Chapter 4

Hydrochlorothiazide Increases Plasma or Tissue ACE Inhibitor Drug Levels in Rats with Myocardial Infarction: Differential Effects on Lisinopril and Zofenopril.

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Abstract

Background: Sodium depletion with diuretics augments the efficacy of ACE inhibitor (ACE-I) therapy for hypertension and renal dysfunction, and possibly for LV dysfunction after myocardial infarction. Underlying mechanisms may involve altered ACE-I pharmacokinetics. We hypothesized that the diuretic hydrochlorothiazide (HCTZ) causes increased steady-state levels of the ACE-inhibitors lisinopril (LIS) and zofenopril (ZOF) in rats with myocardial infarction.

Methods: Rats were subjected to coronary ligation to induce myocardial infarction. After 1 week, rats were randomized to 50 mg/kg/day HCTZ or control treatment for 3 weeks. The last week, rats received LIS or ZOF in equipotent dosages (3.3 and 10 mg/kg/day, respectively. Rats were sacrificed at $T_{max}$ after the last dose of ACE-I, and tissues were collected for analysis of drug concentrations.

Results: LIS concentrations in plasma were significantly increased by HCTZ, at unchanged tissue concentrations. This increase could be fully explained by decreased renal function, as evidenced by increased plasma creatinine levels (LIS-HCTZ; 82±5 versus LIS 61±5 µM, p<0.001). In contrast, zofenoprilat levels in kidney and non-infarcted LV were markedly increased by HCTZ, whereas plasma concentrations were unchanged. Although HCTZ tended to increase plasma creatinine in zofenopril-treated rats as well, this increase was less pronounced (ZOF-HCTZ; 61±3 versus ZOF 54±2 µM, p=0.15).

Conclusions: HCTZ increases steady-state ACE-I drug levels, most likely by affecting their renal clearance. Notably, the lipophilic ACE-I zofenopril accumulated in tissue, whereas the hydrophilic lisinopril increased in plasma. Whether combining different ACE inhibitors with HCTZ translates into distinct clinical profiles requires further study.
Introduction

Left ventricular dysfunction after myocardial infarction (MI) is characterized by progressive cardiac remodeling eventually leading to chronic heart failure. Activation of the renin angiotensin aldosterone system is thought to play a central role in this process. Consequently, angiotensin-converting enzyme inhibitor (ACE-I) therapy effectively prevents this remodeling and reduces mortality, although the therapeutic effects of ACE inhibitors may be at least partially independent from inhibition of the ACE enzyme in itself. Angiotensin II levels can even be elevated during ACE inhibition therapy.\(^1,2\)

Diuretic-induced sodium restriction can enhance the effects of ACE-I in renoprotective and antihypertensive therapy.\(^3-6\) Whether this can be extended to cardioprotection by ACE inhibition is less well established, but experimental data from animal studies suggest that adding a diuretic may improve ACE-I treatment in the early chronic phase after myocardial infarction.\(^7-9\) The mechanism underlying diuretic-induced enhanced efficacy of ACE-I therapy is unknown, but a pharmacokinetic interaction may play a role. Interestingly, we previously observed in MI-rats augmented inhibition of cardiac ACE activity by zofenopril during dietary sodium restriction, whereas dietary sodium intake in itself had no effect on ACE activity.\(^10\) Potentially, sodium restriction affected pharmacokinetics of the ACE-I, for instance by affecting its renal clearance. Accordingly, we studied the influence of hydrochlorothiazide (HCTZ) on steady-state plasma, cardiac and renal tissue ACE-I drug levels and ACE activity after ACE-I therapy in an experimental setting of LV dysfunction after MI in rats.

Methods

Study design

The present study was performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. The animal research committee of the University of Groningen approved the study protocol. Male Sprague Dawley rats were subjected to coronary artery ligation (n=163) or sham operation (n=9), as described before.\(^11\)

MI-rats were randomly allocated to one of five experimental groups. One group of MI-rats, as well as the group of sham-operated rats, received no active treatment (i.e. untreated control groups). The other four groups of MI-rats were allocated to one of four different treatment regimens, as summarized in figure 1. We chose to design a protocol with established HCTZ treatment preceding the start of ACE-I treatment. Thus, after a recovery period of one week, MI-rats were randomized to either HCTZ or control treatment. HCTZ was dissolved in the drinking water to achieve a final dosage of 50 mg/kg/day. We and others previously showed that in rats with MI this dose results in diuresis and RAAS activation without blood pressure reduction.\(^7,12\) As initiation of HCTZ affects water intake, HCTZ was initiated two weeks before ACE-I therapy, to ensure stable water intake and HCTZ dosing at the onset of ACE inhibition.
Against this background of established HCTZ-treatment, ACE-I treatment with either lisinopril or zofenopril was started at day 21, i.e. 3 weeks after induction of MI. The treatment regimen of zofenopril (10 mg/kg/day) and lisinopril (3.3 mg/kg/day) was based on previous experiments in our laboratory. The dose of lisinopril was chosen relative to zofenopril based on the clinical defined daily doses for both ACE-I (10 and 30 mg, for lisinopril and zofenopril, respectively; ATC index, 1998). Until day 26, a steady-state of both ACE-I was achieved by dissolving appropriate amounts of drug in the drinking water. Steady-state is achieved after 4-5 half-lives, which is in total 20-35 hours for zofenopril and and 48-60 hours for lisinopril. The required amounts of food and water were determined as guided by measurements of body weight and daily water intake. This was done by measuring water intake and average body weight per cage weekly, and calculating the required drug concentration in the drinking water (per cage) for the week after.

On days 27 and 28 of the study protocol, the ACE-I as well as HCTZ was administered by means of oral gavage (total volume of 1 µl/g body weight nitrocellulose containing the drugs, or without the drugs in case of untreated controls) as to ensure a synchronized, accurate drug intake in all rats. During the last night before sacrifice, all rats were fasted.

Harvesting of tissues and plasma
At the day of sacrifice, rats were terminated exactly at $T_{\text{max}}$ after administration of zofenopril (0.5 hours) or lisinopril (4 hours). $T_{\text{max}}$ was found to be unaffected by HCTZ,
both for zofenoprilat (unpublished studies at Menarini Ricerche, Firenze) and 
loratadine. Rats were anesthetized with isoflurane (2.0-2.5%), heparin (1000 IE) was injected into 
the tail vein for anticoagulation, arterial blood was drawn from the abdominal aorta and 
collected in separate tubes for analysis of plasma ACE activity and ACE-I drug levels; 
one mL of arterial blood was mixed with N-ethylmaleimide (5mg/mL) to prevent 
oxidation of zofenoprilat. Tubes were immediately centrifuged at 1600G for 10 minutes 
at 4° C. Subsequently, plasma was frozen in liquid nitrogen, and stored at -80° C until 
assay.

After blood collection, rats were perfused with saline to remove remnant blood, as to 
avoid contribution from the blood compartment in assessment of tissue drug levels and 
tissue ACE activity. To this end, 10 mL cold 0.9% NaCl solution was gently injected into 
the aorta, and a hole was pinched into the vena cava inferior to let out the rinsing 
solution. Thereafter, organs were quickly removed, weighed, divided and frozen in 
liquid nitrogen for measurement of either tissue concentrations of 
loratadine/zofenoprilat or lisinopril, or tissue ACE activity. For measurement of renal 
drug levels and ACE activity, small pieces of cortical tissue were used. In case of cardiac 
measurements, both ACE activity and drug concentrations were measured in small 
pieces of non-infarcted free LV wall. Moreover, a mid-ventricular slice of the LV was 
stored in 2% paraformaldehyde for histological assessment of infarct size using 
planimetry on Sirius Red/ Fast Green-stained slides as described previously11. Only rats 
with MI sizes comprising over 20% of the LV were included for analysis.

Drug measurements
Before shipment, tissues were homogenized on dry ice in a 0.5 M K2HPO4 buffer 
containing 5 mg/mL N-ethylmaleimide to prevent oxidation of zofenoprilat. For 
measurement of lisinopril concentrations, homogenates were shipped on dry ice to 
Analytical Laboratories R&D, Berlin-ChemieLab (Berlin, Germany) for HPLC analysis. 
The determination of lisinopril and internal standard (Enalapril-Diketopiperazin DKP) 
in rat plasma was performed by means of a validated HPLC-MS/MS analytical method. 
The lower limit of quantification (LOQ) for lisinopril was 2.0 ng/mL of rat plasma 
The lisinopril and the internal standard were extracted from rat plasma by a solid-phase 
extration method. Briefly, after addition of 0.2 N HCl to the samples, the samples were 
vortexed and transferred into a solid phase extration cartridge (Waters Oasis 30 mg), 
that had been conditioned with 1 mL methanol and 1 mL HPLC water. After loading, 
the cartridge was washed with 1 mL water and 1 mL methanol/ water (5:95; v/v). 
Subsequently, both drugs were eluated with 3 x 1 mL methanol. The methanolic eluates 
were evaporated to dryness; the residue was redissolved in mobile phase and injected 
into the chromatographic system.

The HPLC-MS/MS system consisted of a model 200 solvent delivery pump (Perkin 
Elmer) equipped with an autosampler Gilson 234, a column oven Agilent (G1316A), an 
HPLC-column (Symmetry Shield RP-8, 2.1 x 150 mmn and 5 μm particle size (Waters)), 
an HPLC precolumn (Symmetry Shield RP-8, 2.1 x 10 mmn and 3.5 μm particle size
For measurement of zofenopril and zofenoprilat samples were shipped on dry ice to the research lab of Menarini Richerche (Roma, Italy). The assay was performed by liquid chromatography coupled with tandem mass spectrometry as described previously. Briefly, analytes were extracted by liquid-liquid extraction with toluene. The organic phase was separated, dried, reconstituted with 200 µL of a methanol/water mixture (1:1), and injected through an autosampler. The extract was chromatographed on a reverse phase column coupled to a triple quadruple mass spectrometer.

ACE activity
ACE activity in the plasma and spared myocardial tissue was determined according to the Hip-His-Leu method, as has been described before. In short, tissues were homogenized in a 50 mM KPO₄ buffer. Of the homogenates 100 µl was pipetted in a 0.5 M K₂PO₄ buffer. Then the ACE substrate Hippuryl-His-Leu 12.5 nM (Sigma) was added and incubated at 37°C for exactly 10 minutes. The conversion of the substrate was stopped by adding 1.45 ml 280 mM NaOH. Thereafter, 100 µl phtaldialdehyde was added for the labeling of free His-Leu. The amount of labeled His-Leu was fluorimetrically determined at excitation and emission wavelengths of 364 and 486 nm, respectively. Control samples were included in which the conversion of substrate was prevented by adding NaOH before the substrate Hippuryl-His-Leu.

Statistical analysis
For comparison of zofenopril and lisinopril effects in the infarct model, ANOVA with post hoc least square difference correction was performed. For analysis of drug levels in MI rats, group averages of HCTZ+ACE-I were compared to ACE-I alone with a student’s t-test in case of normal distribution. If distribution was not normal, log transformation was used to achieve normality. If log transformation did not result in a normal distribution, a non-parametric Mann-Whitney test was used. Levels of zofenoprilat in some of the LV and kidney tissue samples were undetectably low. In case of detection problems, zofenoprilat levels were given the value of the detection limit in statistical analysis, to be sure not to overestimate any difference.

Results
General characteristics
Mortality during the first 24 hours was 33% after MI induction; during the rest of the follow-up period, none of the rats died. None of the rats died after sham operation. After exclusion of data from rats with infarcts smaller than 20% of the LV circumference, infarct sizes were comparable in the MI groups, except for a small, yet significant difference between rats with lisinopril + HCTZ and lisinopril only (table 1). Both zofenopril and lisinopril significantly reduced body weight gain, as compared to untreated MI-rats. HCTZ further reduced body weight gain, in both zofenopril- and lisinopril-treated rats (table 1).
Table 1. Effects of experimental myocardial infarction and of 10 mg/kg/day zofenopril and 3.3 mg/kg/day lisinopril on LV hypertrophy.

<table>
<thead>
<tr>
<th></th>
<th>No treatment</th>
<th>MI - Lisinopril</th>
<th>MI - Zofenopril</th>
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<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>Vehicle</td>
<td>HCTZ</td>
</tr>
<tr>
<td></td>
<td>MI</td>
<td>HCTZ</td>
<td></td>
</tr>
<tr>
<td>N=</td>
<td>9</td>
<td>14</td>
<td>15</td>
</tr>
<tr>
<td>BW (g)</td>
<td>358 ± 6</td>
<td>336 ± 6 *</td>
<td>317 ± 3 **</td>
</tr>
<tr>
<td>Δ BW (g)</td>
<td>21 ± 5</td>
<td>15 ± 2 †</td>
<td>2 ± 1 †</td>
</tr>
<tr>
<td>Infarct size (%)</td>
<td>-</td>
<td>32 ± 2 †</td>
<td>29 ± 2</td>
</tr>
<tr>
<td>Plasma K⁺</td>
<td>3.8±0.1</td>
<td>4.4±0.1 *</td>
<td>3.9±0.1 †</td>
</tr>
<tr>
<td>LV:BW (mg/g)</td>
<td>2.4 ± 0.1</td>
<td>2.9 ± 0.1 *</td>
<td>2.5 ± 0.0 †</td>
</tr>
</tbody>
</table>

Data are shown as mean±sem. The Δ-BW is the difference in bodyweight (g) between time of operation and sacrifice, giving an indication of treatment on body weight development. Infarct size is expressed as percentage of infarct-to-total left ventricular circumference. * p<0.05 versus Sham; † p<0.05 versus MI, ‡ p<0.05 versus MI+lisinopril; § p<0.05 versus MI+zofenopril. HCTZ; hydrochlorothiazide. LV:BW; left ventricle-to-body weight ratio.

Both lisinopril and zofenopril treatment alone caused a moderate, but highly significant increase in plasma K⁺ concentrations. HCTZ treatment did not alter this increase in lisinopril-treated rats, whereas HCTZ in zofenopril-treated rats normalized plasma K⁺ concentrations.

MI resulted in LV hypertrophy, as indicated by increased LV/BW ratios in untreated MI rats compared to sham-operated rats. Zofenopril and lisinopril treatment similarly reduced LV/BW ratios, without additional effects of HCTZ (table 1).

Drug concentrations

Plasma lisinopril concentrations were significantly higher (60%, p=0.03) in rats instituted with HCTZ as compared to those not, whereas lisinopril concentrations in cardiac and renal tissue were not different (figure 2a).

Plasma levels of the prodrug zofenopril were very low (approximately 4.5% of total plasma zofenopril plus zofenoprilat levels) compared to the plasma levels of zofenoprilat, but neither plasma zofenopril (152±14 vs. 125±15 ng/mL, p=ns) nor plasma zofenoprilat levels (figure 2b) differed between rats treated with HCTZ and vehicle, respectively. Contrary to lisinopril, however, tissue levels of the active metabolite zofenoprilat were significantly higher in rats treated with HCTZ as compared to those not; 3-fold and 20-fold increases in median values for LV and renal tissue, respectively (figure 2c). Finally, the prodrug zofenopril could not be detected in these tissues.
To assess whether alterations in drug levels could be explained by altered renal drug clearance, kidney function was determined, as measured by plasma creatinine concentrations. Myocardial infarction alone did not alter plasma creatinine levels. Lisinopril alone caused a moderate increase in plasma creatinine, and treatment with HCTZ caused a significant further increase (figure 3). Treatment with zofenopril alone did not significantly increase plasma creatinine. Zofenopril treatment in rats treated...
with HCTZ caused a moderate increase in plasma creatinine (p<0.05 versus untreated sham- and MI-rats).
The HCTZ-induced increase in plasma lisinopril was related to a decrease in renal function, as is shown by the highly significant correlation between plasma creatinine and lisinopril concentrations (figure 4A). Tissue concentrations of lisinopril were positively correlated with plasma creatinine as well (figure 4C). Trends were similar for LV and kidney. Plasma concentrations of zofenoprilat were not significantly associated with creatinine (figure 4B). However kidney as well as LV concentrations of zofenoprilat were significantly correlated with plasma creatinine (figure 4D).

ACE activity
Both ACE inhibitors caused a nearly complete reduction (±90%) in plasma ACE activity compared to untreated MI- and sham-rats, and this effect did not differ between rats treated with or without HCTZ (figure 5).
Renal ACE activity tended to be slightly decreased in MI-rats compared to sham operated rats. ACE-I treatment further decreased ACE activity in the kidneys, and consequently all ACE-I treated groups displayed significantly decreased renal ACE activity compared to the group of untreated sham-rats. As all ACE-I treated groups displayed very low renal ACE activity no differences were observed between MI-rats treated with or without HCTZ. LV ACE activity was significantly increased in MI-rats compared to untreated sham-controls, but both ACE-I failed to reduce LV ACE activity, regardless whether or not rats were treated with HCTZ (figure 5).
No significant correlation between ACE-I drug levels (neither zofenoprilat nor lisinopril) and ACE activity was observed in plasma or tissue.
Figure 4. Relation between kidney function and plasma ACE-I concentrations in rats with myocardial infarction, either or not treated with hydrochlorothiazide. HCTZ; hydrochlorothiazide. A) Lisinopril concentrations in plasma were highly correlated with plasma creatinine concentrations. B) Zofenoprilat and creatinine concentrations in plasma did not correlate significantly. C) and D) Correlation between plasma creatinine and (kidney) tissue concentrations of zofenoprilat.
Figure 5. ACE activity in plasma, left ventricle, and kidney in rats with myocardial infarction, and effects of the ACE inhibitors zofenopril and lisinopril, alone or combined with hydrochlorothiazide. Data are shown in boxplots in case data were not normally distributed. HCTZ; hydrochlorothiazide. *p<0.05 versus sham; †p<0.05 versus MI control.
Discussion

Aim of the present study was to investigate the effects of hydrochlorothiazide (HCTZ) on steady-state plasma, cardiac- and renal tissue ACE-I drug levels and ACE activity after ACE-I therapy in an experimental setting of LV dysfunction after MI in rats.

ACE-I accumulation by diuretic treatment
Lisinopril, the lysine derivate of enalaprilat, is highly hydrophilic, does not require metabolic transformation to become active, and is cleared unchanged via the kidneys. We found that plasma drug levels of this ACE-I were higher in rats treated with HCTZ. This was explained by decreased renal clearance of lisinopril, as plasma concentrations of lisinopril showed a highly significant relation with plasma creatinine, a measure for glomerular filtration rate (GFR). Most likely, volume depletion accounted for this decrease in GFR, thereby resulting in drug accumulation. Others have reported no or marginal interactions in terms of clearance between HCTZ and lisinopril, but note that these were all studies testing effects of HCTZ on plasma lisinopril concentrations after one single dose. To our knowledge, this study is the first to address the effects of HCTZ on steady-state ACE-I levels in a setting of repeated drug dosing.

Infarct sizes were significantly different between lisinopril- and lisinopril + HCTZ-treated rats. However this difference in infarct size between the two lisinopril groups does not interfere with our findings, as we found no relation between infarct size and ACE-I concentrations. We also tested effects of HCTZ on ACE-I concentrations in sham-operated rats (data not shown for reasons of clarity), and results were very similar, confirming that presence of myocardial infarction did not play a role in the observed effects of HCTZ.

To compare ACE inhibitors with different kinetic properties, we also investigated the effects of HCTZ on zofenopril/zofenoprilat drug levels. Zofenopril is a lipophilic ACE-I prodrug which undergoes hydrolyzation to its active metabolite zofenoprilat in tissue. Plasma prodrug levels represented only 4.5% of the total circulating levels of zofenopril plus zofenoprilat in the present study, indicating a near total conversion of zofenopril into its active metabolite. Zofenoprilat is hydrophobic, and cleared predominantly via the kidneys (±70%), but also via the bile and feces.

In contrast to lisinopril, plasma zofenoprilat levels were only marginally increased by HCTZ, whereas tissue zofenoprilat levels increased markedly, indicating ACE-I drug accumulation in the tissue. These findings may be related to volume depletion by addition of HCTZ to zofenopril treatment. Rather than leading to increased plasma levels, the lipophilic nature of zofenopril would favor drug penetration of the ACE-I into the tissue, with a subsequent tissue accumulation of the active metabolite zofenoprilat. Note that we observed a significant correlation between plasma creatinine and tissue zofenoprilat concentrations, potentially as both depend on volume status. Surprisingly, zofenoprilat concentrations in plasma did not increase significantly along with tissue concentrations during HCTZ treatment. Thus, mechanisms independent of volume status may be involved as well in the observed accumulation of zofenoprilat in renal and cardiac tissue.
Relevance/implications of increased drug levels.

Increased ACE inhibitor levels were not associated with a significant reduction in ACE activity in the current study. In case of plasma and kidney, this may be explained by maximal inhibition as a result of high dosing: both zofenopril and lisinopril caused nearly complete inhibition of circulating and renal ACE. For cardiac ACE activity, we observed no effect of zofenopril or lisinopril, and also no effects of HCTZ treatment. This finding is consistent with previous studies employing the rat coronary ligation model of LV dysfunction post-MI. An explanation may be upregulation of cardiac ACE expression under ACE inhibition, although this has not unambiguously been shown for cardiac tissue.

It has long been recognized that the local rather than the circulating RAAS is involved in pathophysiology of cardiovascular disease. Thus, increasing the tissue penetration of the ACE-I may potentiate its beneficial effects. Although we found no further reduction of ACE activity, the HCTZ-induced increase in tissue zofenoprilat levels may have local beneficial effects independent from ACE inhibition per se. Firstly, increased levels of tissue ACE-I could lead to further reduction in oxidative stress. Zofenoprilat contains a sulphydryl-group, which may scavenge reactive oxygen species, thereby reducing inflammation and increasing NO bioavailability.

Secondly, ACE-inhibitors have zinc-chelating properties, which may interfere with cardiac remodeling via reduction of matrix metalloproteinase activity.

Renal effects HCTZ on lisinopril versus zofenopril

We used equipotent dosages of ACE inhibitors, based on previous results, and the ratio between clinically used dosages. Dose equivalence was illustrated by similar reductions in LV hypertrophy and ACE activity, and a comparable rise in serum K⁺. Notably, effects of HCTZ treatment on zofenopril and lisinopril were differential, substantiating the view that not all ACE inhibitors are by definition interchangeable within their class.

Firstly, we observed differential effects of HCTZ on lisinopril and zofenoprilat concentrations, as discussed above. Furthermore, we observed that HCTZ decreased (i.e. normalized) plasma K⁺ levels in zofenopril- but not lisinopril-treated rats. We have no explanation for this finding, but as plasma electrolyte disturbances are of great importance in patients with heart failure, this matter deserves further study.

Combining HCTZ with lisinopril increased plasma creatinine concentrations. Although plasma creatinine may not be the most accurate indicator of renal function, it corresponds with a substantial drop in glomerular filtration rate GFR by 25% compared to lisinopril monotherapy and 50% compared to untreated rats. Importantly, decreased renal function by combining RAAS inhibition with diuretic treatment has also been reported in humans. This effect of combining ACE inhibition with HCTZ treatment could have clinical implications, as even mildly impaired renal function is strongly and independently associated with worsened prognosis after MI.
Conclusion
In the present study diuretic treatment with HCTZ significantly influenced steady-state plasma and tissue ACE-I drug levels in rats with experimental MI. The effect of HCTZ differed for the different ACE-I employed, resulting in increased ACE-I drug levels in the plasma in case of the hydrophilic ACE-I lisinopril versus increased ACE-I drug levels in renal and cardiac tissue in case of the lipophilic ACE-I zofenopril. Increased tissue ACE-I drug levels may contribute to the enhanced organ-protective effects of ACE-I therapy. However, decreased renal function by combining HCTZ with ACE inhibition may have adverse effects.

References

