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Captopril inhibits pp60$^{c\text{-}src}$ tyrosine phosphorylation in cultured human mesangial cells

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Abstract

The present experiments were devoted to analyzing the mechanisms involved in the captopril-dependent inhibition of human mesangial cell proliferation. Studies were performed in cultured human mesangial cells incubated with captopril, an angiotensin II-converting enzyme inhibitor with antioxidant properties, lisinopril, a non-antioxidant angiotensin II-converting enzyme inhibitor, and tocopherol, a pure antioxidant. Both angiotensin II-converting enzyme inhibitors significantly inhibited fetal calf serum-induced [^{3}H]thymidine uptake by human mesangial cells, in a dose- and time-dependent manner, an effect which was not observed with tocopherol. The antiproliferative effect of captopril and its ability to block tyrosine phosphorylation of human mesangial cells proteins were significantly greater than those of lisinopril. Moreover, captopril significantly prevented the fetal calf serum-induced tyrosine phosphorylation of pp60$^{c\text{-}src}$. The present results suggest that the antiproliferative ability of captopril does not completely depend on its angiotensin II-converting enzyme inhibitor properties, pointing to a possible interaction of the drug with the intracellular mechanisms responsible for the transmission of the proliferative signals. © 1997 Elsevier Science B.V.

Keywords: Mesangial cell; Proliferation; Captopril; Angiotensin II-converting enzyme inhibitor; Tyrosine phosphorylation; pp60$^{c\text{-}src}$

1. Introduction

Angiotensin-converting enzyme inhibitors have proven to be useful in the prevention of myointimal proliferation after vascular injury (Powell et al., 1989; Prescott et al., 1991). The study of the mechanisms involved in this inhibition has led to the conclusion that these drugs block smooth muscle cell proliferation by mechanisms not completely defined (Hadrava et al., 1991; Uehara et al., 1993). More recently, it has been demonstrated that this group of drugs may also inhibit the proliferation of other contractile cells, including glomerular mesangial cells (Bakris et al., 1994), as well as the proliferation of non-contractile cells (Nguyen et al., 1994; Reddy et al., 1995; Volpert et al., 1996). In consequence, it has been proposed that these drugs may exert a general antiproliferative effect, and their use has even been suggested as a coadjuvant treatment in some proliferative disorders (Kleinman and Ponce, 1996).

The mechanisms of these antiproliferative effects have not been elucidated. Two main actions have been proposed for these drugs, namely the blockade of angiotensin II synthesis, and the blockade of local kinin degradation (Brogden et al., 1989). As these biological mediators have been proposed to regulate smooth muscle cell proliferation (Farhy et al., 1992; Nickening et al., 1996), the beneficial action of angiotensin II-converting enzyme inhibitor, at least in the progression of myointimal proliferation, could be reasonably explained. However, the drugs have also a clearcut antiproliferative action in cell cultures, even on non-contractile cells (Bakris et al., 1994; Nguyen et al., 1994; Reddy et al., 1995; Volpert et al., 1996), suggesting that the mechanisms of the inhibition of proliferation could be more general. In this regard, recent experimental work has contributed to the elucidation of a rather general

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mechanism of transmission of proliferative signals from the cell membrane to the nucleus, which includes the quick and successive phosphorylation of cytoplasmic proteins at specific amino acids, especially tyrosine (Anderson et al., 1990). One of these proteins, $pp60^{c\text{-}src}$, has been recently proposed to be involved in the transmission of the proliferative signals induced by angiotensin II in smooth muscle cells (Ishida et al., 1995) and hydrogen peroxide in mesangial cells (González-Rubio et al., 1996).

The present experiments were designed to analyze the antiproliferative activity of captopril, an angiotensin II-converting enzyme inhibitor containing a sulfhydryl group, on serum-stimulated cultured human mesangial cells. In order to adequately separate the angiotensin II-converting enzyme inhibitor and the antioxidant effects of the captopril molecule, these effects were compared with those evoked by lisinopril, a non-antioxidant angiotensin II-converting enzyme inhibitor, and tocopherol, a pure antioxidant. Moreover, the effect of the drug on serum-stimulated protein tyrosine phosphorylation was also tested. As captopril shares angiotensin II-converting enzyme inhibitor and antioxidant effects, special attention was paid to $pp60^{c\text{-}src}$. The hypothesis was that the antiproliferative effect of captopril could be the consequence of direct interference with this system of transmission of proliferative signals.

2. Materials and methods

2.1. Human mesangial cell culture

Human glomerular mesangial cells were cultured according to a previously described procedure (Díez-Marqués et al., 1995). Portions of macroscopically normal, cortical tissue were obtained from human kidneys immediately after nephrectomy for renal cell carcinoma. After preparation, the material was pushed through 180 and 105 μm stainless steel sieves and washed, in order to obtain isolated glomeruli free from tubular contamination. The glomeruli were then treated with collagenase, plated in plastic culture flasks and maintained in RPMI 1640, supplemented with 10% fetal calf serum, L-glutamine (1 mM), penicillin (0.66 μg/ml), streptomycin sulfate (60 μg/ml), and buffered with HEPES and bicarbonate, pH 7.4. Culture media were changed every two days and experiments were performed at passages 3–5.

2.2. Proliferation experiments

Cell proliferation was assessed by measuring [^{3}H]thymidine uptake according to previously described techniques (González-Rubio et al., 1996). Experiments were performed with confluent cells, plated on 24-well dishes and maintained in serum-free medium for 48 h. Afterwards, cells were incubated in standard culture medium, with or without 15% fetal calf serum for 24 h, and were pulsed with [^{3}H]thymidine (1 μCi/well) 16 h after the start of the experimental period. Captopril, lisinopril, and vitamin E were added to the incubation media at different concentrations (1–100 μM) and for different times (6–48 h) before cell collection. Following the incubations, the media were siphoned off and the cells were washed (3×) with phosphate-buffered saline (PBS, 1 ml) and precipitated directly in the wells by addition of 0.5 ml (3×) of 10% trichloroacetic. Cell suspensions were collected and the trichloroacetic-precipitable material was pelleted by centrifugation at $2000 \times g$, redissolved in 0.2 ml NaOH 1 M, and neutralized with HCl 1 M and the radioactivity was measured in a scintillation counter. As an index of toxicity, the ability of cells to exclude Trypan blue dye under the different experimental conditions was evaluated.

2.3. Measurement of tyrosine phosphorylation

Measurement of tyrosine phosphorylation was performed according to previously described techniques (González-Rubio et al., 1996). In short, quiescent human mesangial cells (48 h of serum deprivation) were incubated for 30 min in standard culture medium containing 15% fetal calf serum, with or without captopril, or lisinopril (the drugs, at a concentration of 100 μM, were added 24 h before fetal calf serum). Afterwards, cell cultures were chilled on ice and washed three times with cold PBS supplemented with 0.2 mM vanadate. Subsequently the culture flasks were incubated for 30 min with 0.7 ml radioimmunoprotein assay (RIPA) lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride, 0.2 mM vanadate, pH 7.2). The resulting solution was centrifuged (1200 rpm, 30 min at 4°C). The protein concentration in each lysate was determined spectrophotometrically (BCA Protein Assay Reagent, Pierce, Rockford, IL, USA). The extracted proteins from mesangial cell lysates were solubilized by boiling in SDS loading buffer and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (20 μg/lane). Subsequently, the proteins were transferred to a 0.45 μm pore nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) by electrophoresis (Polyblot, American Binuclear, Emeryville, CA, USA). Molecular mass markers (Pharmacia, Uppsala, Sweden) were detected by staining with Amido black. The nitrocellulose membranes were blocked (PBS/3% bovine serum albumine with 0.02% NaN_3) overnight at 4°C. The membranes were then washed three times in Tris-buffered saline–Tween 20 (TBS-T) (0.05% Tween 20 in 50 mM Tris, 150 mM NaCl, 0.02% NaN_3, pH 7.4) and incubated for two hours at room temperature with an antiphosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY) diluted in TBS/1% bovine serum albumine, under gentle agitation. Thereafter the nitrocellulose membranes were

washed three times with TBS-T and incubated for one hour at room temperature with an alkaline phosphatase goat anti-mouse antibody (Promega Biotech, Madison, WI, USA) diluted 1:10 000 in TBS-T. After three final washes, the nitrocellulose membranes were incubated in alcaline phosphatase buffer (10 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5). Secondary antibody bound to the nitrocellulose membranes was detected by incubation with a substrate solution (Sigma, St. Louis, MO, USA), which consisted of nitroblue tetrazolium (330 ng/ml) and 5-bromo-chloro-3-indolyl phosphate (165 ng/ml). Color development was stopped after approx. 5 min by washing the nitrocellulose membranes with 20 mM Tris, 5 mM EDTA, pH 8.0.

2.4. Measurement of $pp60^{c\text{-}src}$ tyrosine phosphorylation

The incubations for the measurement of specific $pp60^{c\text{-}src}$ tyrosine phosphorylation were the same as those for tyrosine phosphorylation, but only captopril was used. The immunoprecipitation Western blot technique has been described previously (González-Rubio et al., 1996). After extraction of the proteins from the cells, as mentioned above, 300 μg of protein was preabsorbed with non-immune IgG and Pansorbin (Calbiochem, San Diego, CA) for 60 min at 4°C. Incubations with the primary antibody, a specific anti-$pp60^{c\text{-}src}$ monoclonal antibody (Oncogene, Uniondale, NY) diluted 1/1000 in TBS-T/BSA 1%/NaN_3 0.02%, were performed overnight at 4°C with gentle agitation. The antigen–antibody complexes were precipitated with 200 μl of Pansorbin, and the beads were pelleted and washed five times with RIPA buffer. Pellets were solubilized by boiling in SDS loading buffer and analyzed as described above, by SDS-PAGE electrophoresis, nitrocellulose transfer and blotting with the antiphosphotyrosine antibody. In order to determine the total amount of $pp60^{c\text{-}src}$ in cells, extracted non-immunoprecipitated proteins were processed as for the tyrosine phosphorylation measurement, but the nitrocellulose filters were incubated with the anti-$pp60^{c\text{-}src}$ antibody.

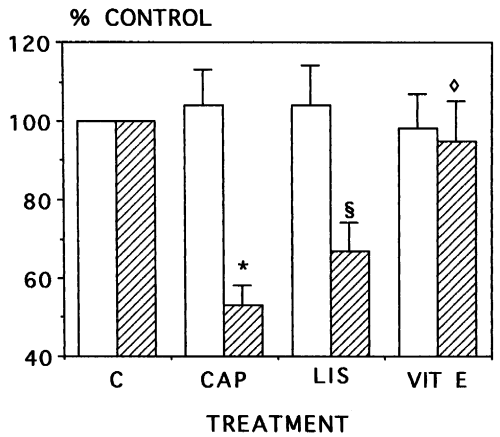

Fig. 1. [^{3}H]thymidine uptake by cultured human mesangial cells in the presence of angiotensin-converting enzyme inhibitors or exogenous antioxidants. Quiescent cells were incubated without (open bars) or with (closed bars) 15% fetal calf serum for 24 h, in the presence of captopril (CAP), lisinopril (LIS) and vitamin E (VIT E). The drugs were added to the incubation media with fetal calf serum (24 h of incubation), and their final concentrations were the same (100 μM). Results are the means ± S.E.M of 10 different experiments, with 3 wells each, and they are expressed as percentages of the control values (C). * $P < 0.05$ vs. C. § $P < 0.05$ vs. C and CAP. ◇ $P < 0.05$ vs. CAP and LIS.

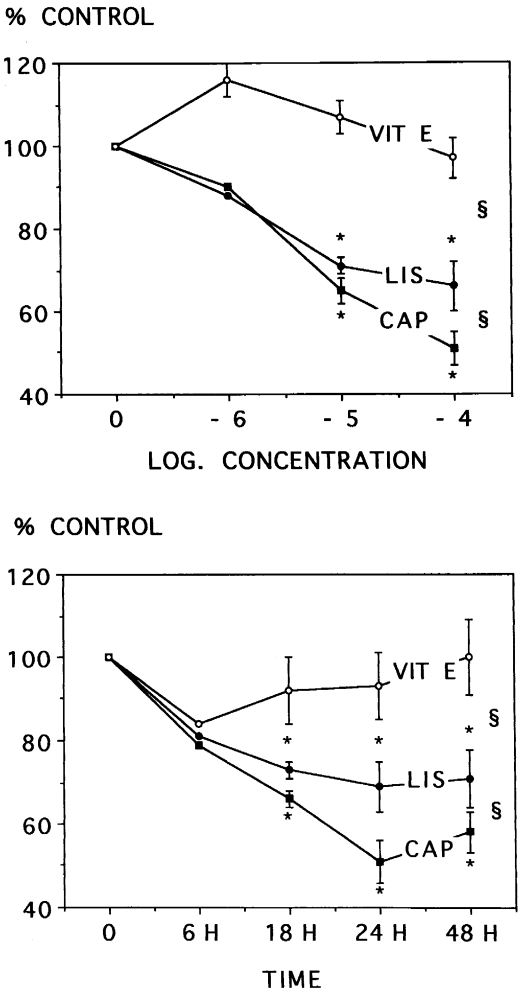

Fig. 2. Dose- and time-dependence of [^{3}H]thymidine uptake by cultured human mesangial cells, in the presence of angiotensin-converting enzyme inhibitors or exogenous antioxidants. Quiescent cells were incubated with 15% fetal calf serum for 24 h, in the presence of different concentrations of captopril (CAP), lisinopril (LIS), and vitamin E (VIT E), or for different times. In the dose–response experiments (upper panel), the drugs were added to the incubation media with fetal calf serum (24 h of incubation), and their final concentrations ranged between 1–100 μM. In the time–response experiments (lower panel), 100 μM of the different drugs was added to the incubation media for different times (the times are referred to the end of the incubation period). Results are the means ± S.E.M of 6 different experiments, with 3 wells each, and they are expressed as percentages of the control values (C). * $P < 0.05$ vs. 0 (within the same treatment). § $P < 0.05$ vs. the other treatments.

2.5. Statistical methods

Results are expressed as the means ± S.E.M of the different experiments (the number is provided in the leg-

ends to the figures). Comparisons were performed by the Friedman test or ANOVA. A $P < 0.05$ was considered statistically significant.

3. Results

Fig. 1 shows the effect of the same concentration (100 μM) of captopril, lisinopril and vitamin E on human mesangial cells proliferation. In the absence of fetal calf serum, neither angiotensin II-converting enzyme inhibitors nor tocopherol modified the proliferation rate of cultured cells (Fig. 1, open bars). However, in the presence of 15% fetal calf serum, both captopril and lisinopril significantly inhibited [^{3}H]thymidine uptake by the cells, there being no detectable effect of vitamin E on cell proliferation (Fig. 1, closed bars). The captopril-dependent inhibition of human mesangial cells proliferation was significantly greater than that of lisinopril (Fig. 1, closed bars). The dose and time dependency analysis of the above-mentioned inhibition of fetal calf serum-induced human mesangial cells proliferation is considered in Fig. 2. The selected drug concentrations ranged between 1 and 100 μM (Fig. 2, upper panel), whereas incubation times were between 6 and 48 h (Fig. 2, lower panel). Vitamin E did not modify cell proliferation at any concentration or time selected. Both captopril and lisinopril inhibited human mesangial cells proliferation in a dose- and time-dependent manner, but the inhibition was again significantly greater with captopril.

The upper panel of Fig. 3 shows the characteristic pattern of human mesangial cells proteins incubated with a specific antiphosphotyrosine antibody, after incubation with captopril or lisinopril, in the presence of 15% fetal calf serum. The increased tyrosine phosphorylation induced by

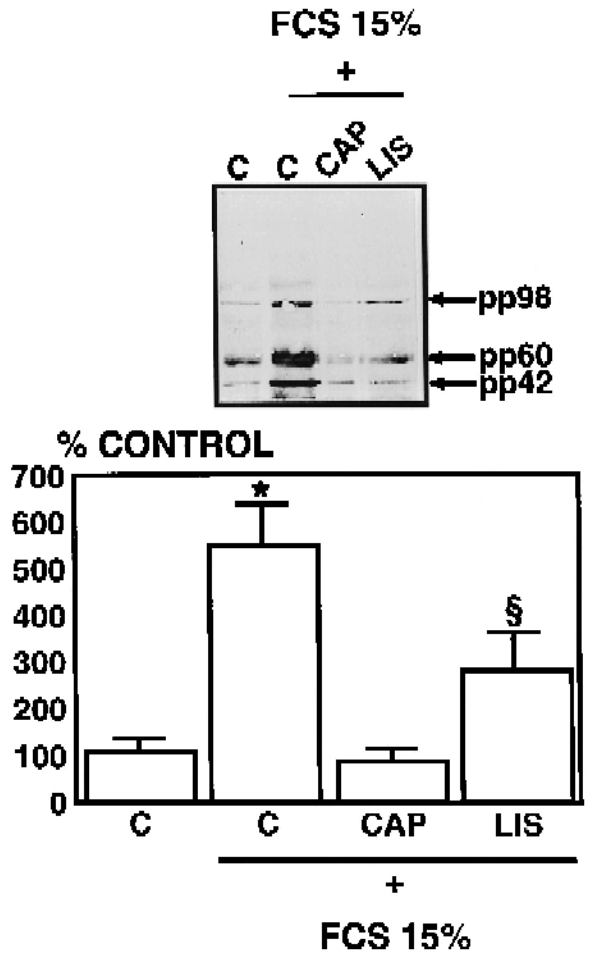

Fig. 3. Effect of angiotensin-converting enzyme inhibitors on serum-induced tyrosine phosphorylation of cytoplasmic proteins in cultured human mesangial cells. Quiescent cells were incubated for 30 min with 15% fetal calf serum (FCS), after 24 h of treatment with 100 μM captopril (CAP) or lisinopril (LIS). The upper panel of the figure shows a characteristic Western blot experiment with a specific antiphosphotyrosine antibody. The arrows show the proteins which changed significantly upon phosphorylation of tyrosine. In the lower panel of the figure, the mean values of the densitometric analysis of the 60 kDa protein in four different experiments are shown, and they are expressed as percentages of the control values (C). * $P < 0.05$ vs. C without FCS and CAP. § $P < 0.05$ vs. C, with or without FCS, and CAP.

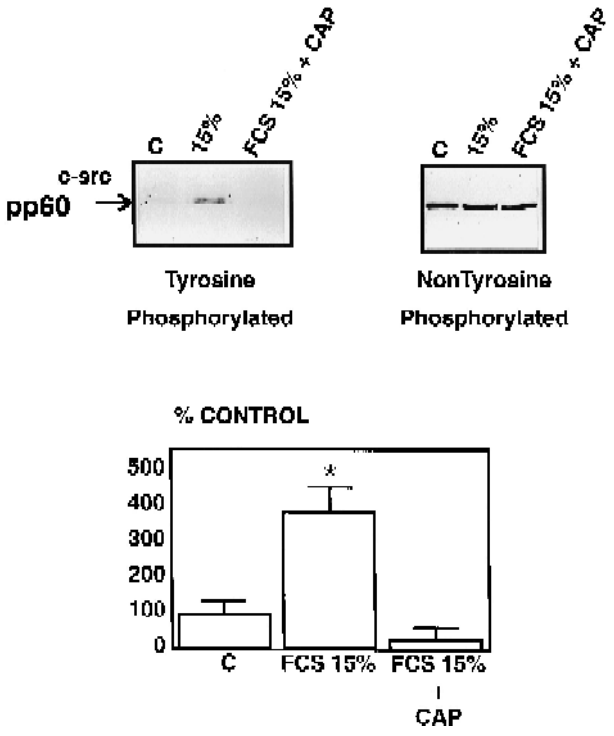

Fig. 4. Effect of angiotensin-converting enzyme inhibitors on serum-induced tyrosine phosphorylation of pp60$^{c\text{-}src}$, in cultured human mesangial cells. Quiescent cells were incubated for 30 min with 15% fetal calf serum (FCS), after 24 h of treatment with 100 μM captopril (CAP). The upper left panel of the figure shows a characteristic Western blot experiment with a specific antiphosphotyrosine antibody, after specific immunoprecipitation of cell proteins with anti-*src*. The upper right panel of the figure shows a characteristic Western blot experiment with a specific anti-*src*. In the lower panel of the figure, the mean values of the ratios between the densitometric signal of the phosphorylated and non-phosphorylated *src* in three different experiments are shown, and they are expressed as percentages of the control values (C). * $P < 0.05$ vs. the other groups.

fetal calf serum (lane 2) in proteins with apparent molecular masses of 98, 60 and 42 kDa was completely inhibited by 100 μM captopril (lane 3), but only partially inhibited by the same concentration of lisinopril (lane 4). The mean results of the densitometric analysis of four different experiments, with respect to the band with an apparent molecular mass of 60 kDa, are shown in the lower panel of Fig. 3, showing again the intense, captopril-dependent inhibition of tyrosine phosphorylation, whereas lisinopril only partially inhibited this effect.

In order to analyze the nature of the 60 kDa tyrosine-phosphorylated protein, and the specific effect of captopril on its phosphorylation, cell extracts were immunoprecipitated with a specific anti-*src* antibody and, after separation, proteins were incubated with the antiphosphotyrosine antibody. The upper left panel of Fig. 4 shows that the specific *src* phosphorylation induced by fetal calf serum was almost completely blunted by previous treatment with captopril. This effect did not depend on a decreased *src* content in cells, as the amount of this protein was similar under the different experimental conditions tested (Fig. 4, upper right panel). The statistical analysis of three different experiments, considering the ratio between the tyrosine-phosphorylated *src* and the total *src* content, confirmed the captopril-mediated inhibition of *src* tyrosine phosphorylation (Fig. 4, lower panel).

4. Discussion

The present experiments clearly demonstrated that captopril and lisinopril inhibit serum-induced human mesangial cells proliferation, but they do not modify the proliferative pattern of quiescent cells. Similar results have been previously published for the same cell type (Bakris et al., 1994), but the experimental design chosen was slightly different, mainly because most of the experiments were performed in the presence of insulin, which was not included in our experiments. Insulin magnifies the angiotensin II-converting enzyme inhibitors-dependent inhibition of proliferation, a fact which can possibly account for the quantitative differences between our results and those from Bakris et al. (1994).

As proposed by these authors (Bakris et al., 1994), angiotensin II-converting enzyme inhibitors probably blocks cell proliferation in the presence of serum by a mechanism related to its inhibition of angiotensin convertin-enzyme/kininase II, as sera added to culture media revealed significant angiotensin II-converting enzyme activity, which could generate significant amounts of angiotensin II, with subsequent cell proliferation. After blockade of this enzyme, the synthesis of angiotensin II in the incubation media of the cells would be negligible, thus explaining the antiproliferative effect of the drug. Alternatively, in these angiotensin II-converting enzyme inhibitors-treated cells, the amount of active kinins could increase with respect to that in the non-treated cultures. Kinins have been proposed as antiproliferative local mediators (Farhy et al., 1992) and they could contribute to the observed angiotensin II-converting enzyme inhibitors-dependent inhibition of proliferation. However, it has been recently demonstrated that rat mesangial cells proliferate in the presence of kinins (El Dahr et al., 1996), a fact which does not support a role for these metabolites as mediators of the antiproliferative effects of angiotensin II-converting enzyme inhibitors.

As previously stated, cell proliferation seems to be a very complex phenomenon which involves the interaction of growth factors with cell membranes, the successive phosphorylation of various proteins in the cytoplasm, and the regulation of the cell cycle at the nuclear level (Anderson et al., 1990; Taylor and Shalloway, 1993; Erpel and Courtneidge, 1995). One of these mechanisms, tyrosine phosphorylation of proteins, was investigated in the present study, because it is a well-known fact that different mediators may act as antiproliferative stimuli by preventing tyrosine phosphorylation or by promoting dephosphorylation of these proteins (Prius et al., 1996; Reardon et al., 1996; Sugimoto et al., 1996). In addition, previous studies have demonstrated that angiotensin II induces tyrosine phosphorylation of various proteins in different cell types (Schieffer et al., 1996). In mesangial cells, captopril and lisinopril inhibited tyrosine phosphorylation of different proteins, including pp42, pp60 and pp98. Moreover, the degree of blockade of this phosphorylation, in the case of captopril and lisinopril, paralleled the inhibition of cell proliferation. Thus, it can be suggested that, at least partially, the angiotensin II-converting enzyme inhibitors-dependent inhibition of mesangial cell growth may depend on the ability to decrease tyrosine phosphorylation.

The antiproliferative action of captopril cannot be solely explained by its ability to block angiotensin II-converting enzyme, as its effect on [^{3}H]thymidine incorporation and tyrosine phosphorylation in mesangial cells was more marked than that of lisinopril, even though its affinity for angiotensin II-converting enzyme is lower (Cushman et al., 1989). The additional antiproliferative effect of captopril could be the consequence of the antioxidant properties of the drug. Under some experimental conditions, reactive oxygen species could stimulate mesangial cell proliferation (Duque et al., 1993), and it could be proposed that antioxidants may have antiproliferative properties under the same conditions. However, vitamin E, an exogenous antioxidant, did not modify the proliferation rate of mesangial cells in the presence of serum, thus it seems unlikely that the antiproliferative effect of captopril depends on its antioxidant ability.

The marked ability of captopril to inhibit cell proliferation must have alternative explanations. The drug has proven to be antiproliferative in different cell types (Powell et al., 1989; Hadrava et al., 1991; Prescott et al., 1991; Uehara et al., 1993; Bakris et al., 1994; Nguyen et al.,

1994; Reddy et al., 1995), including tumor-derived cell lines (Volpert et al., 1996). In this context, a more general antiproliferative mechanism, besides the more explored angiotensin II-converting enzyme inhibition, could be proposed for the drug. The present experiments addressed this problem. As shown in Section 3, captopril prevented the tyrosine phosphorylation of different cell proteins, including $pp60^{c\text{-}src}$. This protein has been shown to be involved in the growth of different types of cells (Twanley-Stein et al., 1993; Maa et al., 1995), and it could be the target of the antiproliferative effect of the drug. However, other mechanisms that regulate the cell cycle could be modulated by captopril, and they must be investigated in future experiments.

The molecular basis of the action of captopril on cell tyrosine phosphorylation is unknown. Some authors have proposed that the antiproliferative effect could be linked to the presence of the thiol group, as penicillamine, another thiol-containing drug, also elicits an antiproliferative effect on lung fibroblasts (Nguyen et al., 1994). None of our data support this contention, but this possibility must be explored in future experiments.

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