BASIC SCIENCE

Diverse factors influencing angiotensin metabolism during ACE inhibition: insights from molecular biology and genetic studies

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Reduction of angiotensin converting enzyme (ACE) activity can profoundly benefit a proportion of patients receiving cardiac care by improving cardiovascular function, reducing the risk of myocardial infarction, and extending life expectancy.^{1,2} The long term response to ACE inhibition, however, remains unpredictable in patients, and issues currently requiring attention are whether and how ACE inhibition might be improved such that a larger proportion of patients can be effectively treated.

Although plasma ACE activity can be essentially abolished pharmacologically, enzyme activity is not permanently reduced to negligible values in all human tissues.³ The existence of pathophysiologically important, patient specific differences in angiotensin II or bradykinin production during ACE inhibition might account, in part, for the outcome in different subjects.

New insights into the relevance of residual or altered angiotensin metabolism during treatment with ACE inhibitors might be close at hand following advances in the molecular biology of ACE, cardiac chymase, and other components of the renin-angiotensin and bradykinin systems.

Current evidence indicates that activation of the plasma renin-angiotensin system in heart failure is a secondary line of endocrine response enlisted after increases in plasma atrial natriuretic peptide and arginine vasopressin have already occurred.⁴ The activation is usually transient, largely reversible, and associated with phases of acute decompensation followed by stabilisation, unless end stage disease develops or diuretics are introduced. It is suspected, however, that localised tissue specific systems such as the intracardiac renin-angiotensin system might be significantly activated from an early stage during the progression of heart failure, although it is not known precisely what factors acting within the heart or other organs might influence the sequence of events leading to this change.

Progress towards the identification of important modifications in ACE production and function of the renin-angiotensin system in heart failure has been made in animal models of cardiovascular disease. Determining which of the molecular changes seen in animals are most relevant to humans must

now be evaluated after taking several complicating human specific factors into account.

Response of renin-angiotensin system to ACE inhibitors

Several predisposing factors in humans influence the response of the renin-angiotensin system to ACE inhibitors.

GENETIC INFLUENCES

So far, a tentative molecular-genetic link between ACE and the occurrence of human cardiovascular disease has been established. A common, simple genetic variation existing within intron 16 of the human ACE gene is linked to determination of plasma, and perhaps tissue, activities of ACE.^{5,6} Plasma ACE activity in subjects with the ACE DD genotype can be twofold higher than that in those with the ACE II genotype. The ACE D allele is apparently associated with increased risk of myocardial infarction and perhaps in the development of end stage disease through different aetiologies in white people.⁷⁻¹⁰ Around 30% of white patients with heart failure attending hospital clinics are homozygous for the ACE D allele, the proportion increasing according to the severity of disease. Raised baseline plasma ACE activity in some of these patients may have contributed to the generation of damaged or inappropriately repaired cardiovascular tissue through long term increased production of angiotensin II around the primary lesion, though a contribution from closely linked and unrelated genes cannot be ruled out.¹¹

The precise molecular mechanism through which possession of the D allele contributes to raised plasma ACE activity is not yet understood; a structural change located elsewhere within the ACE gene might provide the answer. Theoretically, the expression of the D allele may be raised in some tissues, the release of the protein it encodes from the plasma membrane may be increased, or the degradation of excess enzyme may be reduced. Studies aimed at understanding the biochemical-genetic regulation of ACE production and its relation to local haemodynamic, endocrine, and histological reorganisation during ACE inhibition will be of fundamental importance for drug targeting in

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the future, but progress may be delayed by the current absence of similar ACE genetic variants in animal models.

It has been difficult to establish a clear role for human plasma or tissue concentrations of renin¹²⁻¹⁴ or aldosterone¹⁵⁻¹⁷ in the generation of predisposition to cardiovascular disease. The existing biochemical data suggest that ACE activity is not the only determinant governing the cascade but that interaction of various protein and peptide components determines net angiotensin production. The discovery of a common allelic variant of human angiotensinogen is thought to be significant.¹⁸ This common molecular variation specifies the substitution of a methionine (Met) residue by a threonine (Thr) residue at position 235 of the mature protein. Individuals homozygous for the Thr²³⁵ residue usually have high baseline plasma concentrations of angiotensinogen, and there may be large ethnic differences in allele frequencies.¹⁹

Uncertainties about the importance of raised plasma angiotensinogen concentration and its consequences for the response to ACE inhibition are raised by the discovery of this angiotensinogen variant which parallel those associated with the ACE gene dimorphism. Does a combination of alleles specifying raised angiotensinogen and ACE result in a high risk group of patients? About 5–10% of patients with heart failure in the United Kingdom are homozygous for both the angiotensinogen and ACE alleles (K Morgan *et al*, unpublished data on 300 patients). Are plasma or tissue angiotensinogen, renin, and angiotensin I concentrations raised during treatment with ACE inhibitors to an extent that might outweigh any genetically influenced interindividual differences in baseline angiotensin II production? Further clinical studies are required to resolve these questions.

ENDOTHELIAL FUNCTION

Although further investigation is required to determine how subjects respond to ACE inhibition according to their genetic make up, molecular interactions within different organs at the blood-endothelial interface are likely to be important in all patients.²⁰ Whereas coronary endothelial angiotensin receptors colocalise with ACE binding, they are also found at lower concentrations in the medial layer of the vessel wall.²¹ In addition, though residual angiotensin-forming activity occurs in the vascular beds of a range of different organs during ACE inhibition, it is currently unclear which locations within the coronary tree are most important with respect to occurrence of deleterious vascular events mediated by angiotensins. The fine details of the molecular organisation of the renin-angiotensin system along the coronary vascular tree, where ACE is found associated predominantly with coronary endothelium and perivascular nerves, require further molecular investigation.²²

Perturbation of endothelial cell ACE turnover is presumably an early event in the response to ACE inhibition. Enzyme-inhibitor complexes may be internalised and degraded

or ACE secretase activity, which releases membrane bound ACE, may be altered.²³ Theoretically, endothelial cells might respond to reduced circulating angiotensin II concentrations by upregulating cell surface angiotensin receptor concentrations or down-regulating angiotensin degradation.

In response to raised circulating concentrations of angiotensinogen, prorenin, and activated renin secreted from the liver and kidneys, respectively, ACE inhibited endothelial cells might begin sequestering and metabolising these extra circulating components. Although specific cell surface receptors mediating uptake of angiotensinogen and renin have not been identified, alterations in their post-translational glycosylation states known to occur during ACE inhibition might affect their interaction with the endothelial cell surface and their metabolism in some organs.²⁴⁻²⁵

A localised tissue specific contribution to vascular angiotensin production by initiation of de novo synthesis of ACE, angiotensinogen, and renin within certain endothelial cells as a result of activation of gene transcription remains a distinct possibility²⁶⁻²⁸ (despite considerable difficulties associated with its in vivo and in vitro analysis²⁹⁻³⁰), provided that there is efficient translation of the messenger RNA (mRNA) and secretion of the correctly processed mature proteins.³¹ Does induction of endothelial gene expression result in resistance to ACE inhibition in certain patients?

EXTENT OF VASCULAR DISEASE

Dysfunctional endothelial cells surrounding atherosclerotic plaques may well respond differently to ACE inhibition than do normal cells. Activated endothelial cells and vascular smooth muscle cells in injured arteries express various trophic factors capable of modulating cell function. Paracrine or autocrine induction of angiotensin production may already exist in the diseased coronary arteries of some patients before treatment with ACE inhibitors. Parallel changes in the generation of bradykinin within the damaged vessel wall by tissue kallikrein activity³²⁻³⁵ might also occur.

The regulation of vascular wall angiotensin and bradykinin peptide concentrations might be modulated at several levels.³⁶⁻³⁸ The local balance of angiotensin and bradykinin peptide may be influenced by the leucocyte chemo-attractant and proadhesive properties of angiotensin and the subsequent production of angiotensinogen and ACE by infiltrating cells.³⁹⁻⁴²

The high ligand binding density of ACE localised in perivascular nerves suggests that operation of extrinsically controlled neural mechanisms might also be important in determining the vascular response to ACE inhibition. How vascular innervation relates to cardiac angiotensin metabolism requires further research. Within the myocardium, nerve fibres, blood vessels, fibroblasts, and mast cells⁴³ are juxtaposed within the cardiac interstitium, which consists of an extracellular

glycoprotein network supporting myofibril bundles anchored in parallel array. Nerves containing neuropeptide synthase and nitric oxide synthase are distributed throughout the atria and ventricles.^{44 45} The nerves are associated with blood vessels and myocytes, with the atria having more nerve fibres than the ventricles. Around coronary blood vessels there is an inverse relation between external vessel diameter and the extent of innervation. The number of nerve fibres containing substance P, a potential ACE substrate, is low. Ganglion cells containing nitric oxide synthase in the subepicardium and interatrial regions are surrounded by terminals positive for substance P. Connective tissue mast cells occur in close proximity to interstitial nerve fibres in some tissues, and it has been hypothesised that they metabolise peptides released into the interstitium from nerves. Whether the cardiac innervation contributes to the release of components of the renin-angiotensin system into the interstitium and modulation of peptide and vasodilator action is uncertain.

REGIONAL DIFFERENCES IN INTRACARDIAC DISTRIBUTION OF COMPONENTS OF RENIN-ANGIOTENSIN SYSTEM

Atria

Human atrial cardiac myocytes manufacture angiotensinogen, renin, ACE, and angiotensin receptors and show a positive inotropic contractile response to angiotensin II.⁴⁶⁻⁴⁸ Neural reflex mediated activation of atrial renin-angiotensin activity might occur in response to failing cardiac output in an attempt to improve the dynamics of ventricular filling. Raised atrial pressure leading to increased ventricular filling pressure has been hypothesised to have deleterious effects on damaged ventricles by contributing to end diastolic stretch of ventricular sarcomeres. Atrial ACE inhibition is therefore thought to be beneficial, at least mechanically. There remains, however, a poor understanding of potential interactions between atrial renin-angiotensin activity and the dysregulated production of atrial natriuretic peptide seen in heart failure.

Impulse conduction system

Concentrations of angiotensinogen, renin, and angiotensin receptors in cardiac conduction tissue differ significantly from the distribution of ACE in rat hearts. The concentration of ACE in the sinoatrial and atrioventricular nodes is relatively low compared with the concentrations of angiotensinogen and angiotensin receptors. This observation has raised speculation concerning the mode of angiotensin II production and function in conduction tissue.

The function of the sinoatrial node is often disturbed in patients with heart failure, with about 30% of patients showing atrial fibrillation, and particularly high concentrations of angiotensin receptors and nerve fibres have been noticed in the atrioventricular node in rats. Conduction cells, neural tissue, and the nodal artery might be involved in angio-

tensin II metabolism in these histologically specialised locations.⁴⁹ Although atrioventricular node dysfunction is not a common persistent problem in patients with heart failure, the conduction system running through the ventricular endocardium may, nevertheless, represent an important site of residual intracardiac angiotensin generation affecting intercellular communication during ACE inhibition.^{50 51}

Ventricles

Experimental evidence suggests that myocardial ACE gene expression is increased in rats with augmented ventricular wall stress and heart failure produced as a result of overload.⁵² A chamber specific fourfold increase in the levels of ACE mRNA, a twofold elevation of ACE protein, and a fourfold increase in ACE activity occurs in the free wall, septum, and ventricular apex.⁵³

After induction of left ventricular infarction in rats there is a 1.8-fold increase in the density of myocyte angiotensin II receptors associated with myocyte hypertrophy⁵⁴ while left ventricular angiotensinogen expression is only transiently increased, about 1.7-fold, subsiding to control levels at some point between five days and 25 days after infarction.^{55 56} Increased ACE expression can remain detectable one month after infarction.⁵⁷ These observations are consistent, perhaps, with a role for tissue angiotensinogen production in the acute phase separate from the function of chronically raised ACE activity and angiotensin receptor density. Induction of scar formation and peri-infarct inflammation and angiogenesis may be important processes affecting activation of the cardiac renin-angiotensin system soon after infarction, whereas localised release of growth factors in mechanically stressed tissues might account for induction and maintenance of ACE and angiotensin receptor expression in hypertrophy and scar expansion over the long term. Raised cardiac ACE and angiotensinogen concentrations can be induced in the absence of ischaemia or increases in ventricular mass⁵⁸ by heart failure induced by increased heart rate.

It is not clear whether changes in gene expression of the ventricular renin-angiotensin system at different times after infarction or hypertrophy or in response to ACE inhibitors are beneficial, detrimental, or ineffectual. Most experimental evidence indicates that ACE inhibitors can limit infarct size and ventricular dilatation and slow hypertrophy, but it is not certain whether these effects are mediated by indirect haemodynamic changes or by direct cardiac structural-functional changes mediated by gene expression.⁵⁹

Analysing the human cardiac renin-angiotensin system

Detection of raised ACE gene expression in the human left ventricle (up to twofold increases) has so far been described only in a proportion of patients with end stage heart failure⁶⁰; distinct mechanisms might still be

involved in the induction of raised ventricular ACE expression in human heart failure according to disease aetiology.

It is now possible to analyse ACE gene expression in small cardiovascular tissue samples derived from different regions of the heart using reverse transcription-polymerase chain reaction amplification (fig 1). Although qualitative studies are straightforward, quantitative studies are more time consuming and require application of rigorous controls using a competitive polymerase chain reaction strategy (fig 2).⁶¹

Localised cardiac chymase production

Human cardiac chymase is fairly abundant in ventricular tissue.⁶² It is synthesised as a pre-

protein⁶³ and secreted into the interstitium.⁴³ The purified enzyme has a restricted substrate specificity for angiotensin I and neurotensin.⁶⁴ It generates angiotensin II more efficiently than does ACE, whereas it cleaves neurotensin somewhat less efficiently in the same micromolar range. Chymase mediated generation of interstitial angiotensin II within the ventricles is potentially of great importance, provided that local angiotensin I production is sufficient in view of its potential inotropic, hypertrophic, and tissue remodelling effects.

Chymase activity has been analysed in different chambers of the heart. Likewise, we have analysed chymase gene expression in different regions of the heart. Using reverse transcriptase-polymerase chain reaction amplification we confirmed that the highest human cardiac chymase gene expression is restricted to ventricular tissue, although spliced mRNA can be detected in atrial tissue at lower levels in a proportion of patients.⁶¹ The analysis of mechanisms modulating cardiac chymase gene expression in different patients and its relation to disease progression should have important implications for understanding the role of angiotensins in heart failure in the future.

Figure 1 Polyacrylamide gel autoradiograph showing qualitative analysis of the expression of genes coding for angiotensinogen (1), renin (2, negative), angiotensin converting enzyme (3), cardiac chymase (4), glyceraldehyde 3-phosphate dehydrogenase (5), and transferrin receptor (6) in a single human tissue sample taken from the free wall of the right ventricle of an explanted heart; reverse transcriptase-polymerase chain reaction was used to amplify mRNA (M, markers).

Figure 2 Polyacrylamide gel autoradiograph showing a competitive polymerase chain reaction method for measuring ACE gene expression in human tissues by titration of endogenous message (lower band) against a competitor template (upper band). Quantification is possible after titration when the two cDNA molecules are amplified to the same extent (lane 4) following the addition of a predetermined amount of competitor molecule. (M, markers.)

Interpreting the importance of altered cardiac ACE gene expression

Cardiac ACE might have distinct roles in the peptide hormone response to ischaemia, damage repair, wall stress, and maintenance of hypertrophy according to its intracardiac distribution and activity. Determining which intracardiac sites respond favourably to intervention with ACE inhibitors may require a detailed understanding of regional intracardiac ACE production and intracardiac signal transduction pathways.

The activated angiotensin II receptor subtype 1 (AT₁) and its associated heterotrimeric GTP binding protein (Gq subtype) are linked to increased intracellular production of inositol triphosphate, raised intracellular calcium ion concentrations, generation of diacylglycerol (DAG),⁶⁵ and inhibition of adenylate cyclase activity.⁶⁶ Angiotensin dependent increases in intracellular calcium are associated with stimulation of tyrosine kinase activity,⁶⁷ whereas release of DAG activates members of the protein kinase C family of enzymes capable of phosphorylating serine and threonine residues within target proteins, including intracellular structural proteins, contractile proteins, components of the protein biosynthesis machinery, and gene transcription factors relevant to cardiac pathology.⁶⁸⁻⁷²

Exogenous angiotensin II upregulates net cardiomyocyte gene expression via a protein kinase C-fos/jun dependent pathway,⁷³ but whether this pathway normally stimulates feedback inhibition of ACE production by activation of repressors which recognise the ACE gene remains to be determined.

Ventricular hypertrophy in spontaneously hypertensive rats is associated with a threefold increase in AT_{1A} gene expression and a

corresponding twofold increase in receptor density.⁷⁴ The relative concentrations of AT₁ and AT₂ subtypes does not seem to be changed and upregulation of expression is reversible. Although the human AT_{1A} receptor gene has been characterised,⁷⁵ no alterations in the levels of its expression have been described so far in failing hearts.⁷⁶

The distribution and modulation of cardiac bradykinin receptors is less well characterised. Blunted intracellular responsiveness to raised plasma concentrations of atrial natriuretic peptide secreted from atria and ventricles correlates with altered cyclic GMP (cGMP) metabolism. Cardiac responsiveness to other agents which activate increases in intracellular cGMP, such as the bradykinin-nitric oxide pathway, might also be compromised in heart failure. ACE inhibition does, however, seem to have cardioprotective effects through the bradykinin and nitric oxide pathways in some experimental systems.⁷⁷⁻⁷⁹

Disease related regional disturbances of cardiac phospholipid, intracellular calcium, and cyclic nucleotide metabolism in failing hearts may exert an important influence on ACE gene expression. Uncoordinated hyperstimulation of cardiac intracellular signal transduction probably occurs, in part, as a result of compromised energy metabolism. Changes in cardiac energy metabolism in heart failure are thought to be largest in the sub-endocardium, where increased wall stress and ventricular filling pressure contribute to decreased perfusion.⁸⁰ An infarction covering 40% of the left ventricle in rats is associated with a prolonged 15% decrease in ATP content in the remaining viable tissue. Changes in the septum and right ventricle are also prolonged but are less pronounced.⁸¹ Hypoxic cardiomyocytes release high concentrations of AMP, which can be metabolised to adenosine and inosine at the extracellular surface of endothelial cells.⁸² Sustained increased extracellular concentrations of adenosine might modify myocardial and endothelial cell function by activation of adenosine receptors and inhibition of the effects of β adrenergic stimulation either at the level of adenylate cyclase or adrenergic receptor phosphorylation. Although negative feedback by extracellular adenosine may primarily be a protective mechanism operating against excessive β adrenergic stimulation in the endocardium, it might seriously compromise other processes dependent on signal transduction through the cyclic AMP (cAMP) pathway. Unravelling the relation between altered cAMP status, ACE production, and altered cardiac angiotensin II responsiveness⁸³ may identify cardiovascular regulatory pathways important during ACE inhibition.

The proximal 5' promoter regions of the human and mouse ACE genes have been described,⁸⁴ but precise molecular mechanisms regulating cardiac ACE gene expression are currently undefined. Molecular control of renin gene expression has been characterised more extensively than the other components

of the renin-angiotensin system.⁸⁵⁻⁹⁴ Induction of renal renin expression is intimately associated with the intracellular level of cAMP, and expression of the gene in extra-renal tissues is normally repressed by a nuclear protein which blocks the cAMP responsive DNA sequence element located in the 5' promoter region of the gene. Regional myocardial renin expression might be expected to remain repressed in heart failure, in which stimulation through the adenylate cyclase cascade may be defective. However, increases in atrial renin gene expression have been shown by dietary salt deprivation.⁹⁵ This may represent an atrial autocrine or paracrine response aimed at reinforcing angiotensin mediated downregulation of atrial natriuretic peptide production in the salt depleted state. It is unknown whether this mechanism is defective in heart failure.

Intracardiac levels of angiotensinogen gene expression are higher than those of ACE or renin and they increase in aging rat hearts.⁹⁶ Molecular mechanisms governing its expression in the liver have been defined in terms of DNA sequence elements involved in cytokine mediated acute phase transcription activation and the response to glucocorticoids and angiotensin II.⁹⁰⁻⁹²

Mechanisms controlling the activity of the cardiac kallikrein-kinin system have not been studied extensively, although the existence of kallikrein and kininogen in rat heart and blood vessels has been proved.⁹⁷⁻⁹⁹ Concentrations of bradykinin peptides and their response to ACE inhibition have been measured in a range of rat tissues, including the heart, where there is a significant increase in their concentration after drug treatment.¹⁰⁰ A corresponding activation of bradykinin receptors presumably occurs, but whether there is parallel modulation of local kininogen and kallikrein activation is uncertain. Expression of the kininogen gene displays differential 3' terminal exon usage for the production of high molecular weight and low molecular weight kininogens.¹⁰¹ In the liver expression of the low molecular weight transcript, designed primarily for bradykinin production, predominates.¹⁰²

Further studies of factors affecting angiotensin metabolism during ACE inhibition

HUMAN GENETIC STUDIES

The long term response to treatment with ACE inhibitors in patients classified according to angiotensinogen and ACE genotype should show the existence of any adverse contribution made by the possession of these alleles. If there is an adverse effect then studies of ethnic variations in allele frequencies and the effects of dose modifications or use of alternative ACE inhibitors may be required to improve the targeting of existing treatment.

PATHOLOGICAL TISSUE SAMPLES

The demonstration of abnormal concentrations or unusual isoforms of components of

the renin-angiotensin system and their localisation within atherosclerotic arteries or particular regions of hypertrophied hearts would be of mechanistic importance. In a survey of 30 samples from the right atrial appendage we found an unusual cDNA species encoding angiotensinogen in one sample.⁶¹ Interpreting the relevance of such findings will require more investigation at the molecular level.

NEW COMPONENTS OF THE RENIN-ANGIOTENSIN SYSTEM

Further study of several recently characterised components of the renin-angiotensin system should provide new insights into the functional organisation of the system in different organs. More details about the molecular biology of prorenin converting enzyme,¹⁰³ renin binding protein,¹⁰⁴ ACE secretase, the angiotensin II type 2 receptor,^{105 106} and angiotensinases^{107 108} and their responses to ACE inhibition should be forthcoming in the near future.

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