thymidine incorporation by fibroblasts during a 24-hr incubation (Fig. 4). In contrast, lisinopril was superior to captopril as an inhibitor of ACE activity in rat and human serum (data not shown).

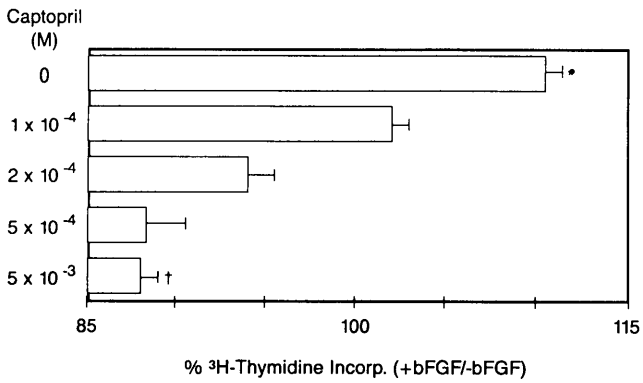

Figure 2. ^{3}H-thymidine incorporation during 24 hr in bFGF-stimulated (10 ng/ml) relative to unstimulated human lung fibroblasts as a function of captopril concentration. At a value of 100%, stimulated and unstimulated cells incorporate ^{3}H-thymidine at the same rate. Mean ± SEM, *N* = 8 wells per group. *greater than unstimulated, $P < 0.05$. †less than unstimulated, $P < 0.05$.

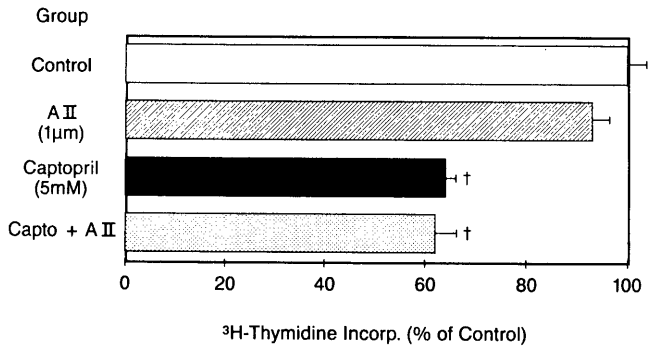

Figure 3. Exogenous angiotensin 2 (A2) fails to reverse captopril-induced inhibition of ^{3}H-thymidine incorporation in human lung fibroblasts incubated for 24 hr. Mean ± SEM; N = 8–12 wells per data point. †less than control, $P < 0.05$.

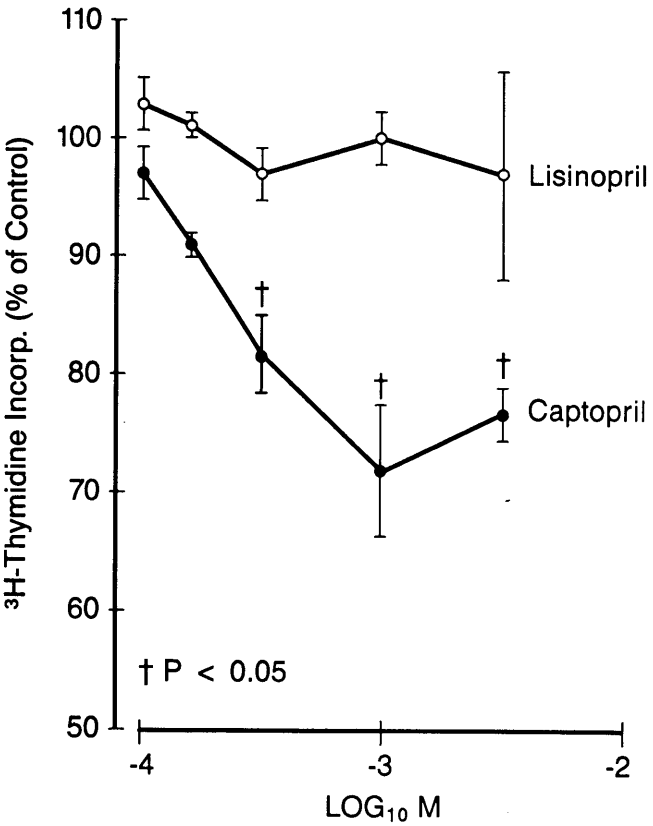

Figure 4. Comparison of the effect of captopril, a free-thiol ACE inhibitor, and lisinopril, a nonthiol ACE inhibitor, on ^{3}H-thymidine incorporation by human lung fibroblasts during a 24 hr incubation. Mean ± SEM; N = 8–16 wells per group. †less than control, $P < 0.05$.

Effect of Penicillamine. Penicillamine also inhibited ^{3}H-thymidine incorporation in fibroblasts incubated for 24 hr, although this effect was significant ($P < 0.05$) only at a concentration of 5 mM, and not at 1 mM or lower (data not shown). Nevertheless, after exposure of cells to 5 mM of captopril or penicillamine for 24 hr, ^{3}H-thymidine incorporation was reduced to 79.6 ± 3.2 and 75.8 ± 1.4 percent of control, respectively (Table I). Both values were significantly ($P < 0.05$) lower than control, but not different from one another. Penicillamine (5 mM) did not increase ^{51}Cr release from prelabeled cells (data not shown).

Discussion

These data demonstrate that captopril inhibits human lung fibroblast proliferation and ^{3}H-thymidine incorporation *in vitro*. Maximum inhibition of ^{3}H-thymidine incorporation, approximately 30% below the control rate, occurs at a captopril concentration of 1 mM. At this dose, captopril appears to be cytostatic but not cytotoxic. The data also indicate that bFGF stimulates ^{3}H-thymidine incorporation, even in the presence of 10% serum, and that bFGF-stimulated cells are more sensitive to captopril inhibition than are unstimulated cells. In the absence of captopril, bFGF-stimulated cells incorporate significantly more ^{3}H-thymidine than do unstimulated cells, whereas in the presence of 0.5 mM captopril or higher they incorporate significantly less (Fig. 2). The observations that exogenous angiotensin 2 does not reverse the ability of captopril to inhibit ^{3}H-thymidine incorporation, and that penicillamine (Table I) and GSH (13) but not lisinopril (Fig. 4) mimic captopril, suggests that the thiol group and not ACE inhibition is the structural basis of captopril's action in this model. Likewise in irradiated rat lung it appears that the presence of a free sulfhydryl and not ACE inhibition is essential for the antifibrotic activity of captopril (20).

The ability of captopril and penicillamine to inhibit ^{3}H-thymidine incorporation in human lung fibroblasts is not restricted to that cell type, rather reflects a general cytostatic activity of the two compounds (6–8, 15–18). Recent studies suggest that the antimitotic action of captopril and penicillamine in vitro requires copper (18, 21). The present study did not test the copper dependency of captopril action, but this ion was present in the serum-supplemented culture medium and would, of course, be present *in vivo*.

It is not likely that the captopril concentrations required for significant inhibition of fibroblast proliferation within 24 hr *in vitro* (0.5–1.0 mM) would be achieved in the lungs of irradiated captopril-treated rats (50 mg/kg body weight/day, p.o.) whose pulmonary fibrosis is spared by the drug (4). On the other hand, subtle inhibition of fibroblast mitotic activity over the long term *in vivo* might compound into a significant antifibrotic mechanism in irradiated lung. The same captopril regimen does inhibit mitosis in radiation-induced fibrosarcomas (Ward *et al.*, unpublished data) and nitrosamine-induced preneoplastic hepatic foci in rats (22), suggesting that cytostatic concentrations of the drug are attainable *in vivo*. Other relevant differences between the *in vivo* and *in vitro* models as they might impact effective captopril doses include extracellular matrix metabolism, matrix metalloproteinase activity, cytokines, and other paracrine interac-

tions involving the fibroblast. The action of captopril on these phenomena has yet to be determined. Furthermore in irradiated rat lung, captopril spares other pathophysiology, including pulmonary edema (23), pulmonary hypertension (23), endothelial dysfunction (3), and mast cell accumulation (4), and these actions may reduce lung fibrosis indirectly.

The salutary effects of captopril on fibroproliferative disorders are not restricted to lung, but can be seen in the heart as well. In rats, captopril reduces cardiac fibrosis arising spontaneously with age (24), as well as that induced by pressure overload (25) or radiation (26). Recently, captopril also has been reported to decrease morbidity and mortality in patients with left ventricular dysfunction after myocardial infarction (27).

In conclusion, these data demonstrate that the widely used antihypertensive drug captopril inhibits proliferation and ^{3}H-thymidine incorporation in human lung fibroblasts *in vitro*. Thus the well-documented sparing effect of captopril on experimental pulmonary (4, 5) and cardiac (24–26) fibrosis may result in part from a direct cytostatic action on fibroblasts, particularly on fibroblasts responding to mitogenic stimuli.

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