Specific Delivery of Captopril to the Kidney with the Prodrug Captopril-Lysozyme

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ABSTRACT
Low-molecular-weight proteins (LMWPs) accumulate in the proximal tubular cells of the kidney, which makes these proteins interesting tools for renal drug targeting. We studied this approach using the LMWP lysozyme as a carrier for the angiotensin-converting enzyme inhibitor captopril. Captopril was conjugated to lysozyme via a disulfide bond. The pharmacokinetics of the captopril-lysozyme conjugate was studied in the rat. Only intact conjugate could be detected in the circulation. The total amount of conjugate disulfides in the kidney was six times higher after administration of the conjugate than after the administration of an equivalent amount of free captopril. The conjugate was recovered in the urine partially as intact conjugate and partially as low-molecular-weight disulfides. The excretion of conjugate in the urine was not a consequence of the coupling of captopril to lysozyme because an equivalent bolus dose of native lysozyme was similarly excreted into the urine. By determination of the renal angiotensin-converting enzyme activity, we showed that the conjugate was degraded to the pharmacologically active captopril in vivo. We conclude that the coupling of captopril to the LMWP lysozyme results in increased captopril concentrations in the kidney and reduced captopril concentrations in the circulation.

In the past years, angiotensin-converting enzyme (ACE) inhibitors have been used to treat patients with hypertension and congestive heart failure. Furthermore, ACE inhibitors effectively reduce urinary protein excretion in renal disease and exert a long-term renoprotective effect (Navis et al., 1996). Whether the beneficial effects of ACE inhibiting drugs on the kidney are the result of a reduction of circulating angiotensin II or of the inhibition of local tissue renin-angiotensin system is unknown. The current ACE inhibitors display only minor differences in their selectivity toward different tissues (Cushman et al., 1989). We wanted to study the effect of a selective inhibition of the local renal ACE via drug delivery of an ACE inhibitor to the kidney. For this purpose, we synthesized a conjugate of the ACE inhibitor captopril (CAP) and the low-molecular-weight protein (LMWP) lysozyme (LZM) (Fig. 1) (R. J. Kok, F. Grijpstra, G. W. Somsen, F. Moolenaar, D. de Zeeuw and D. K. F. Meijer, submitted for publication). Because LMWPs accumulate in the kidney, drug-LMW conjugates can be used for renal drug targeting (Franssen et al., 1994; Haas et al., 1996). After glomerular filtration, these conjugates are reabsorbed by the proximal tubular cells and degraded in the lysosomes. After release of the free drug from the lysosomal compartment, the now-activated drug can diffuse to other parts of the kidney (Fig. 2). In this study, we present the pharmacokinetic data for the CAP-LZM conjugate. The pharmacokinetic profile of the conjugate was compared with those of unconjugated CAP and LZM. We studied the plasma disappearance and urinary excretion of the conjugate and its metabolites in freely moving Wistar rats. In another set of experiments, we determined the CAP accumulation in the kidney and the renal tissue ACE inhibition that resulted from the CAP conjugate.

Materials and Methods
Chemicals. CAP, LZM (3 × crystallized, grade I), and Micrococcus lysodeicticus were purchased from Sigma Chemical (St. Louis, MO). Succinimidylcarboxyl-α-methyl-α-(2-pyridyl)toluene (SMPT) was purchased from Pierce (Rockford, IL). Tributylphosphine (TBP) (purum) was obtained from Fluka (Buchs, Switzerland). All solvents for high-performance liquid chromatography (HPLC) were of HPLC quality (Labscan, Dublin, Ireland). Water was purified with an Elgastat Maxima-HPLC water system (High Wycombe, UK).

Synthesis and Characterization of CAP-LZM. The preparation and characterization of the CAP-LZM conjugate have been described in detail elsewhere (R. J. Kok et al., submitted). The conjugate that was used in this study had a protein content of more than 95% and a degree of drug substitution of 0.4, which indicates that the product consisted a mixture of native LZM and 1:1 CAP-LZM conjugates.

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ABBREVIATIONS: ACE, angiotensin-converting enzyme; LMWP, low-molecular-weight protein; CAP, captopril; HPLC, high-performance liquid chromatography; LZM, lysozyme; SMPT, succinimidylcarboxyl-α-methyl-α-(2-pyridyl)dithio)toluene; TBP, tributylphosphine.
The homogenates were stored at phosphate buffer (pH 7.4) was prepared by Turrax homogenization. A 1:5 homogenate in ice-cold potassium phosphate buffer, pH 7.4, and TBP solution (1% in methanol; n = 6). Blood samples for four of the CAP-LZM rats and all of the CAP plus LZM rats were drawn at several time points via the cannula. Blood samples were heparinized and immediately centrifuged (5 min at 13,000 rpm) to obtain plasma. The rats voided spontaneously, after which the urine was collected per fraction into preweighed plastic tubes. The collection of the urine was continued up to 10 h after the administration of the drugs. The plasma and urine samples were analyzed as described in the text.

Distribution of CAP to the Kidney and ACE Inhibition in the Kidney. Under a light anesthesia of halothane, the rats (280–300 g) received an i.v. bolus dose of the conjugate ([33 mg·kg⁻¹] dissolved in approximately 0.5 ml of 5% glucose, n = 4 for each time point) via the dorsal penile vein. A control group of rats received an i.v. bolus dose of LZM alone (33 mg·kg⁻¹). Blood samples for four of the CAP-LZM rats and all of the CAP plus LZM rats were drawn at several time points via the cannula. Blood samples were heparinized and immediately centrifuged (5 min at 13,000 rpm) to obtain plasma. The rats voided spontaneously, after which the urine was collected per fraction into preweighed plastic tubes. The collection of the urine was continued up to 10 h after the administration of the drugs. The plasma and urine samples were analyzed as described in the text.

Pharmacokinetic Experiments. Male Wistar rats (280–300 g) were obtained from Harlan (Zeist, the Netherlands). The plasma disappearance and urinary excretion of the conjugate were determined in freely moving rats that were equipped with a permanent cannula in the jugular vein 1 week before the experiment (Steffens, 1969). The distribution of CAP to the kidney and the ACE inhibition in the kidney were determined in rats that had received an i.v. bolus dose via the dorsal penile vein under halothane anesthesia.

Plasma Disappearance and Excretion in the Urine. A day before the experiment, the rats were placed in metabolic cages, in which they had free access to food and water. From this moment on, the rats received an infusion of 5% glucose at a rate of 2 ml·h⁻¹. At the start of the experiment, the rats received an i.v. bolus dose via the cannula of either the conjugate ([33 mg·kg⁻¹] dissolved in approximately 0.5 ml of 5% glucose, n = 10), or equivalent doses of uncoupled CAP and LZM (n = 8). Another group of rats received a bolus dose of LZM alone (33 mg·kg⁻¹; n = 6). Blood samples for four of the CAP-LZM rats and all of the CAP plus LZM rats were drawn at several time points via the cannula. Blood samples were heparinized and immediately centrifuged (5 min at 13,000 rpm) to obtain plasma. The rats voided spontaneously, after which the urine was collected per fraction into preweighed plastic tubes. The collection of the urine was continued up to 10 h after the administration of the drugs. The plasma and urine samples were analyzed as described in the text.

Fig. 1. Schematic representation of the CAP-LZM conjugate. CAP is coupled via a disulfide bond to one side of the SMPT-spacer molecule. The other end of the spacer is linked to a lysine residue of LZM.

Fig. 2. Principle of drug targeting to the proximal tubular cell with LMWP. The glomerularly filtered drug-LMWP conjugate is resorbed by the proximal tubular cells and degraded in the lysosomes. The free drug diffuses from the lysosomes into the cytosol and subsequently to the urine or blood stream. For some drugs, the transport out of the proximal tubular cell might also be mediated by carrier proteins.

The urine samples of the rats that had received a bolus dose of 33 mg·kg⁻¹ LZM were also analyzed for the LZM activity in the urine. This analysis was based on the turbidimetric method of Atassi and Habeeb (1969). A 30-μl aliquot of the sample was added to 1 ml of a suspension of M. lysodeicticus in a 0.1 N borate buffer, pH 7.4, and TBP solution (1% in methanol; reacted for 30 min, and deproteinized with 3 volumes of methanol (150 or 300 μl, respectively). To discriminate between conjugate-bound CAP and released CAP, an additional analysis was performed on plasma and urine samples that had been deproteinized immediately on collection. The protein-bound CAP was calculated by subtracting the amount of CAP in the deproteinized samples from the total CAP amount.

Calculations and Statistics. Pharmacokinetic analysis of the total CAP plasma disappearance data was performed with the Multifit program (Department of Pharmacokinetics and Drug Delivery, University Center for Pharmacy, Groningen, the Netherlands) using the Simplex algorithm, assuming a constant relative error. The initial plasma clearance, terminal half-life, and volumes of distribution at steady state (Vdₘₐₓ of central and peripheral compartment) were calculated with a two-compartment model, using the combined data of each treatment in one curve fit. The area under the curve of the renal distribution curves was calculated with the Multifit program (trapezoidal rule, up to last data point).
Because freely moving rats were used in this experiment, the animals voided their bladders at different time points; therefore, the averaged excretion curves were calculated by interpolation with the urinary excretion rate at fixed time points.

The statistical significance of differences was tested at a significance level of $p < .05$ (*), $p < .01$ (**), or $p < .001$ (***), using the two-sided Student’s $t$ test.

**Results**

Figure 3 shows the plasma disappearance curves of the CAP-LZM conjugate and CAP plus LZM. Plasma concentrations were followed during 240 min; after that time point, total CAP concentrations dropped below the detection limit of the applied analytical method (25 ng · ml$^{-1}$). In the group of rats that had received CAP plus LZM, the relative amount of protein-bound CAP ranged from 24 ± 3% at $t = 5$ min to 53 ± 4% at $t = 240$ min. No unbound CAP could be detected in the plasma samples of the rats that had received CAP-LZM conjugate. The calculated kinetic parameters of the plasma disappearance curves are shown in Table 1.

Figure 4 shows the concentration of total CAP that was measured in the kidney. To estimate the amount of CAP that had been accumulated in the kidney, we calculated the area under the curve of the renal distribution curves. These values averaged 97 and 580 (percent dose-minute) for CAP and the CAP-LZM conjugate, respectively.

The excretion of CAP in the urine was followed up to 10 h (Fig. 5). After administration of free CAP plus LZM, only non-protein-bound CAP was excreted in the urine. In contrast, the administration of CAP-LZM resulted in excretion of both protein-bound and non-protein-bound CAP disulfides into the urine. After the administration of free CAP plus LZM, most of the excreted CAP was recovered in the first 2 h after administration. The CAP-LZM conjugate resulted in a sustained excretion of non-protein-bound CAP into the urine with a constant excretion rate of 4.5% · h$^{-1}$. The protein-bound CAP was excreted during the first 5 h after administration (Fig. 6). Figure 6 shows that the urinary excretion of protein-bound CAP was similar to the excretion of intact LZM in the rats that had received an equivalent dose of native LZM. Table 2 gives a summary of the data on the cumulative urinary excretion of the CAP-LZM conjugate and the unconjugated CAP and LZM.

To test whether the accumulation of CAP in the kidney would lead to renal ACE inhibition, we performed a pilot experiment in which we measured the renal tissue ACE activity. At 30 min after administration of the CAP-LZM conjugate, the renal ACE activity was significantly lower than the renal ACE activity of control rats (Fig. 7).

**Discussion**

The aim of the present study was to increase the renal selectivity of the ACE inhibitor CAP. This can be accomplished either by increasing the amount of drug that accumulates in the kidney or by preventing the extrarenal effects of CAP. Our results show that the CAP-LZM conjugate influences the renal selectivity via both strategies.

The free thiol group of CAP is used for the conjugation of CAP to LZM, but it is also essential for its ACE-inhibiting properties (Ondetti and Cushman, 1981). Therefore, the
The synthesized CAP-LZM conjugate is an inactive prodrug. Previous experiments have shown that the parent drug can be released by incubation of the conjugate with reduced glutathione (R. J. Kok et al., submitted). Because LZM accumulates rapidly in the kidney and high levels of glutathione are present in the proximal tubular cells but not in the plasma, we anticipated that the release of CAP should predominantly take place in the kidney. This hypothesis is confirmed by the observation that only protein-bound CAP was found in the plasma after administration of the conjugate. Consequently, the total CAP plasma curve of the conjugate reflects the distribution of CAP-LZM. This plasma disappearance curve is similar to the curves of native LZM and of other drug-LZM conjugates (Hysing and Tolleshaug, 1986; Franssen et al., 1991). When the plasma curves of the conjugate and the control group are fitted in a two-compartment pharmacokinetic model, the differences between the curves are reflected in the calculated clearance and volumes of distribution. The plasma clearance of uncoupled CAP is much higher than of the conjugate, which can be explained by the different routes of elimination of the two compounds: free CAP is actively secreted by the kidney into the urine, whereas the LZM conjugate is only eliminated by glomerular filtration.

### Table 2

Cumulative urinary excretion data

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<th>Non-protein-bound CAP</th>
<th>Protein-bound CAP</th>
<th>LZM</th>
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<tr>
<td>% dose</td>
<td>84 ± 1.3</td>
<td>30 ± 4</td>
<td>33 ± 2</td>
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Fig. 5. Cumulative excretion of CAP disulfides in the urine. CAP concentrations were determined as total CAP (CAP-SH + CAP-disulfides); all values are expressed as mean ± S.E.M. •, Total CAP excretion after administration of CAP plus LZM (n = 6). ○, Dotted line, total CAP excretion after administration of CAP-LZM conjugate (n = 10); ○, solid line, excretion of non-protein-bound CAP after administration of CAP-LZM conjugate (n = 10).

Fig. 6. Cumulative excretion of protein-bound CAP and of LZM in the urine. The protein-bound CAP excretion was calculated from the total and non-protein-bound CAP excretion; the LZM excretion was determined with an LZM activity assay. □, Excretion of protein-bound CAP after CAP-LZM administration (n = 10). ▲, Excretion of LZM after LZM administration (n = 6).

Fig. 7. Renal tissue ACE activity. ACE activities were measured in kidney homogenates of rats that had received i.v. bolus injections of vehicle (5% glucose, control rats) or CAP-LZM 30 min before the extirpation of the kidneys. Open column, control rats. Closed column, CAP-LZM. Values are expressed as mean ± S.E.M. (n = 4).
As mentioned before, an increase in the renal selectivity of CAP could be obtained by increasing the amount of drug that accumulates in the kidney. Because the amount of total CAP that has distributed to the kidney was increased 6-fold by administering CAP as the CAP-LZM conjugate, we succeeded in delivering more CAP to the kidney. The accumulated conjugate can be degraded and activated in vivo to free CAP, as can be concluded from the observed ACE inhibition in the kidney homogenates. Experiments in which the renal and extrarenal ACE inhibitions of the CAP-LZM conjugate are compared with those of free CAP are ongoing and must reveal whether the administration of the conjugate results in a more renal-selective ACE inhibition. Furthermore, the comparison of the effects of the conjugate and free CAP on blood pressure and renal physiological parameters such as sodium excretion and renal hemodynamics must corroborate the present pharmacokinetic data.

After glomerular filtration, the CAP-LZM conjugate can be either reabsorbed by the proximal tubular cells or excreted into the urine. The tubular reabsorption process of LMWP's is a receptor-mediated endocytotic process with a low affinity and a high capacity (Maack et al., 1979). Our data show that one third of the injected doses of both CAP-LZM and native LZM is not reabsorbed in the kidney. We conclude that the protein-bound CAP in the urine represents conjugate that has been filtered but not reabsorbed in the kidney and that this partial loss of targeted CAP is a consequence of the protein that is used as drug carrier in this study.

The administration of CAP-LZM results in the excretion of non-protein-bound CAP in the urine with a slow and relatively constant excretion rate. This excretion rate should reflect the degradation of the conjugate and subsequent clearance of CAP from the kidney. In contrast, the excretion of non-protein-bound CAP in the urine after administration of free CAP reflects the plasma clearance of this drug. The recovery of total CAP in the urine is in accordance with the recovery of CAP in other studies in the rat (Park et al., 1982; Matsumoto et al., 1986).

In conclusion, this pharmacokinetic study demonstrates that the renal delivery of CAP with the prodrug CAP-LZM resulted in an increased renal selectivity by both a reduced extrarenal distribution and an increased renal accumulation of the drug. We conclude that the delivery of CAP to the kidney has been achieved successfully.

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References


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