Captopril (SQ 14,225): In vitro and in vivo influence on the proliferative response of rat lymphocytes[1]

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Summary. Captopril in vitro (50–500 µg/ml) increased ^{3}H-TdR incorporation in unstimulated and mitogen-stimulated cultures of rat lymphocytes. Unseparated spleen and lymph node cells of rats orally treated with captopril (50 mg/kg/day × 4) showed decreased basal and mitogen stimulated ^{3}H-TdR incorporation. The removal of macrophages abrogated this inhibitory effect. Leucine aminopeptidase activity of macrophages was reduced – in vivo and in vitro – by captopril.

Captopril (D-3-mercapto-2-methylpropanoyl-L-proline, SQ 14,225), an orally active inhibitor of angiotensin-I converting enzyme, has recently been introduced as a highly specific antihypertensive drug[2]. The most common side-effects of captopril include the development of maculopapular and urticarial skin eruptions, taste disturbances and membranous glomerulonephritis[3]. These side-effects are similar to those seen in rheumatoid arthritic patients treated with D-penicillamine[4]. The chemical structures of captopril and D-penicillamine show certain similarities, each compound having a highly reactive sulfhydryl group as part of a specific stereochemical configuration. These considerations raise the possibility that captopril, in analogy with D-penicillamine, may interfere with the function of the cells participating in the immune response. The present experiments describe the effects of captorpril in vitro and in vivo on the response of rat lymphocytes to mitogens.

Materials and methods. Suspensions of lymph node cells and spleen cells were prepared from Lewis rats as previously described[5]. Aliquots of 0.5 ml cell suspension (10×10^6 nucleated cells/ml) in medium RPMI 1640 (Gibco), supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% fetal calf serum (Gibco) were cultured at 37 °C in 5% CO_2 in air in flat-bottomed tissue-culture-treated multidishes (Nunc No. 14568357, Denmark). To obtain cell suspensions of nonadherent cells, spleen cells were incubated on tissue-culture-treated plastic Petri dishes (Nunc No. 150350, Denmark) at 37 °C. 2 h later the nonadherent cells were collected, counted and cultured as described for the unseparated cells. Smears of unseparated and nonadherent cells were made for cytological examination. The smears were stained with Giemsa-May-Grünwald for the identification of lymphocytes; monocytes and macrophages were identified by the presence of membrane-associated non-specific esterase[6]. Concanavalin A (Con A, 2.5 µg/ml, Sigma, grade IV) or lipopolysaccharide (LPS, 10 µg/ml, *Escherichia coli* 055:B5, Difco) were added at the start of culture and the cultures were incubated for

Table 1. Effect of captopril in vitro on the response of rat lymph node cells to Con A and LPS

Captopril (µg/ml)	Mitogen added	^{3}H-TdR incorporation (dpm/10^6 cells ± SEM)	Change in ^{3}H-TdR incorporation (%)	p
0	None	3,248 ± 28		
50	None	4,019 ± 79	+ 24	< 0.001
250	None	13.881 ± 615	+ 327	< 0.001
500	None	8,779 ± 245	+ 170	< 0.001
0	Con A	15,742 ± 347		
50	Con A	13,682 ± 567	− 13	< n.s.
250	Con A	25,746 ± 7	+ 64	< 0.001
500	Con A	24,239 ± 1,702	+ 54	< 0.01
0	LPS	6,647 ± 281		
50	LPS	8,625 ± 160	+ 30	< 0.005
250	LPS	17,492 ± 322	+ 163	< 0.001
500	LPS	14,078 ± 294	+ 112	< 0.001

Lymph node cells were prepared and pooled from 5 Lewis rats. The cell suspensions were adjusted to 10×10^6 cells/ml and cultured for 48 h in the presence of captopril and Con A (2.5 µg/ml) or LPS (10 µg/ml). 1 µCi/ml of ^{3}H-TdR was added during the last 4 h of the incubation and the incorporation of ^{3}H-TdR into cellular DNA was calculated as dpm/10^6 cells ± SEM, representing the mean of triplicate cultures. n.s., not significant.

Table 2. Effect of treatment with captopril (50 mg/kg/day p.o. for 4 days) on the response of rat spleen cells to Con A and LPS

Treatment with captopril (mg/kg/day)	Mitogen added	Unseparated spleen cells dpm/10^6 cells (mean ± SEM)	% change	p	Spleen cells depleted of adherent cells dpm/10^6 cells (mean ± SEM)	% change	p
0	None	5,811 ± 274			8,956 ± 408		
50	None	2,143 ± 587	− 63	< 0.005	5,926 ± 547	− 34	< 0.02
0	Con A	13,574 ± 828			25,833 ± 3,312		
50	Con A	5,881 ± 1,732	− 57	< 0.02	27,867 ± 1,900	+ 8	n.s.
0	LPS	8,332 ± 538			16,207 ± 767		
50	LPS	1,674 ± 143	− 80	< 0.001	15,541 ± 4,607	− 4	n.s.

Spleen cell suspensions were prepared individually from 3 Lewis rats and adjusted to 10×10^6 cells/ml (unseparated cells). Spleen cells depleted of adherent cells were obtained by incubation on plastic Petri dishes for 2 h. The cultures were incubated for 48 h with Con A (2.5 µg/ml), LPS (10 µg/ml) or without mitogen. ^{3}H-TdR incorporation was assessed as described in the legend to table 1, and expressed as dpm/10^6 cells ± SEM. n.s., not significant.

48 h. ^{3}H-thymidine (^{3}H-TdR, 1 µCi/ml, 5000 mCi/mmole, The Radiochemical Centre, Amersham, England) was added during the last 4 h of culture, and incorporation of ^{3}H-TdR into cellular DNA was determined as previously described[5]. Captopril (SQ 14,225) was added to the cultures at the start of incubation (0–500 µg/ml) or administered p.o. to Lewis rats (50 mg/kg/day) for 4 days. The viability of the cells was determined before culture and at the end of incubation by the eosin Y exclusion method. The results were expressed as dpm/10^6 living cells ± SEM. All determinations were performed in triplicate. Student's t-test was used for statistical analysis.

Leucine aminopeptidase activity was determined using peritoneal macrophages from Lewis rats as the source of enzyme. Macrophage suspensions were adjusted to 2×10^6 cells/ml and aliquots of 1 ml were plated in 3-cm Petri dishes (Nunc No. 153066, Denmark). After 1 h of incubation, nonadherent cells were removed and the macrophage monolayers were incubated with 1 ml Hank's solution (pH 7.2), containing 100 µmoles Tris-HCl and 1 µmole L-leucine-β-naphtylamide · HCl (Sigma Chemical Company), according to the method described by Suda et al.[7]. After 30 min of incubation at 37 °C, the medium was removed, centrifuged for 10 min at 2500 rpm, and 0.3 ml fast Garnet GBC (1 mg/ml, Sigma Chemical Company) in 1 M acetic acid buffer (pH 4.2) was added and allowed to react for 15 min at room temperature. The effect of captopril on leucine aminopeptidase activity was assayed using macrophages preincubated with the drug for 1 h prior to addition of the reaction mixture or by using macrophages from Lewis rats treated with captopril (50 mg/kg/day p.o.) for 4 days. The results are presented as the absorbance at 530 nm, mean ± SD of at least triplicate determinations.

Results and discussion. The addition of captopril (50–500 µg/ml) to cultures of rat lymph node cells significantly increased the basal incorporation of ^{3}H-TdR into cellular DNA (table 1). Increased ^{3}H-TdR incorporation was also found when captopril was added to cell cultures stimulated with the T-cell mitogen Con A or the B-cell mitogen LPS (table 1). Cell viability was not affected by doses of captopril up to 500 µg/ml, whereas higher doses were found to impair cell viability. Removal of adherent cells prior to culture with captopril did not abolish the observed increase in ^{3}H-TdR incorporation (results not shown).

The effect of a short oral treatment with captopril (50 mg/kg/day for 4 days) on the responsiveness of rat spleen and lymph node cells to Con A and LPS was next investigated. Table 2 shows that unseparated spleen cells from captopril-treated rats incorporated significantly less ^{3}H-TdR both in unstimulated and in mitogen-stimulated cultures than did the corresponding control cultures from untreated rats. The most pronounced inhibitory effect was observed in LPS-stimulated cultures. The removal of adherent spleen cells prior to incubation with mitogens completely abrogated the inhibitory effects seen in cultures of unseparated spleen cells from rats treated with captopril (table 2). A slight inhibition was however still observed in cultures of unstimulated spleen cells from captopril-treated rats. A similar profile of activity was observed, using unseparated and adherent-cell depleted lymph node cells from captopril-treated rats (results not shown). Cell viability and recovery of nonadherent cells were not affected by treatment with captopril. Regardless of the treatment the unseparated spleen cell suspensions contained more than 80% lymphocytes as assessed by Giemsa-May-Grünwald staining and 10–15% monocytes/macrophages as assessed by esterase-staining. After removal of adherent cells (20% of total cells) less than 2% of the nonadherent cells were esterase-positive. Unseparated lymph node cells contained 5–8% esterase-positive cells, less than 1% was found after depletion of adherent cells.

The addition of captopril (10^{-3} M) to rat peritoneal macrophages was found to inhibit macrophage leucine aminopeptidase activity by 72% (table 3). Lower concentrations of captopril were without effect. Treatment with captopril for 4 days (50 mg/kg/day p.o.) resulted in a small, but significant, inhibition of macrophage leucine aminopeptidase activity (Abs. controls = 1.269 ± 0.027 and Abs. captopril = 0.997 ± 0.049, n = 6, $p < 0.001$).

From these results it is evident that captopril in vitro exerts a stimulatory effect on ^{3}H-TdR incorporation by rat lymphocytes, both in cultures with and without added mitogens. This property is shared by a wide variety of thiol compounds, such as L-cysteine, 2-mercaptoethanol, cysteamine, 3-mercaptopropionic acid and α-thioglycerol[8,9]. Some of these thiols may exert a direct mitogenic effect on cultured lymphocytes, but are without effect when administered in vivo[10]. In addition, thiols have been shown to exert growth-promoting effects when added to cultures of lymphocytes or lymphoma cells[9]. A similar mechanism of action may account for the observed stimulatory effects of captopril on ^{3}H-TdR incorporation by lymphocytes in vitro. In vivo, however, captopril significantly inhibited lymphocyte proliferation when administered p.o. for 4 days. This effect may be a consequence of prolonged exposure to the drug, or alternatively a metabolite with immunosuppressive activity may be formed in vivo. The suppressive effects on lymphocytes were possibly mediated by macrophages, as removal of adherent cells prior to culture abrogated the suppression. An increase in the plasma concentration of PGE_2 has recently been observed after administration of captopril[11] and it is tempting to speculate that the observed inhibition of lymphocyte reactivity may be related to the immunosuppressive properties of PGE_2[12], produced by the major prostaglandin-producing cell type, the macrophage[13]. Macrophages have also been implicated in the mechanism of action of D-penicillamine[5,14], an antirheumatic drug that induces immunological side-effects similar to these observed after treatment with captopril[4]. However, the present experiments do not exclude the existence of adherent suppressor lymphocytes in cultures from captopril-treated rats, and further experiments are required in order to establish the exact nature of the suppressive cells.

The present experiments also show that captopril, in vitro and in vivo, moderately inhibits macrophage leucine aminopeptidase activity. These results are in agreement with

Table 3. Effect of captopril in vitro on macrophage leucine aminopeptidase activity

Concentration of captopril	Absorbance at 530 nm (mean ± SD)	Inhibition of leucine aminopeptidase activity (%)
None	1.280 ± 0.033	–
10^{-6} M	1.184 ± 0.151	8
10^{-5} M	1.137 ± 0.081	11
10^{-4} M	1.075 ± 0.019	16
10^{-3} M	0.359 ± 0.246	72

Peritoneal macrophages were obtained from 5 Lewis rats, pooled and adjusted to 2×10^6 cells/ml. 1-ml aliquots were incubated in plastic Petri dishes for 2 h, whereafter nonadherent cells were removed and counted. 1 ml fresh medium containing captopril (10^{-6}–10^{-3} M) was added for 1 h. The cultures were washed 3 times and reincubated with 1 ml reaction mixture containing 1 µmole L-leucine-β-naphtylamide for the determination of leucine aminopeptidase activity as described in 'methods'. The results are expressed as the mean ± SD of triplicate determinations.

the previously reported inhibition of porcine kidney leucine aminopeptidase by captopril[2]. Leucine aminopeptidase is a membrane-bound protease which has been implicated in the events that induce resting cells to proceed to the proliferative stage[15]. A potent inhibitor of leucine aminopeptidase activity, bestatin, has previously been shown to possess immunomodulatory activity[16,17]. Further experiments, however, are needed to establish whether a relationship exists between the inhibition of this enzymatic activity and the observed effects of captopril on lymphocyte responsiveness.

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Effects of sphingolipids on erythroblastic maturation in the mouse[1]

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Summary. The changes effected by injection of an extract of phospholipids obtained from the plasma of normal human donors (PLE) or an emulsion of commercially available sphingolipids on erythropoiesis in the mouse were studied. The parameters followed were ^{59}Fe uptake by the erythroid tissue and the number of circulating reticulocytes. It was found that in the 12-24-h period following administration of PLE or purified sphingomyelin a significant increase in ^{59}Fe uptake by circulating RBC and by their hemic fraction takes place. This change was associated with a higher ^{59}Fe utilization by the bone marrow and with an increase in the number of circulating reticulocytes.

In the light of the current theory, erythropoiesis in vivo is governed by erythropoietin (Epo)[2,3]. Since this unified theory does not explain many experimental and physiologic situations, the existence of accessory oxygen-independent mechanisms has been postulated[4-10].

Previous reports have indicated that a lipid factor(s) which probably affects erythroblastic cellular maturation, could participate in the quantitative regulation of erythropoiesis[5,6,11-13]. In this study we report the effects of some major phospholipids of human plasma and various commercial sphingolipids on mouse erythropoiesis as measured by ^{59}Fe uptake by RBC, spleen, bone marrow and by the number of circulating reticulocytes.

Material and methods. Preparation of phospholipids. Blood obtained from normal volunteers no less than 6 months from a previous donation, was received in acid-citrate-dextrosa anticoagulant (ACD) and centrifuged at 3000 × g for 30 min. Phospholipid extracts (PLE) were prepared from the plasma using the technique described by Reed et al.[14] for extraction of phospholipids from red blood cells. Methanol and chloroform were used in the same proportions (1:5) as in the original method. A fraction of the dry final product was emulsified in isotonic saline solution, adjusted to a concentration of 2 mg/ml and kept at 4 °C until used. A portion of the dry extract was separated for determination of total lipid phosphorus according to the technique of Fiske and Subbarow[15]. Another fraction was prepared for TLC in silica gel as described by Marinetti et al.[16].

After identification of sphingomyelin and phosphatidyl choline in the chromatograms the spots were marked, cut out and eluted with 0.5 N methanolic HCl as described by Reed et al.[14]. The phosphorus content in aliquots of the eluates was determined and the rest of each eluate was emulsified in isotonic saline solution at a concentration of 2 mg/ml and stored frozen until use.

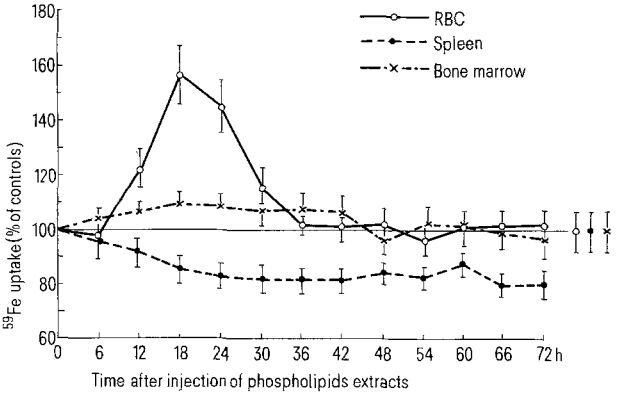

Figure 1. Changes in ^{59}Fe utilization by RBC, —○—; bone marrow —·—·x—·— and spleen ——●——, measured at various times after injection of plasma phospholipid extracts. Values are means ± SE of 8 animals.