Effects of Angiotensinase Inhibitors on Plasma Protein Binding and IC$_{50}$ Determinations of Renin Inhibitors


To establish whether the use of proteinase inhibitors in the routine determination of in vitro plasma renin activity overestimates the potency of renin inhibitors in vivo, we examined the effects of phenylmethylsulfonyl fluoride and 8-hydroxyquinoline sulfate on the binding to plasma proteins and the respective IC$_{50}$ values (50% inhibiting concentrations) of three renin inhibitors. All three renin inhibitors, A-64662, A-65317, and A-74273, bound (>60%) to plasma proteins at both pH 6.0 and 7.4, with slightly greater binding at pH 7.4. Phenylmethylsulfonyl fluoride (1.45 mmol/L) had no significant effect on the protein binding at either pH 6.0 or 7.4; 8-hydroxyquinoline sulfate (3.4 mmol/L) caused a modest dissociation (10–30%) of the renin inhibitors from plasma proteins at both pH values; and the effects of both proteinase inhibitors together were similar to those of 8-hydroxyquinoline alone. At pH 7.4, phenylmethylsulfonyl fluoride increased the potencies of the three renin inhibitors slightly (<43%), whereas IC$_{50}$ values determined in the presence of 8-hydroxyquinoline decreased by 1.5- to 3.7-fold. The greatest increase in potency occurred with the most hydrophilic compound, and with both angiotensinase inhibitors the effect was no greater than that of 8-hydroxyquinoline alone. The results show that any dissociation of the hypotensive activity measured in vivo from the plasma renin activity measured in vitro is not simply an artifact in the plasma renin activity assay stemming from the use of these angiotensinase inhibitors, especially if only phenylmethylsulfonyl fluoride is used.

Additional Keyphrases: renin · blood pressure · variation, source of

In the search for a specific target for the rationally designed blockade of the renin-angiotensin system (RAS), the aspartic proteinase renin would seem the perfect candidate. In its role as the first and rate-limiting step in the RAS, renin acts on a single, unique substrate—angiotensinogen—to produce angiotensin-I (ANG-I). This product has no potent bioactivity itself, but serves as the precursor for the production of the highly potent vasoconstrictor angiotensin-II (ANG-II) through the action of the multifunctional enzyme kinase II, or angiotensin-converting enzyme (ACE). ACE, the first target of successful, rationally designed, orally active inhibitors of the RAS, cleaves several biomolecules in addition to ANG-I, including bradykinin and substance P (1). This characteristic of ACE may enhance the efficacy of its inhibitors in the treatment of hypertension and congestive heart failure, but may also cause some of the side effects associated with its inhibitors (2). Thus, the development of potent, bioavailable renin inhibitors (RIs) may be anticipated to result in a specific antihypertensive therapy that would have minimal potential for side effects.

The most widely used method for quantifying RI potency and the inhibition of plasma renin activity (PRA) is the enzyme kinetic assay coupled with radioimmunoassay (RIA). The antigen detected in the RIA is the renin-cleavage product ANG-I; however, because plasma contains endogenous proteolytic enzymes that can degrade ANG-I (angiotensinases), as well as ACE, proteinase inhibitors are routinely added during the ANG-I-generating enzyme kinetic assay.

When in vivo studies with RIs began, an observation was made that has not been adequately explained: upon in vivo administration of RIs, the decrease in blood pressure (BP) did not correlate fully with the inhibition of the plasma renin activity measured in vitro. In fact, the decrease in PRA was induced by subdepressor doses of RIs. Furthermore, the BP continued to decrease with higher doses of RI, even after PRA had been completely inhibited (3). This dissociation of BP from PRA is seen in many different mammalian models with the use of various peptidic RIs (4–10).

One possible explanation for the lack of correlation between the inhibition of PRA and the hypotensive response involves the binding of RIs to plasma proteins (11) and their subsequent displacement from these proteins during the in vitro measurement of PRA (10, 12–17). If such displacement occurred, the PRA measured in vitro would be less than is physiologically present in vivo during RI treatment. Clinical doses predicted from these artificially lowered PRA values would elicit a weaker hypotensive response in vivo, requiring higher doses of drug to attain the same degree of PRA inhibition observed in vitro.

The logical candidates for compounds that might alter potential RI–plasma protein(s) interactions would be the angiotensinase inhibitors added in the PRA to preserve ANG-I. The proteinase inhibitors commonly


1Nonstandard abbreviations: ACE, angiotensin-converting enzyme (kinase II); ANG-I or -II, angiotensin-I or -II; BP, blood pressure; 8-OHQ, 8-hydroxyquinoline sulfate; PMSF, phenylmethylsulfonyl fluoride; PRA, plasma renin activity; RAS, renin–angiotensin system; and RI, renin inhibitor.

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used in the enzyme kinetic assay include EDTA, an ACE inhibitor; phenylmethylsulfonyl fluoride (PMSF), a serine protease inhibitor; and 8-hydroxyquinoline sulfate (8-OHQ), a metalloprotease inhibitor. For this reason, several groups have used an antibody trapping assay (18) to measure PRA. For this assay, no angiotensinase inhibitors are used other than EDTA, which improves the correlation (8, 10, 12, 14, 19). Because EDTA must be present in any current PRA assay to inhibit ACE, we examined the effects of PMSF and 8-OHQ on the interactions of three of our peptidic RIs with plasma protein at pH 6.0 and 7.4 (the usual pH values for PRA assays). We also determined the effects of the two angiotensinase inhibitors on the potency of the three RIs, as measured by IC$_{50}$ values (concentrations inhibiting PRA by 50%).

**Materials and Methods**

**Materials**

Figure 1 shows the structures of the three RIs used. Each compound is a peptidomimetic of the Phe-His-Leu triplet in positions P$_3$ to P$_1$ of the renin cleavage site of angiotensinogen. When used in the plasma binding studies, these compounds were labeled with $^{14}$C at the positions indicated to the following specific activities: A-64662, 1.07 Ci/mol; A-65317 and A-74273, 50 Ci/mol.

Human plasma was obtained from donors given 50 mg of furosemide 4 h before donation. The plasma was filtered through glass wool and adjusted to pH 7.0 with ice-cold 0.3 mol/L HCl immediately before use.

PMSF was obtained from Calbiochem Corp. (La Jolla, CA), 8-OHQ and EDTA from Sigma Chemical Co. (St. Louis, MO). All other reagents used were of reagent-grade quality or better.

**Plasma Binding Studies**

The percentage of RI bound to plasma proteins during a routine, 2-h PRA assay was determined by dialysis. Drug binding was investigated at pH 6.0 and 7.4. The same procedure was followed at these two pH values, except for the use of 0.05 mol/L maleic acid or 0.1 mol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid to buffer the plasma samples at pH 6.0 or 7.4, respectively. Buffered plasma (87.0% ± 0.7% of total sample volume) containing 3 mmol/L EDTA, and either 1.46 mmol/L PMSF (in ethanol), 3.4 mmol/L 8-OHQ, or both, was added to the top half-cell of a 1-mL equilibrium dialysis chamber, while buffered isotonic saline (87.7% ± 0.7% of total sample volume; Abbott Hospital Products, Abbott Park, IL) containing the same combination of protease inhibitors was added to the bottom half-cell. The pH of the plasma and saline samples was adjusted to the value required, with microliters of 1 mol/L NaOH or small amounts of solid maleic acid; in addition, the pH was monitored at the start of experiment after the addition of the angiotensinase inhibitors, and at the end of the 2-h incubation to guarantee that the pH was maintained at 6.0 or 7.4. One of the three RIs (in ethanol) was then added to the top half-cell to give a concentration of 3.1 ± 0.2 μmol/L; the volume of drug added never exceeded 0.5% of the total sample volume, so the total proportion of ethanol in the system was always <20 mL/L. We added the same volume of ethanol to the bottom half-cell. The dialysis chamber was separated by a sheet of cuprophane type "C" dialysis membrane (Technicon Instruments Corp., Tarrytown, NY) with a molecular mass cutoff of ~10 000 Da. The dialysis cells were placed horizontally in a rotary water bath shaker (New Brunswick Scientific Co., Inc., Edison, NJ) equilibrated at 37°C. After 2 h of shaking, 750-μL portions were removed from the top and bottom half-cells and diluted into scintillation cocktail (Insta-Gel; Packard Instrument Co., Inc., Meriden, CT), and radioactivity was measured for 10 min in a scintillation counter (Beckman Instruments, Inc., Fullerton, CA) to determine the total counts remaining in each half-cell. Separate experiments demonstrated that equilibrium had been established by this time. The percentage of each RI bound to plasma was then calculated as 100 x the difference between 1.0 and the ratio of counts in the saline divided by the counts remaining in the plasma. Each measurement was made in duplicate, and the mean value ± SD was reported.

**Assay of PRA for the Determination of IC$_{50}$**

The measurement of PRA in the presence of the RIs and different combinations of the angiotensinase inhibi-
Effects on IC₅₀ Values

The effects of the angiotensinase inhibitors on the IC₅₀ values of the three RIs, as determined by RIA, followed the same general trends as their effects on plasma binding (Table 2). PMSF caused no change in the IC₅₀ of A-74273, whereas the potency of A-64662 increased by 3.7-fold. As in the plasma protein binding studies, the combination of both PMSF and 8-OHQ in the assay exhibited no further effect on renin inhibitory potency (within experimental error) than did 8-OHQ alone. None of the RIs cross-reacted with the ANG-I antibody used in the RIA at the concentrations of the RIs used in the IC₅₀ determinations.

The potencies of the three RIs in plasma were determined at both pH 6.0 and 7.4 by using our standard PRA protocols [EDTA and PMSF only (23, 24)]. The effects of the angiotensinase inhibitors on IC₅₀ values in plasma at pH 6.0 were not examined because all our IC₅₀ values in plasma are conducted at the physiologically relevant pH of 7.4. These results are given in Table 3 and demonstrate the profound effect that pH control has on IC₅₀ determination.

Table 3 shows the angiotensinase-protective effects of PMSF and 8-OHQ on ANG-I during the PRA assay at pH 7.4. All samples, including the control, contained 3 mmol/L EDTA to inhibit ACE; otherwise, very little ANG-I would have been detectable at all. EDTA is also

<table>
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<tr>
<th>Table 1. Effects of Angiotensinase Inhibitors (Mean ± SD) on the Binding of Three Renin Inhibitors to Plasma Proteins</th>
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<tbody>
<tr>
<td>Renin Inhibitor</td>
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<tr>
<td>-----------------</td>
</tr>
<tr>
<td>A-64662</td>
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<tr>
<td></td>
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<tr>
<td>A-65317</td>
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<tr>
<td>A-74273</td>
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<td>* Percent change = (% drug bound, control) – (% drug bound with angiotensinase inhibitor).</td>
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Results

Plasma Binding Studies

As shown in Table 1, all three of the RIs examined bound appreciably to plasma proteins at pH 6.0 and 7.4, with the percentage of bound drug ranging from 61% to 93%. PMSF had a negligible effect on the binding of any of the three RIs at pH 6.0, and no effect at pH 7.4. In the presence of 8-OHQ, however, some dissociation of the RIs from plasma proteins was observed. The percentage of drug not bound to plasma proteins (i.e., free) increased slightly with pH for all three RIs; A-74273 was least affected at both pH values, whereas the amount of free A-64662 and A-65317 increased 20–23% at pH 6.0, and 27–33% at pH 7.4. The addition of both angiotensinase inhibitors had no further effect on RI binding than did the addition of 8-OHQ alone.

Table 2. Effects of Angiotensinase Inhibitors on IC₅₀ Values of Three Renin Inhibitors in Plasma, pH 7.4

<table>
<thead>
<tr>
<th>Renin Inhibitor</th>
<th>IC₅₀ nmol/L</th>
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<tr>
<td>A-64662</td>
<td>2.3 ± 0.2</td>
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<tr>
<td>A-65317</td>
<td>0.68 ± 0.03</td>
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<tr>
<td>A-74273</td>
<td>16.0 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>9.2 ± 2.1</td>
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<tr>
<td></td>
<td>2.3 ± 0.4</td>
</tr>
<tr>
<td>+PMSF</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td>+8-OHQ</td>
<td>0.35</td>
</tr>
<tr>
<td>+Both</td>
<td>4.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>0.53 ± 0.10</td>
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<tr>
<td></td>
<td>3.5 ± 0.1</td>
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</table>

CLINICAL CHEMISTRY, Vol. 38, No. 11, 1992 2241
routinely used in the PRA trapping assay for the same reason (18). Little to no additional inhibition of ANG-I degradation is afforded by 8-OHQ, either alone or in combination with PMSF. PMSF, in contrast, increased the amount of ANG-I detected by ~20%. These results are given for human plasma samples only; the use of 8-OHQ in the determination of animal PRA values may be warranted.

Discussion

Among the proposed explanations for the dissociation of the inhibition of PRA in vitro from the in vivo hypotensive effect of RIs (3-10) is one implicating the binding of these inhibitors to plasma proteins (11) and their subsequent displacement during the plasma renin activity assay (12, 19). Recently, Derkx et al. (19) reported that the angiotensinase inhibitors PMSF and 8-OHQ caused a decrease of 2.3- to 14-fold in the IC50 values of four RIs from three different pharmaceutical companies (19), with 8-OHQ causing the greatest displacement of bound RI. Although the general trends in their data corroborate those reported here, their data were obtained in nonacified, unbuffered plasma, pH 7.4 ± 0.4—a range over which renin activity itself can change more than twofold (25, 26). In addition, when the IC50 and plasma binding data were determined for one of the RIs in plasma buffered at pH 6.0, no effect was seen on either index in the presence of any combination of the angiotensinase inhibitors (19). Therefore, the effects of angiotensinase inhibitors on the binding of RIs to plasma proteins and on PRA measurements will depend on experimental conditions.

To control the maintenance of pH, and because the binding of RIs to plasma proteins would be expected to be highly structure-dependent, we investigated the plasma protein binding affinities and renin inhibitory potencies of three RIs in the presence and absence of the same angiotensinase inhibitors. The relatively dramatic decrease in the IC50 of A-64662 in the presence of 8-OHQ (Table 2) was not solely a function of binding to plasma proteins, because A-74273 bound just as avidly to plasma proteins (Table 1), but the IC50 value of A-74273 was only minimally affected by 8-OHQ. Although A-64662 is significantly more hydrophilic than are the other two RIs tested (as judged by log P values; unpublished data), A-74273 is the only completely nonpeptidic RI tested. These results suggest that the inclusion of 8-OHQ in human PRA determinations may overestimate the potencies of some RIs. The importance of pH control in the accurate and reproducible determination of PRA was stressed by Preibisz et al. (27) in their review of PRA measurements (27). From the pH profile of the renin-catalyzed cleavage of angiotensinogen (25, 26), it is clear that renin activity decreases by 40-60% between pH 6.0 and 7.4. The IC50 of A-64662 in plasma (no 8-OHQ present) increased by more than an order of magnitude over this same pH range (Table 3). If A-64662 were administered in vivo at a dose that would predict 50% inhibition of PRA at pH 7.4, but the in vitro determinations of PRA from treated animals were made at pH 6.0 (the optimum pH for renin activity), the PRA data would reflect total inhibition of renin activity, because of the 14-fold increase in potency of the drug at the pH of the assay. Indeed, the nonuniformity between the pH at which in vitro PRA and in vivo efficacy values are determined has been postulated as a potential source for the lack of correlated BP vs PRA data (13, 16).

Several researchers have suggested the use of the ANG-I trapping assay of Poulsen and Jørgensen (18) as an alternative to the more traditional enzyme kinetics assay for the determination of PRA, because the trapping assay correlated more closely than the standard PRA assay with plasma angiotensin concentrations and the hypotensive effects of the RIs (8, 10, 12, 14, 19). Examination of the methodologies used by these researchers to determine PRA shows that the conditions used might confound the interpretation of the results. First, some of the studies assayed PRA at pH 5.7, whereas the antibody trapping assay was performed at pH 7.4; results from both of these assays were then compared with the plasma ANG-II concentrations (8, 14). One might expect assays conducted at the same pH as that in vivo to correlate better with plasma ANG-II concentrations than data measured at the optimum pH for renin activity. In another study, PRA measurements were made at neutral pH but 2,3-dimercaptopropanol was used in the assay; this compound reportedly inhibits the activity of renin and interferes with antibody–antigen interactions, resulting in artifactual PRA determinations (26). In yet another study, PRA was determined at pH 7.5 in the presence of PMSF, 8-OHQ, and aprotinin (10)—all (except aprotinin) being suggested components for the determination of plasma renin activity at this pH (26); however, as this study has shown, 8-OHQ can cause a significant increase in the in vitro potency of certain RIs. If one were comparing PRA values determined with the antibody trapping assay or with the standard enzyme kinetic assay in the presence of 8-OHQ, the potency of the test compound would be overestimated in the presence of 8-OHQ. Consequently, one might observe a recovery in BP after RI-induced hypotension with a concomitant recovery in the PRA based on the trapping assay, whereas a more sluggish recovery in PRA would be seen with data obtained with the standard assay in the presence of 8-OHQ. From these results, one might conclude that the PRA trapping assay results more closely correlated with BP effects than did the standard PRA assay; however, the same conclusion would not have been reached in the absence of 8-OHQ. In fact, our results and those of others (18) have shown that the amount of ANG-I formed during a standard human PRA assay at pH 7.4 (in the absence of any renin inhibitors) is the same (within experimental error) with or without 8-OHQ (Table 4). Therefore, because 8-OHQ may result in the overestimation of RI potencies, we recommend its omission in human PRA determinations. Furthermore, we suggest a standardization of the conditions under which PRA determinations are performed. If PRA measurements were per-
formed routinely with adequate and appropriate buffers at pH 7.4 containing EDTA and PMSF, antibody trapping assays could more properly be compared with the standard PRA methodology. In addition, the comparison of PRA data, plasma angiotensin concentrations, and in vivo hypotensive effects of RIs could be made under similar conditions.

In conclusion, the lack of correlation between the state of the RAS, as measured by PRA, and BP reductions is not eliminated by the use of PRA trapping assay methodology (19, 28); however, any dissociation of BP effects from the degree of inhibition of PRA measured in vitro (pH 7.4, no 8-OHQ) with the three RIs studied here will not be due solely to the use of angiotensinase inhibitors in the determination of PRA.

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References
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