Pathways for Angiotensin II Generation in Intact Human Tissue
Evidence From Comparative Pharmacological Interruption of the Renin System

Norman K. Hollenberg, Naomi D.L. Fisher, Deborah A. Price

Abstract—Multiple lines of evidence have suggested that alternative pathways to the angiotensin-converting enzyme (ACE) exists for angiotensin II (Ang II) generation in the heart, large arteries, and the kidney. In vitro studies in intact tissues, homogenates, or membrane isolates from the heart and large arteries have repeatedly demonstrated such pathways, but the issue remains unresolved because the approaches used have not made it possible to extrapolate from the in vitro to the in vivo situation. For our in vivo model, we studied young and healthy human volunteers, for the most part white and male; when these subjects achieved balance on a low salt diet to activate the renin system, the response of renal perfusion to pharmacological interruption of the renin system was studied. With this approach, we studied the renal vasodilator response to 3 ACE inhibitors, 2 renin inhibitors, and 2 Ang II antagonists at the top of their respective dose-response relationships. When these studies were initiated, our premise was that a kinin-dependent mechanism contributed to the renal hemodynamic response to ACE inhibition; therefore, the renal vasodilator response to ACE inhibition would exceed the alternatives. To our surprise, both renin inhibitors and both Ang II antagonists that were studied induced a renal vasodilator response of 140 to 150 mL/min/1.73 m², ~50% larger than the maximal renal hemodynamic response to ACE inhibition, which was 90 to 100 mL/min/1.73 m². In light of the data from in vitro systems, our findings indicate that in the intact human kidney, virtually all Ang II generation is renin-dependent but at least 40% of Ang I is converted to Ang II by pathways other than ACE, presumably a chymase, although other enzyme pathways exist. Preliminary data indicate that the non-ACE pathway may be substantially larger in disease states such as diabetes mellitus. One implication of the studies is that at the tissue level, Ang II antagonists have much greater potential for blocking the renin-angiotensin system than does ACE inhibition—with implications for therapeutics. (Hypertension. 1998;32:387-392.)

Key Words: renin-angiotensin system ■ angiotensin II ■ angiotensin-converting enzyme

The renin-angiotensin-aldosterone system is one of the longest recognized hormonal systems, having been described about 100 years ago in remarkable detail.1 Few would argue about the role of pharmacological interruption of the renin-angiotensin system (RAS) in the rekindling of interest in the renin system over the past 2 decades.2 Fewer still would debate the contribution of angiotensin-converting enzyme (ACE) inhibition to therapeutics in processes ranging from hypertension to congestive heart failure, and from ventricular remodeling after myocardial infarction to diabetic nephropathy. The use of ACE inhibitors has provided unambiguous benefit, well beyond even the most enthusiastic imagining 2 decades ago.3

Despite this extraordinary record of success, there is an undeniable basic fact that emerges from pharmacological principles: ACE inhibition would not be the first or even the second choice of a pharmacologist prospectively planning a strategy to block the renin system. ACE inhibition was an unexpected product of snake venom toxicology and not of a systematic approach to blocking the renin system. Far more attractive choices would have been the interaction of renin with its substrate, which is the rate-limiting step,4 or equally attractive (perhaps more attractive if alternative pathways for angiotensin II [Ang II] generation exist), blockade at the final step of the cascade, at the level of the Ang II receptor. The purpose of this essay is to review evidence from multiple lines of investigation that indicates, in the aggregate, that alternative pathways to the formation of Ang II indeed exist and contribute substantially to its formation. Evidence for quantitatively important species variation in these pathways makes it necessary to pay special attention to evidence obtained from studies in humans.5

Evidence From Isolated Arteries

Among the early studies in this area, one stands out because of its title, an unambiguous declarative statement. In that
Ang I–induced contractile responses in human gastroepiploic arteries. Note the 40% inhibition of the response to Ang I induced by captopril (Capt) and the 80% to 90% blunting of the response to Ang I induced by chymostatin (Chym). The combination blocked the response completely. Arrowheads (A1) indicate application of Ang I. Reproduced with permission from Okunishi et al.5

Figure 1. Ang I-induced contractile responses in human gastroepiploic arteries. Note the 40% inhibition of the response to Ang I induced by captopril (Capt) and the 80% to 90% blunting of the response to Ang I induced by chymostatin (Chym). The combination blocked the response completely. Arrowheads (A1) indicate application of Ang I. Reproduced with permission from Okunishi et al.5

report, titled “Direct Evidence for the Presence of a Different Converting Enzyme in the Hamster Cheek Pouch,” Cornish et al8 found that vasoconstriction induced by Ang I in the blood vessels of the hamster cheek pouch was inhibited only partially by ACE inhibitors in high concentration, but it was completely inhibited by either an Ang II receptor antagonist or by an antiserum directed against Ang II. The character of the enzyme or enzymes responsible for conversion of Ang I to Ang II remained unclear.

Between 1984 and 1990, Okunishi and Toda with their coworkers (Okunishi et al5,8 and Okamura et al) described evidence from studies of blood vessels of humans, monkey, and dogs of a unique enzyme that converts Ang I to Ang II but differs from ACE. Their observation that this conversion was catalyzed by an enzyme that was inhibited by several serine protease inhibitors, including chymostatin, provided a clue as to the nature of the enzyme. In these studies, chymostatin in high concentration provided partial blockade of the conversion of Ang I to Ang II; captopril or other ACE inhibitors also provided partial inhibition, although somewhat less than that induced by chymostatin, and the combination of chymostatin and ACE inhibition led to total blockade of Ang II formation in primate and canine blood vessels.5 Their primary experimental end point was the contractile response of isolated blood vessels to Ang I in vitro (Figure 1).

These authors designated the newly found enzyme responsible for converting Ang I to Ang II as “CAGE,” an acronym obtained from the description “chymostatin-sensitive Ang II–generating enzyme.”5 Evidence was assembled that this enzyme represented a chymase derived from passenger mast cells located in the adventitia of the arterial segments studied in vitro, presumably a cellular passenger.10 These unambiguous facts led to one area of investigative concern: Was it likely that an enzyme derived from mast cells plays a role in normal physiology? As a second concern, in the in vitro experiment in which Ang I is injected into the tissue bath surrounding the artery, the resultant hormone concentrations in the adventitia at the antiluminal surface are as high as they are in the lumen, near the media where the contractile apparatus operates. In vivo, if Ang I is generated primarily in the circulation rather than locally, the Ang I concentration in the adventitial interstitium might be too low for CAGE to make an important functional contribution.

Even more fundamentally, in a series of reports during that same time interval, other investigators11–13 were unable to confirm the findings of Okunishi et al. Each study failed to demonstrate any evidence for the presence of non-ACE enzymatic pathways in the vasculature, as the responses to Ang I were completely abolished by ACE inhibition.11–13 In view of the simplicity and wide use of the preparations, it seemed unlikely that technical factors were responsible.

In a more recent report, Okunishi et al10 accounted for the differences in an elegant study that has raised crucial issues for future investigators. They noted that the studies that failed to confirm their original observations had all been performed with rat or rabbit blood vessels.11–13 Their follow-up study, which was designed to address the issues raised by that difference in study design, is once again well described in the title of their report, “Marked Species-Difference in the Vascular Angiotensin II–Forming Pathways: Humans Versus Rodents.” In isolated arteries, they demonstrated a marked difference in the pathways for Ang II formation between human, rat, and rabbit arteries. In human gastroepiploic arterial strips, treatment with captopril blocked only 30% to 40% of the conversion of Ang I to Ang II. Treatment with chymostatin blocked ≈60% of Ang II generation. A combination of captopril and chymostatin was required to produce 100% blockade (Figure 1). In rabbit arteries, on the other hand, captopril induced over 60% of Ang II formation. A combination of captopril and chymostatin was required to produce 100% blockade (Figure 1). In rabbit arteries, on the other hand, captopril induced over 90% inhibition, and chymostatin had little or no effect (Figure 2). One technical concern was that the smaller arteries from rabbits would suffer more endothelial damage or loss, but Okunishi et al provided both morphological and functional evidence for the integrity of endothelium in all of their preparations. They made the interesting speculation that their observation might account for the disturbing inability of ACE inhibitors to prevent the arterial response to injury in primates.14,15 Despite their ability to prevent neointimal hyperplasia in rat injury models,16,17 Should that speculation be correct, the therapeutic implications of the alternative pathways are obvious.
Evidence From Isolated Myocardial Preparations

Investigation in this field achieved new momentum with the identification by Urata et al.\(^{18}\) of a chymostatin-sensitive pathway for conversion of Ang I to Ang II in the human heart. Human myocardial chymase has since been isolated, cloned, and expressed.\(^{19}\) The efficiency of human heart chymase as an Ang II–forming enzyme exceeds that of ACE in kinetic studies.\(^{20}\) Studies on the distribution of chymase in human tissue have revealed a distribution involving not only the heart but also the lung, kidney, and blood vessels.\(^{21}\) Chymase-like immunoreactivity is localized in the cardiac interstitium and in several cell types, including cardiac mast cells and endothelial cells, which serve as sites of chymase biosynthesis and storage.\(^{22}\)

The original observation on a serine protease–inhibitable conversion of Ang I to Ang II in myocardial membrane preparations, a capacity that far exceeded Ang II production blocked by an ACE inhibitor,\(^{18}\) was not confirmed in some studies\(^{23}\) but was confirmed in others.\(^{24}\) An apparently satisfying explanation for the difference, involving techniques for tissue handling and membrane preparation,\(^{24}\) underscores the problem in this area. Although the studies on myocardial chymase in isolated membranes have been elegant, they provide no assurance that the observations apply to the intact system. To address that issue, alternative experimental approaches are required.

Hemodynamic Studies in Intact Canine Kidney

DiSalvo et al.\(^{25}\) showed that a converting enzyme inhibitor infused into the renal artery blocked the local action of Ang I but not of Ang II in dogs. Ang I therefore must require conversion to have a renal action, and that conversion must occur within the kidney. This observation, of course, documented the potential for production and not production itself, and it clearly did not indicate whether all of the Ang II that was formed came via ACE.

To assess the effects of Ang II generated by ACE-independent pathways on renal hemodynamics in the dog, Murakami et al.\(^{26}\) used a novel substrate: this artificial peptide, pro 11, d-ala 12,Ang I is inactive but yields Ang II on digestion by chymase but not by ACE.\(^{27}\) Renal vasoconstriction induced by infusion of either Ang I or Ang II into the renal artery was blocked by an Ang II antagonist. The renal hemodynamic response to the artificial substrate paralleled that for Ang I, indicating that in the in vivo system, chymase-like activity could contribute to Ang II production (Figure 3). This was supported by in vitro studies which revealed that the substantial Ang II–forming activity in the renal cortex was about 80% ACE-dependent and 20% chymase-like-dependent.\(^{26}\) These percentages differ radically from those in the heart, where chymase-like activity is the dominant action. Unfortunately, Murakami et al did not pursue comparative pharmacological studies on the renal blood supply. Consequently, we do not know whether the in vitro ratio of ACE and chymase-like activity applies in vivo.

Serine Protease–Dependent Ang II Formation During Exercise in Humans

Miura et al.\(^{28}\) used exercise to stimulate the RAS and used as their end point the relation of Ang I to Ang II in plasma, measured by radioimmunoassay. Captopril increased plasma Ang I concentration and reduced plasma Ang II concentration as anticipated (Figure 4). During exercise, a significant increase in Ang II was induced despite captopril treatment, but the rise was blunted. They also investigated the effects of a serine protease inhibitor, nafamostat, under the same conditions. The effect was almost complete obliteration of Ang II formation (Figure 4). Indeed, the blockade of conversion was so complete that the intriguing possibility that nafamostat blocks both ACE- and non-ACE–dependent serine protease pathways must be considered. Although it is unlikely that the investigators’ decision to use assays for immunoreactive Ang I and Ang II rather than the authentic molecule contributed to the finding, the very striking reduction in Ang II formation during nafamostat treatment suggests that fragments contributed to the very high Ang II levels during exercise in

\(^{\text{References}}\)
the other parts of the study. Nafamostat clearly requires more detailed investigation.

Studies on Intact Human Kidney: Evidence From Pharmacological Interruption

During the past decade, pharmaceutical science has provided an alternative approach to this problem with the development of renin inhibitors and novel Ang II antagonists that are free of partial agonist activity.29,30 Thus, the logic of our approach to exploring alternative pathways of Ang II formation was straightforward. If all of the Ang II acting on the intrarenal circulation was formed through the classic pathway, with Ang I conversion to Ang II occurring only in the transit of blood through the pulmonary circulation, one would anticipate that ACE inhibition, renin inhibition, and Ang II antagonists would induce an identical increase in renal plasma flow (RPF). To facilitate that comparison, we initiated studies in a familiar model, healthy young males who were in balance on a 10-mEq sodium intake daily to activate the renin system. We chose renin inhibition as the initial pathway for exploring the control of renal perfusion for several reasons. First, the remarkable substrate specificity of the renin reaction made mechanistic specificity of the renin inhibitor very likely. Second, the fact that both ACE and renin inhibition would lead to a fall in plasma Ang II concentration facilitated comparison of the degree of blockade achieved. Finally, the identification of multiple Ang II receptor subtypes29 added another layer of complexity to the interpretation of studies that used Ang II antagonists to interrupt the system.

The anticipated result in our first study was that the renal hemodynamic response to ACE inhibition under these circumstances would reflect not only a fall in local Ang II formation but also reduced kinin degradation. The result would be the accumulation of vasodilator products including bradykinin and kinin-dependent prostaglandin formation or activation of endothelial nitric oxide release. To our surprise, the renal vasodilator response to the renin inhibitor enalkiren was remarkable (Figure 5), exceeding expectations from our experience with ACE inhibitors.31 In a follow-up 3-arm study that compared placebo, captopril, and the same renin inhibitor (enalkiren), placebo did nothing and captopril and enalkiren both led to renal vasodilation.32 The response to enalkiren was larger than the response to captopril in 6 of 9 healthy subjects, confirming our earlier observation. These findings with enalkiren in 2 studies were supported by a third study that used zankiren as the renin inhibitor in the same model.33

Although renin is a fastidious enzyme with great substrate specificity, one possible interpretation of our findings was that the renin inhibitors acted via a mechanism unrelated to renin. Several lines of investigation make this unlikely. Ang II administration into the renal arteries in dogs after renin inhibition completely reversed the diuresis and natriuresis induced by the renin inhibitor.34 In accord is the observation in humans of blunting of the renal vascular response to renin inhibition by a high salt diet31 and in low renin hypertension32 and concordance in the primary renal vasodilator response to ACE and renin inhibition. Despite all of these considerations, the possibility existed that renin inhibition led to an overestimate of the contribution of the RAS to renal vascular tone because of a lack of specificity, reflecting an action unrelated to renin.
In this context, the development of the Ang II antagonist class created the possibility of a “tiebreaker.” If the renin inhibitor acted via an alternative non–angiotensin-dependent mechanism, one would anticipate that Ang II antagonists would provide a different renal vascular response under the conditions of our study. Conversely, if the renin inhibitor acted only through blockade of renin-dependent Ang II formation, one would anticipate an identical response to the renin inhibitor and Ang II antagonist. We have studied 2 Ang II antagonists in that model, eprosartan and irbesartan, and in each case have defined the relationship between Ang II antagonist dose and response. At the top of the dose-response relationship, both Ang II antagonists induced a response that slightly exceeded the response to renin inhibition (Figure 6).35,36

The most parsimonious interpretation of our finding—multiple renin inhibitors and Ang II antagonists induce an almost identical renal vascular response in humans that exceeds substantially the response to ACE inhibition—suggests that a renin-dependent but ACE-independent pathway for Ang II generation is involved. From the blood flow ratios, one can calculate that about two thirds of Ang II formation in the healthy human kidneys in which Ang II formation has been stimulated by a low salt diet occurs via the ACE pathway and about one third occurs via non–ACE-dependent pathways. Thus, the non–ACE-dependent pathway would exceed that for the dog kidney substantially25 but would be less than that in intact isolated human arteries.3 At the moment, in light of the studies reviewed in this essay, it is reasonable to attribute those responses to chymase or CAGE, a chymase-like enzyme.

Perhaps most important, these observations have implications for therapeutics. If Ang II is a toxin under some circumstances, the possibility that blocking the system by renin inhibition or Ang II antagonism will provide greater efficacy than ACE inhibition requires exploration. Moreover, our studies in diabetes raise the interesting possibility that blocking the system by renin inhibition or Ang II antagonism will provide greater efficacy than ACE inhibition requires exploration. More-


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