Renal-specific delivery of antifibrotic drugs using lysozyme
Prakash, Jai
Chapter 3

Renal Targeting of Captopril using Captopril-Lysozyme Conjugate Enhances its Antiproteinuric Effect in Adriamycin-Induced Nephrosis

Willemijn A.K.M. Windt, Jai Prakash, Robbert J. Kok, Frits Moolenaar, C. Alex Kluppel, Dick de Zeeuw, Richard P.E. van Dokkum, Robert H. Henning

Abstract

High sodium intake blunts the renoprotective efficacy of ACE inhibitors (ACEi). We investigated whether targeting the drug to the kidney may attenuate the inferior response to ACEi under a high sodium condition. The ACEi captopril was coupled to the low molecular weight protein (LMWP) lysozyme, yielding captopril-lysozyme conjugates that accumulate specifically in the proximal tubular cells of the kidneys. We compared the antiproteinuric efficacy of captopril to that of the captopril-lysozyme conjugate in adriamycin induced proteinuric rats fed with a high sodium diet. Rats with adriamycin (single injection 2mg/kg) induced proteinuria were put on a high sodium diet (HS; 3% NaCl). When stable proteinuria developed at 5.5 weeks, animals were assigned to the following subcutaneous treatments: (1) vehicle (n=7); (2) lysozyme (equivalent to the amount in conjugate) (n=7); (3) captopril (5mg/kg/24h) (n=8); (4) captopril-lysozyme conjugate (captopril content equivalent to 1mg captopril/kg/24h) (n=7). Blood pressure and proteinuria were monitored. After ten days of treatment the rats were sacrificed and kidneys and plasma were taken out. After injection with adriamycin at t=0, stable proteinuria developed amounting 547 ± 79 mg/24h at week 5.5. Subsequently, after 7 and 9 days of treatment, no reduction of in proteinuria was observed in the captopril treated group. In contrast, a significant reduction in proteinuria amounting 35 ± 4% (day 7) and 25 ± 2% (day 9) was observed in the captopril-lysozyme conjugate group (p<0.05 compared to captopril group). In contrast, blood pressure was reduced in the captopril treated group by 13.9 ± 2.9 mmHg, while in the captopril-lysozyme treated group an increase of 7.9 ± 3.3 mmHg was found. The renal ACE activity was lowered with 30% in the captopril as well as in the captopril-lysozyme conjugate treated group compared with control. Further, the ratio of kidney over plasma levels of captopril doubled almost as a consequence of coupling to lysozyme. In proteinuric rats fed with a high sodium diet, captopril induced a reduction in blood pressure without an effect on proteinuria. In contrast, renal targeting of a 5 times lower dose of the ACEi with the captopril-lysozyme conjugate reduced the proteinuria without reducing blood pressure. Therefore, renal targeting of ACEi may be a promising strategy to optimize the therapy response of ACEi.
Introduction

Angiotensin Converting Enzyme inhibitors (ACEi) are a frequently used therapy for patients with hypertension. Apart from their antihypertensive effect, ACEi also display cardiovascular and renoprotective effects, both in human and experimental renal diseases (1,2). These renoprotective effects can be monitored well by studying the antiproteinuric effect of the drug. Unfortunately, ACEi treatment does show side effects such as cough, hypotension (3) and hyperkalemia (4), and only a limited number of patients show an optimal antiproteinuric and renoprotective response. This non response can (partly) be overcome by several manners amongst which the dosing and the dietary sodium intake are important. Increasing the dose of the ACEi will increase the antiproteinuric efficacy. However this may go at the expense of side effects. The efficacy of ACEi can also be enhanced by lowering the dietary sodium intake (5-8). However, compliance with a strict dietary sodium restriction is difficult for many patients in western cultures.

We have previously found that renal selective drug targeting offers a way to gain high drug concentrations in the kidneys. For this purpose, we synthesized a conjugate of the ACEi captopril with the low-molecular-weight-protein lysozyme which was accumulated in the kidney paralleled by an increased renal ACE inhibition in comparison with systemically applied captopril (9,10).

To investigate whether the response to ACEi under high sodium condition may be enhanced by targeting the drug to the kidney, we studied the effect of captopril-lysozyme conjugate on adriamycin induced proteinuria in rats fed with a high sodium diet, and compared that to the efficacy of the untargeted ACEi.

Materials and Methods

Synthesis and characterization of captopril-lysozyme

The process of synthesis and characterization of captopril-lysozyme is described elsewhere (11). Briefly, lysozyme (8.3 g, 0.58 mmol) was dissolved in 0.1 M borate buffer pH 7.5 at a concentration of 20 mg.mL⁻¹. Succinimidylloxycarbonyl-α-methyl-α-(2-pyridyldithio)toluene (SMPT; Pierce, Rockford, IL, USA; 450 mg, 1.17 mmol) was dissolved in 8.3 mL of acetonitrile and added drop-wise to the lysozyme solution and stirred for 30 minutes. Then, captopril (277 mg, 1.29 mmol; Sigma-Aldrich) solution in 8.3 mL of absolute ethanol was added drop-wise and further stirred for 2 hours. The purification of the conjugate was done by using cation exchange fast protein liquid chromatography (HiTrap™ SP XL, Amersham Biosciences AB, Uppsala, Sweden). Afterwards the conjugate was dialyzed extensively against water at 4°C. The purified conjugate was lyophilized and stored at -20°C. The estimation of the amount of captopril in the conjugate was done by using high performance liquid chromatography (HPLC; Waters, Milford, MA, USA) (12). The degree of substitution in captopril-lysozyme conjugate was
found to be 1.14 mol of captopril per mol of lysozyme. No free captopril was found in the final preparation.

**Experimental animals**

Animal experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The Animal Experimental Committee approved all protocols. Male Wistar rats (310 – 325 g; n=29) were housed under standard conditions with free access to food and drinking water.

**Experimental procedure**

After one week of adaptation, baseline 24 hour urine samples were collected in metabolic cages. Rats were injected with adriamycin (2 mg/kg; Adriblastina R.T.U., 2 mg/ml) in the tail vein under anesthesia with isoflurane 3% in N₂O/O₂ (2:1) to induce proteinuria. After three weeks the animals were put on a high sodium diet (3.0% NaCl) (Hope Farms, Woerden, The Netherlands). From that time onwards, 24h-urine was collected in metabolic cages weekly. In addition, systolic blood pressure was measured weekly using tail-cuff plethysmography (IITC Life Science, Woodland Hills, CA) in awake restrained animals. Animals with proteinuria less than 80 mg/24h were excluded from the experiment. After stratification for proteinuria, from t=5.5 weeks onwards the animals were given the following treatments by once daily subcutaneous injections for 10 days (all dissolved in 5% glucose): (1) vehicle (5% glucose; n=7); (2) lysozyme (76 mg/kg/24h, Sigma-Aldrich, St. Louis, MO, USA; n=7); (3) captopril (5mg/kg/24h, Sigma-Aldrich; n=8); (4) captopril-lysozyme conjugate (equivalent to 1mg/kg/24h captopril and 76 mg/kg/24h lysozyme; n=7). After 1, 4, 7 and 9 days of treatment proteinuria was measured. After 3, 6 and 9 days of treatment blood pressure was measured at 30 (n=2) or 60 (n=2), 120 (n=2) and 240 (n=2) minutes after the last injection. Animals were sacrificed after 10 days of treatment at 60 (n=2 or 3) or 240 (n=5) minutes after the last injection. Laparotomy was performed under anesthesia with isoflurane 3% in N₂O/O₂ (2:1) and a blood sample was taken from the abdominal aorta. Kidneys were flushed with saline, removed and weighed. Kidney samples were homogenized using turrax homogenizer for the estimation of captopril amount and ACE-activity.

**Analytical procedures**

Urine samples were analysed using colorimetric assays for total protein with molybdate red. Captopril concentrations were measured in plasma and kidney homogenate by HPLC as described previously (13). ACE activity was measured in plasma and kidney homogenate at 60 or 240 minutes after the final injection as described previously (14).

**Calculations and statistical analysis**

Data of the captopril and captopril-lysozyme treated groups are corrected with data of their respective controls, vehicle glucose and lysozyme treated animals. All data are
presented as mean ± S.E.M. Differences in mean values between groups were compared using one-way analysis of variance (ANOVA) and a Bonferroni t-test to identify the groups that were different. In all tests, p<0.05 was considered statistically significant.

**Results**

All animals completed the study and no differences in growth curves were observed between the groups (data not shown).

**Proteinuria**

After injection with adriamycin, a stable proteinuria developed amounting 547 ± 79 mg/24h at 5.5 weeks after induction of renal disease. Upon start of treatment with captopril and captopril-lysozyme hardly any effect on proteinuria was found after one and four days of treatment (figure 1). After 7 days of treatment a slightly lowering of proteinuria was observed in the captopril group (-9 ± 10 %), although this was not significant, while after 9 days of the treatment the decrease in proteinuria in the captopril group was completely blunted. In the captopril-lysozyme conjugate treated group a decrease in proteinuria of -35 ± 4 % (7 days) and -25 ± 2 % (9 days) was found. The reduction in proteinuria was significant at both time points compared to captopril (p<0.05). Further, in the lysozyme treated group a slight increase in proteinuria was noticed at day 7 and 9 (6 ± 13 % and 12 ± 8 %, respectively).

![Figure 1](image)

**Figure 1.** Antiproteinuric effect of therapy in % of proteinuria before start of the treatment. Filled triangles: lysozyme (equivalent amount as in captopril-lysozyme conjugate); filled rounds: captopril (5mg/kg/24h); open rounds: captopril-lysozyme conjugate (amount corresponding to 1 mg captopril/kg/24 h). Data is mean ± S.E.M. *p<0.05 represents the significant difference between captopril and captopril-lysozyme treated groups.
**Blood pressure**

The mean of the systolic blood pressure (SBP) measured at 3 and 5 days before treatment was considered as baseline measurement. After the onset of the treatment, SBP was measured after 2, 5 and 8 days, at 30, 60, 120 and 240 minutes after the injection in separate rats. As no differences were observed between these time points (data not shown) the data were pooled. Baseline SBP was compared to the average of the three measurements after the start of the treatments. SBP was significantly reduced in the captopril group compared to control (-13.9 ± 2.9 mmHg) (figure 2). In contrast, the animals in the captopril-lysozyme conjugate group showed a trend to an increase in SBP compared to control (7.9 ± 3.3 mmHg). Accordingly, SBP was significantly decreased in captopril treated animals compared to the captopril-lysozyme conjugate treated group. The animals in the lysozyme group showed a reduction in SBP (-11.5 ± 3.4 %), although this was not significant compared to control.

![Figure 2](image)

*Figure 2. Effect of therapy on Systolic Blood Pressure (SBP) in delta SBP (mmHg). Black bars: lysozyme (equivalent amount as in captopril-lysozyme conjugate); white bars: captopril (5mg/kg/24h); gray bars: captopril-lysozyme conjugate (amount corresponding to 1mg captopril/kg/24h). Data in mean ± S.E.M., * significant difference ($p<0.05$), # significant difference between treatment and control ($p<0.05$).*

**ACE activity**

To obtain insight in the efficacy of renal targeting, ACE activity was measured in kidneys collected 60 and 240 minutes after the final injection (table 1). After 60 minutes the ACE activity seemed inhibited to a larger extend in de captopril group than in the captopril-lysozyme group, although the difference is not significant. In contrast, after 240 minutes renal ACE activity was reduced to a similar extent in the captopril treated group as well as in the captopril-lysozyme conjugate treated group compared to control.
Antiproteinuric effect of subcutaneous captopril-lysozyme

Table 1. ACE activity in the kidneys.

<table>
<thead>
<tr>
<th>Time point after last dose</th>
<th>glucose (nmol HisLeu/g/min)</th>
<th>lysozyme (nmol HisLeu/g/min)</th>
<th>captopril (nmol HisLeu/g/min)</th>
<th>captopril-lysozyme (nmol HisLeu/g/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renal ACE activity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 min</td>
<td>35.5 ± 6.2 (n=2)</td>
<td>48.7 ± 16.6 (n=2)</td>
<td>16.2 ± 3.7 (n=3)</td>
<td>24.3 ± 10.6 (n=2)</td>
</tr>
<tr>
<td>240 min</td>
<td>30.8 ± 5.2 (n=5)</td>
<td>35.2 ± 5.6 (n=5)</td>
<td>25.2 ± 3.1 (n=5)</td>
<td>26.0 ± 3.9 (n=5)</td>
</tr>
</tbody>
</table>

Data represents mean ± S.E.M.

Captopril levels
To further investigate renal delivery of captopril, total captopril levels were determined in plasma and kidneys 60 and 240 minutes after the final injection (table 2). Sixty minutes after the last dose renal captopril levels did not differ between the captopril group and the captopril-lysozyme group. However, after 240 minutes there was a significantly higher amount found in the captopril-lysozyme group compared to the captopril group (Table 2).

Table 2. Total captopril concentration in kidneys and plasma.

<table>
<thead>
<tr>
<th>Time point After last dose</th>
<th>Total captopril concentration in kidneys (µg/g)</th>
<th>captopril (µg/g)</th>
<th>captopril-lysozyme (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 min</td>
<td>11.7 ± 3.7 (n=3)</td>
<td>8.1 ± 0.9 (n=2)</td>
<td></td>
</tr>
<tr>
<td>240 min</td>
<td>2.4 ± 0.6* (n=5)</td>
<td>2.5 ± 0.5 (n=3)</td>
<td>0.32 ± 0.03* (n=5)</td>
</tr>
</tbody>
</table>

Data in mean ± S.E.M., *p<0.05 represents the significance difference between captopril and captopril-lysozyme treated groups at the same time point, # p<0.05 represents the significance difference between the time points for one treatment.

In contrast to the kidneys, the plasma levels of captopril 60 minutes after the final dose were significantly lower in the captopril-lysozyme group compared to the captopril group. After 240 minutes a significant reduction of captopril levels was found in the captopril group while those in the captopril-lysozyme group remained at a similar level as observed at 60 min. Thus, the percentage of captopril levels in plasma compared to the levels in the kidney 240 minutes after the final dosing is about halved in the captopril-lysozyme conjugate group (7.2%) compared to the captopril group (17.3%). Moreover, the percentage
of free plasma captopril was significantly higher in the captopril group compared to the captopril-lysozyme group at 240 minutes (60 minutes: 30.0 ± 6.1% and 18.3 ± 1.6%, 240 minutes and 39.4 ± 4.4% and 21.3 ± 0.8%, respectively). Thus, coupling to lysozyme resulted in an about 50% reduction of the percentage of free captopril in plasma.

**Discussion**

The aim of the present study was to investigate whether an inferior response to ACEi under high sodium condition may be attenuated by targeting the drug to the kidney. Therefore, we studied the effect of captopril-lysozyme conjugate on adriamycin induced proteinuria in rats fed with a high sodium diet. In proteinuric rats fed with a high sodium diet, captopril-lysozyme conjugate significantly reduced the proteinuria without affecting blood pressure. In contrast, captopril treated animals displayed the opposite effect, i.e. a reduction in blood pressure without any effect on proteinuria. Although captopril-lysozyme conjugate was administered in a dose five times lower than captopril, even higher captopril levels were found in the kidneys. These results demonstrate the working profile of the captopril-lysozyme conjugate to be renoselective up to an extent that anti-proteinuric effects were observed, without any effect on blood pressure in nephrotic rats on high sodium diet.

In a previous study employing single injection in healthy animals, it was shown that subcutaneous injection is an adequate route of administration to deliver captopril-lysozyme conjugate (15), as the conjugate was almost completely absorbed from the injection site, remained stable and displayed a prolonged residence time in the kidneys. Furthermore, the single subcutaneous dose of captopril-lysozyme conjugate produced a moderate but sustained inhibition of the renal ACE during the 24-hour observation period, with no effects on plasma ACE. In the present study we extended these findings to chronic subcutaneous administration in nephrotic animals. The levels of total captopril that were measured in the current study are in good agreement with those obtained from previous pharmacokinetic data after single s.c. injection (15). Further, our data demonstrate a substantial decrease in the percentage free captopril in plasma by coupling of the drug to lysozyme. Previously, it has been observed that the conjugate does not inhibit plasma ACE (16). Therefore, it is conceivable that the absence of a blood pressure reduction in the captopril-lysozyme conjugate group is attributable to the lack of inhibition of non-renal ACE. Although, when blood pressure is measured by tail cuff, this implies that no gross changes were observed. Because of this method, minor changes in blood pressure effect may have been unnoticed.

The advantage of targeting captopril over normal dosing is clearly demonstrated by the observation that higher levels of total renal captopril are reached, even following a 5 times lower dose of targeted captopril compared to the parent drug.

Similar to the earlier single dose study with captopril-lysozyme conjugate (15), the present study with a ten-day treatment period shows a moderate inhibition of renal ACE
activity albeit non-significant (Table 1). Accordingly, our data demonstrate an effective targeting of captopril to the kidney of nephrotic animals by conjugating the drug to lysozyme. Therefore it is likely that the antiproteinuric effect observed in the captopril-lysozyme conjugate group results from the selective distribution of captopril to the kidneys.

The higher renal levels of total captopril found following the administration of captopril-lysozyme conjugate compared to administration of captopril were accompanied by an antiproteinuric effect in captopril-lysozyme conjugate treated animals. However, increased renal levels of captopril in the conjugate group did not result in a higher inhibition of renal ACE activity at the time points measured, compared to captopril treated animals. There may be several explanations for the discrepancy between effects on ACE activity and on proteinuria. First, the antiproteinuric effect represents the effectiveness of the drug during a 24 h period, while ACE activity was measured at specific time points. As conjugation of captopril to lysozyme apparently prolongs the half-life in renal tissue, renal ACE activity may simply be inhibited for a longer period of time due to conjugation. Secondly, drug levels measured in the captopril-lysozyme conjugate group represent total captopril levels, i.e. both conjugated and unconjugated drug. The thiol group of captopril is conjugated with lysozyme, however this group is essential for the ACE inhibitor action of captopril (17). So, before the coupled captopril can be active, it must be uncoupled in the kidney, probably by reduced glutathione (18). Kok et al. found that the majority of an intravenous dose of captopril-lysozyme conjugate was accumulated in the kidney in the first hour, while the relative amount of free captopril rose during 3 hours after injection to 21% of the total captopril concentration. This feature of the captopril-lysozyme conjugate may explain the discrepancy between the total renal captopril concentration measured and the renal ACE activity measured. Thirdly, distribution of the drug within the kidney may differ when administered as captopril-lysozyme conjugate. Following glomerular secretion captopril-lysozyme conjugate is selectively taken up by tubular cells by receptor mediated endocytosis (19). The majority of ACE in the kidney is localized in the brush border of the proximal and distal tubular cells of the cortex and in the glomeruli (20,21). In overt proteinuria, ACE and angiotensinogen are both up regulated in the proximal renal tubules (22). As noticed before, captopril-lysozyme conjugate is selectively reabsorbed in the tubular cells and uncoupled by intracellular glutathione. Therefore, the captopril released from the conjugate at the level of tubular cells may be responsible for the antiproteinuric effect observed in the captopril-lysozyme conjugate group. It could be, that ACE present in other cells than the tubular cells, such as in the endothelial cells, is not inhibited in the captopril-lysozyme group, while it is in the captopril group. Therefore, the total ACE inhibition would be lower in the captopril-lysozyme group, while tubular ACE is inhibited to the same extent.

The antiproteinuric effect of ACEi develops slowly (23), and usually takes five weeks of treatment to reach its maximal effect. In the present study, we treated the animals with captopril-lysozyme conjugate for 9 days. However, it is likely that after 9 days of treatment the maximal antiproteinuric effect is not reached yet, indicating the need for future research
Chapter 3

in which the efficacy of long-term treatment with captopril-lysozyme conjugate is investigated.

The results of previous clinical studies and animal experiments showed that an increase in sodium intake blunts the antiproteinuric effect of ACEi (24-27). As sodium intake itself has no influence on proteinuria in adriamycin induced nephrosis (28), the efficacy of captopril-lysozyme conjugate in animals fed a high sodium diet is likely the result of improved pharmacokinetic or dynamic properties of the drug. The most obvious explanation would be a change in kinetic parameters of captopril because of coupling to lysozyme, in view of the prolonged residence time in the kidney. However, in the present study we cannot exclude a different pharmacodynamic profile of the drug.

In this study we demonstrate that drug targeting provides a feasible method to chronically treat nephrosis. Targeting of captopril to the kidney has some important benefits: it is devoid of reduction of blood pressure and it has an antiproteinuric effect, even in conditions of high sodium load. In general, blood pressure reduction is sought in therapy of proteinuric renal disease. In this perspective, conjugation of the ACEi does not seem to have any advantage, as the blood pressure reduction is lost. Recent evidence suggests that the anti-proteinuric effect of the systemic administration of ACEi is enhanced by dose increase or combination with ARB (29). In these situations, reduction of blood pressure may become limiting to obtain an optimal antiproteinuric effect. Then, conjugation of ACEi to lysozyme may be employed to allow renal titration of ACEi in the face of limitations of systemic application because of side-effects such as hypotension.

Because of the chemical limitations of ACE inhibitors with more potent antiproteinuric efficacy, captopril had to be used in the present drug targeting approach. In spite of this, the conjugate displayed an antiproteinuric effect at a very low dose. Nevertheless, future research may/will need to disclose strategies to couple more potent ACEi to a small protein carrier, as to avoid administration of large amounts of lysozyme.

Conclusions

Our results show that in adriamycin nephrotic rats fed a high sodium diet, the subcutaneous injection with captopril-lysozyme conjugate induced an antiproteinuric effect without reduction of blood pressure. In contrast, a five time higher dose of subcutaneously administered captopril did not display an antiproteinuric effect and did have a systemic effect in reducing blood pressure. ACEi-lysozyme conjugate provides a valuable tool to further study the way to enhance efficacy and improve on response variability of RAS intervention for renoprotection.

References


