The history of the renin-angiotensin system (RAS) is one of marvelous discoveries extending from Robert Tigerstedt’s naming renin in 1898 to the present time; biochemists, physiologists, pharmacologists, and practicing clinicians have all combined to describe the physiologic implications of converting angiotensinogen into angiotensin II. Indeed, one may argue that the clinical development of ACE inhibitors and angiotensin II receptor antagonists has benefited humankind to a level seen only with the development of antibiotics and steroids. As we begin the twenty-first century, it is worthwhile to summarize the state of our knowledge concerning ACE and angiotensin II. In doing, we benefit from a whole class of experiments not available to those writing reviews even 10 years ago: the revolution in our ability to genetically manipulate the mouse as an experimental model. This is due to the widespread application of gene targeting by homologous recombination in embryonic stem cells. As is widely appreciated, this technology can produce a knockout mouse lacking any particular gene. Less appreciated are the full capabilities of this methodology which can be summarized as: if it can be dreamed, it can be done. Gene targeting can be used to create point mutations, duplicate a gene, and modify the expression pattern of a protein almost as easily as creating knockout mice null for a particular protein. We, and others, have used gene targeting in mice to create modifications in the renin-angiotensin system of a sort not seen in humans. While any single experiment may be assailed as not representing human disease, the totality of the effort has lead to a depth of understanding of the RAS not possible through other means. In this review, we attempt to integrate the findings of these genetic experiments with physiologic studies to arrive at a series of statements (truisms) concerning the actions of ACE and angiotensin II. After more than 100 years investigating this system, what can we say that is true and unassailable?

**The Renin-Angiotensin System and Angiotensin II Is Central to the Control of Blood Pressure**

Since the development of captopril, all have accepted an important role of the renin-angiotensin system in controlling blood pressure. But while captopril was originally envisioned as a potential treatment for high-renin hypertension, the clinical practice of interrupting the renin-angiotensin system to treat hypertension, heart, and kidney disease has expanded considerably. Perhaps the utility of ACE inhibition is surprising given the many different systems (sympathetic nervous system, nitric oxide, atrial natriuretic peptides, endothelin, prostaglandins, etc) influencing blood pressure. Given the multiplicity of systems controlling pressure, why is it that interruption of the renin-angiotensin system is so useful? Part of the explanation may be the magnitude of the effect mediated by angiotensin II. Studies of mice, in which angiotensinogen, renin, ACE, or all angiotensin II receptors...
are absent, consistently show the same phenotype; reduction of systemic blood pressure by $\approx 35$ mm Hg. Thus, a mouse lacking ACE has a systolic blood pressure of $\approx 73$ mm Hg, as compared with 110 mm Hg in wild-type. Knockout mice experiments do show that other systems participate in blood pressure. As an example, mice lacking endothelial nitric oxide synthase (eNOS) have an $\approx 18$ mm Hg rise in blood pressure. However, it is the magnitude of the change observed in the absence of angiotensinogen or ACE that is so remarkable. The renin-angiotensin system is central to blood pressure control; blockage of this system is not accompanied by effective counter regulation.

A mouse lacking angiotensinogen is a pure experiment investigating the role of angiotensin II. In contrast, the elimination of ACE is more complex because of the role of ACE in degrading bradykinin (a vasodilator) as well as in generating angiotensin II. In the absence of ACE, bradykinin accumulates. To gain insight into the role of bradykinin in ACE knockout mice, my group studied double-knockout mice lacking both ACE and the bradykinin B2 receptor. This is thought to be the principal bradykinin receptor leading to activation of eNOS, potentially important in a mouse lacking ACE. We found that the phenotype of the double-knockout mice was essentially identical to that of mice lacking only ACE. These data, as well as the nearly identical blood pressure levels observed in mice lacking either angiotensinogen or ACE, suggest that the major effect of the RAS on blood pressure is mediated by angiotensin II, at least in rodents. Indeed, mice null for angiotensinogen and mice null for ACE have nearly identical phenotypes: low blood pressure, elevated serum potassium, anemia, and both structural and functional renal defects. Angiotensinogen knockout mice seem to have increased mortality compared with ACE knockout mice for reasons not completely understood. Male ACE knockout mice have reduced fertility due to the lack of the testis ACE isozyme (discussed later).

Though ACE Is Not in Great Excess, ACE Levels Are Typically Not Critical to Blood Pressure Regulation

It is interesting to compare renin and ACE. Renin is only produced by a small group of tissues and is exquisitely specific in substrate specificity. ACE hydrolyzes several different peptides and is expressed by a variety of tissues, including proximal tubular epithelium, areas of the gut and areas within the central nervous system. However, it is the production of ACE by vascular endothelium that is thought to be most important in blood pressure control. In particular, the lung is a rich source of ACE; work many years ago established that most angiotensin I is converted to angiotensin II with a single pass through this organ. While renin levels vary in response to blood pressure, ACE levels are far less variable. That said, the conception that ACE is present in great excess is incorrect. We know this from studying mice heterozygous for an ACE knockout allele. These mice are termed ACE 1/wt, where “1” designates an ACE null allele and “wt” indicates a wild-type functional ACE allele. Due to the null allele, the ACE 1/wt mice express 50% to 60% normal ACE protein levels.

A useful way of characterizing ACE knockout mice is to measure plasma levels of angiotensin I and angiotensin II and then express the data as the angiotensin II/angiotensin I ratio (Figure 1). For wild-type mice, this ratio is 0.13±0.03, which indicates a significantly higher level of angiotensin II as compared with angiotensin II. Not surprising, a knockout mouse lacking all ACE has a marked reduction of plasma angiotensin II, an elevation of plasma angiotensin I, and an angiotensin II/angiotensin I ratio of 0.005±0.001. ACE 1/wt (heterozygous) mice have a normal blood pressure and plasma angiotensin II levels that are indistinguishable from wild-type mice. However, to achieve these normal indices in the setting of a modest reduction of ACE expression, the mice elevate angiotensin I, resulting in a plasma angiotensin II/angiotensin I ratio of 0.07±0.01. Thus, whereas a heterozygous mouse has no difficulty maintaining normal blood pressure, a homozygous knockout mouse has regeneration of a significantly lower level of angiotensin II.
pressure with only one functioning ACE allele and 60% normal ACE expression, the normal blood pressure is the result of homeostatic change in which higher concentrations of angiotensin I compensate for the reduced ACE levels. In a landmark study, Smithies et al investigated mice with 1, 2, 3, or 4 functioning copies of the ACE gene.14 These mice had from 62% (1 ACE gene) to 213% (4 ACE genes) of wild-type plasma ACE activity levels. Despite this wide range of ACE activity (wider than the D/I allelism discussed in humans in which D/D individuals have about 1.67-fold the plasma ACE activity (wider than the D/I allelism discussed in humans)), researchers have marveled at the beauty of a system in which ACE is perfectly positioned to generate angiotensin II in the tissue highest in ACE protein levels (Figure 2B). ACE 3/3 mice lack endothelial and most renal ACE expression. As an example, Huang et al, studied streptozotocin induced diabetes in mice with 1, 2, or 3 functional copies of the ACE gene.16 While under basal conditions, these groups have equivalent blood pressures, 12 weeks of diabetes induced a higher blood pressure and a much higher urinary albumin excretion in mice with 3 ACE genes as compared to mice with 1 or 2 ACE genes. Thus, in this model of disease, ACE levels did influence both blood pressure and disease progression. One important caveat is that, in our studies and those of Smithies et al, blood pressure was measured in healthy mice. Disease states may induce a different physiology. As an example, Huang et al, studied streptozotocin induced diabetes in mice with 1, 2, or 3 functional copies of the ACE gene.16

## Both Local and Systemic Formation of Angiotensin II Play a Role in Blood Pressure Control

Ever since the realization that endothelium is a major source of ACE, researchers have marveled at the beauty of a system in which ACE is perfectly positioned to generate angiotensin II in intermediate proximity to vascular smooth muscle, a critical target organ for the vasoconstrictor angiotensin II. This, and other experimental observations, suggest that tissue-based generation of angiotensin II is important, leading to some to question the relevant importance of the circulating (plasma) generation of angiotensin II versus local (tissue-based) generation. Perhaps a way of simplifying this problem is to restate the question: what is the importance of endothelial ACE in the regulation of blood pressure? To study this, we used gene targeting to create mice in which ACE gene expression was transferred from endothelial cells to the liver (Table).17 In these animals (termed ACE 3/3) ACE is made by hepatocytes and localized on their cell surface, but no vascular ACE is produced. The ACE 3/3 mice were made using gene targeting to position a neomycin cassette and albumin promoter between the endogenous ACE promoter and the coding exons of the ACE gene (Figure 2A). Functionally, the endogenous ACE promoter is blocked by the neomycin cassette, leaving ACE gene expression to be controlled by the albumin promoter. This approach of promoter substitution has certain advantages. First, both temporal and tissue-specific ACE expression are now controlled by the new promoter, enabling ACE to be expressed by a tissue such as the liver that does not normally express the protein. Such a result is different from the typical outcome achieved with Cre-lox manipulation in which gene expression is ultimately controlled by the endogenous promoter. A second advantage of our approach is that, in blocking the endogenous ACE promoter, tissues such as endothelium no longer express ACE protein. In fact, careful evaluation of ACE 3/3 mice showed no detectable expression of ACE by lung, typically the tissue highest in ACE protein levels (Figure 2B). ACE 3/3 mice do express 15% normal renal ACE, due to breakthrough expression of the artificial albumin promoter by renal tubular epithelium. ACE 3/3 mice have 80% normal plasma ACE activity, probably due to shedding of ACE by hepatocytes akin to endothelial shedding present in wild-type mice. Finally, ACE 3/3 mice also continue to make the testis isoyme of ACE.

The ACE 3/3 mice lack endothelial and most renal ACE but do express ACE in the liver. This model is unique in that it allows a direct evaluation of whether endothelial expression of ACE is obligatory for the control of blood pressure. Study of this model showed blood pressure, renal development, and other physiologic measures to be equivalent to wild-type

<table>
<thead>
<tr>
<th>Mutation</th>
<th>ACE Genotype</th>
<th>Description</th>
<th>Distribution of ACE</th>
<th>Blood Pressure</th>
<th>Renal Development</th>
<th>Urine Concentration</th>
<th>Hematocrit</th>
<th>Male Fertility</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>wt/wt</td>
<td>Two normal ACE alleles</td>
<td>Vascular endothelium (lung), Kidney, gut, brain, plasma, testis</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
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<tr>
<td>ACE.1</td>
<td>1/1</td>
<td>Null for all ACE</td>
<td>no ACE</td>
<td>Low</td>
<td>Medullar and papillary dysplasia</td>
<td>Dilute</td>
<td>Low</td>
<td>Low</td>
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<tr>
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<td>Heterozygous for null allele</td>
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<td>Normal</td>
<td>Normal</td>
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<td>Normal</td>
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<tr>
<td>ACE.2</td>
<td>2/2</td>
<td>Stop codon in ACE gene</td>
<td>No tissue ACE 34% plasma ACE activity</td>
<td>Low</td>
<td>Normal renal papilla</td>
<td>Dilute</td>
<td>Low</td>
<td>Low</td>
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<td>ACE.3</td>
<td>3/3</td>
<td>Albumin promoter controlling ACE</td>
<td>Hepatocytes 88%, kidney (14% of wild-type), plasma, testis</td>
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<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>ACE 1/3</td>
<td>1/3</td>
<td>Compound heterozygote</td>
<td>Half the expression of 3/3</td>
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<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>ACE.7</td>
<td>7/7</td>
<td>N-terminal catalytic domain inactivated</td>
<td>Same as wild-type</td>
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<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
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</tr>
<tr>
<td>ACE 1/7</td>
<td>1/7</td>
<td>Compound heterozygote</td>
<td>Half the expression of 7/7</td>
<td>Normal</td>
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<tr>
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<td>8/8</td>
<td>α-MHC promoter controlling ACE</td>
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<td>Nearly normal</td>
<td>Normal</td>
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<td>Normal</td>
</tr>
</tbody>
</table>

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mice. ACE 3/3 mice were also used in a simple breeding strategy to generate ACE 1/3 mice, compound heterozygous mice in which one ACE allele (the "1" allele) is null for ACE expression while the second ACE allele (the "3" allele) targets expression to the liver. ACE 1/3 mice were similar to homozygous ACE 3/3 mice in the tissue pattern of ACE distribution, but levels of ACE protein were 60% of those seen in the ACE 3/3 model (Figure 2B). Specifically, ACE 1/3 mice have substantial ACE expression in the liver (compared with wild-type who make very little hepatic ACE), 7% wild-type ACE expression in kidney, 43% wild-type ACE expression in plasma, and no ACE expression by endothelium or lung. To our surprise, these mice also presented with blood pressures and a renal physiology nearly identical to wild-type mice. Even when stressed by the complete absence of dietary salt for 2 weeks, ACE 1/3 mice were able to concentrate urine and retain urinary sodium to a level only mildly less than that of wild-type mice. These data raise the question of how animals with marked variations in both the level and tissue distribution of ACE can maintain such normal physiologic behavior. We believe the answer lies in Figure 3, which demonstrates that these genetically engineered mice retain a key feature of the renin-angiotensin system, namely the ability to alter renin levels to maintain normal homeostatic balance. In ACE 3/3 mice, sufficient total body ACE is present to necessitate only a mild elevation of plasma renin activity, resulting in levels of plasma angiotensin II equivalent to wild-type mice. In the 1/3 mice, the reduced amount of ACE stimulates higher renin levels. This, in turn, increases the plasma concentrations of both angiotensin I and angiotensin II. The result is that ACE 1/3 mice have a normal blood pressure, despite elevated plasma levels of angiotensin II. In fact, the ACE 1/3 mouse nicely illustrates what we feel is an accurate way of viewing the balance between the local and systemic production of angiotensin II. In a wild-type mouse, levels of angiotensin II present at the angiotensin II receptor are the result of both, the local generation of the peptide and a contribution from the systemic circulation. In the ACE 1/3 mouse, the lack of ACE production by endothelium means that local production of angiotensin II is reduced. In response, the kidney raises renin production and plasma angiotensin II levels to maintain homeostatic conditions. In fact, normal levels of aldosterone in ACE 1/3 mice suggest that, despite elevated plasma angiotensin II, tissue angiotensin II concentrations available to angiotensin receptors are close to normal. Thus, the true way to view the discussion of local versus systemic formation of angiotensin II is that, in a wild-type mouse, both systems participate; it is the total production of angiotensin II, and the concentration of this peptide at receptors within target organs, that is maintained in homeostatic balance. ACE 1/3 mice compensate for the lack of endothelial ACE by elevated systemic angiotensin II formation reestablishing normal blood pressure.
An interesting question is how do the ACE 1/3 mice maintain long-term elevation of renin production. In a sense, this is equivalent to asking how any mouse maintains proper levels of renin expression for steady state blood pressure control. We view the ACE 1/3 mice as mechanistically no different from wild-type mice. In both wild-type and ACE 1/3 mice, the combination of renal afferent arteriole blood pressure and blood flow, local angiotensin II concentration at JG cells, and renal distal tubule salt concentration combine to set the renin “thermostat” and establish a steady-state blood pressure. In ACE 1/3 mice, steady state requires higher plasma renin levels than wild-type mice.

**Hypertension Is Due to Renal Dysfunction**

An increasing number of humans develop hypertension as they age. Perhaps the first question is whether we should consider this process really abnormal or as a need for higher blood pressure as a form of physiologic compensation associated with the aging process. Whether or not high blood pressure plays any type of compensatory role, we know that it is associated with increased mortality. Also, the mechanism underlying blood pressure elevation remains a mystery. The experiments from our laboratory and others demonstrate that mice are exquisitely able to compensate for marked changes in ACE levels and ACE patterns of tissue expression through the regulation of renin. For example, consider mice we term ACE 1/7. Here, gene targeting was used to introduce point mutations into the ACE gene (Figure 4). Normally, ACE is composed of 2 independent catalytic domains roughly equal in their ability to hydrolyze angiotensin I. In the ACE 1/7 model, the ACE “7” allele lacks catalytic activity in the amino terminal catalytic domain, leaving an ACE protein with only 1 active catalytic domain. Further, the ACE 1/7 model is a compound heterozygous for the ACE “1” null allele. The result is that ACE 1/7 mice make roughly half the normal levels of a protein debilitated in converting angiotensin I to angiotensin II (a reduction of both protein levels and protein Kcat). Despite these changes, and despite lung ACE activity levels 46% of normal, ACE 1/7 mice maintain normal blood pressure by elevating renin and angiotensin. Thus, ACE 1/7 mice have plasma angiotensin I levels averaging 3166 pg/mL as compared with 775 pg/mL in wild-type mice. Our data are consistent with a large amount of investigation into genetic forms of human hypertension, as well as experimental manipulation of salt levels in animals, all of which emphasize the centrality of the kidney in controlling blood pressure.

**Protein structure**

#### Wild-type ACE

<table>
<thead>
<tr>
<th>N domain</th>
<th>C domain</th>
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<tbody>
<tr>
<td><img src="Zn" alt="N domain" /></td>
<td><img src="HEMGH" alt="C domain" /></td>
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</table>

#### ACE.7 N-terminal inactivated

<table>
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<tr>
<th>N domain</th>
<th>C domain</th>
</tr>
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<tbody>
<tr>
<td><img src="KEMGK" alt="N domain" /></td>
<td><img src="HEMGH" alt="C domain" /></td>
</tr>
</tbody>
</table>

Figure 3. Plasma peptides in the ACE.3 mice. Plasma angiotensin peptides were measured in wild-type (wt/wt), ACE.3 heterozygous (3/wt), ACE 3/3 and compound heterozygous ACE 1/3 mice as described in Figure 1. We also measured plasma renin activity. A group of ACE 3/wt and ACE 1/3 mice were studied for renin activity following a 2-week period of no dietary sodium. The lack of salt induces renal salt retention via increased renin production. ACE 1/3 mice have a normal blood pressure and near normal renal sodium handling, but achieve this through the elevation of plasma renin, angiotensin I and angiotensin II.

Figure 4. In a wild-type animal, ACE is a single polypeptide chain with 2 zinc (Zn) binding sites and 2 catalytic domains. The ACE.7 mutation introduced point mutations into the ACE gene that changed the amino acid sequence of the N-terminal Zn binding site from HEMGH to KEMGK. This new sequence is unable to bind zinc, eliminating catalytic activity in the N-terminal domain. ACE 7/7 mice are homozygous for the ACE.7 mutation. The ACE made by these mice only has catalytic activity in the C-terminal domain.
pressure.\textsuperscript{20} As has been emphasized by Hall, Guyton et al, elevation of blood pressure is only possible with the complicity of the kidney.\textsuperscript{21} If we accept the regulation of renin as being critical in the regulation of salt and water balance, then factors leading to a changed balance must play a role in hypertension’s onset. While the precise pathobiology of this process is not understood, we hypothesize that abnormal vascular flow, reflecting age-associated deterioration of the physical properties of renal blood vessels, must play an important role in this process.

**ACE Contributes to More Than Just Blood Pressure Control**

One of the most interesting aspects of ACE and angiotensin II is that this system plays an important role in several other physiologic processes apart from blood pressure control. For example, an unexpected finding, noted in ACE null mice, is abnormalities of kidney development characterized by expansion of the renal medulla and a marked thickening of renal arterioles (a paradoxical result in an animal with a marked reduction of blood pressure). While surprising, these finding were not totally unexpected given the renal abnormalities seen when ACE inhibitors were administered to neonatal rats (papillary atrophy and pelvic dilation) or pregnant humans (renal tubular dysgenesis among other abnormalities).\textsuperscript{22,23} While the formation of the renal lesion in ACE knockout mice is not completely understood, significant insight resulted from groundbreaking study by Ichikawa et al who assessed renal pelvic and ureteral function in AT1 receptor knockout mice (mice lacking both AT\textsubscript{1A} and AT\textsubscript{1B}).\textsuperscript{24} Work from this group suggests that angiotensin II is necessary for both the development of the renal pelvis and the peristatic function of the pelvis that normally helps promote the transfer of urine from the kidney to the bladder. In the absence of angiotensin II, this peristaltic action appears disrupted, resulting in elevated pelvic urinary pressures.\textsuperscript{24,25} These conclusions are consistent with work from our laboratory (as well as Ichikawa’s laboratory) demonstrating that, at birth, the kidneys from ACE knockout mice are structurally indistinguishable from those of wild-type littermate controls. Expansion of the renal pelvis was not observed in the knockout animals until \( \approx \)2 weeks of age. The work of Ichikawa et al, defining the role of angiotensin II in renal pelvic development and function, represent a very novel discovery resulting from the analysis of knockout mice.

One of the most consistent findings of ACE knockout mice is that these animals are anemic. While the anemia is not profound (hematocrit of 39\% versus 51\% for wild-type), the observation is very reproducible. In considering a mechanism by which ACE may participate in erythropoiesis, the first goal was to eliminate any causality of renal failure as the etiology of the anemia. ACE null animals do have elevated serum creatine levels and other indices suggesting a significant degree of renal insufficiency. Fortunately, my group developed an animal model (termed ACE.2) in which about one third normal ACE activity is present in plasma despite the near complete elimination of ACE from tissues such as endothelium.\textsuperscript{26} ACE 2/2 mice (animals homozygous for the ACE.2 mutation) are very similar to ACE null mice; the blood pressure and hematocrit are identical to ACE null animals. However, the presence of some ACE activity is sufficient to generate a small increase in plasma angiotensin II concentrations sufficient to correct the marked renal pelvic expansion seen in a complete ACE knockout. Evaluation of serum creatinine in the ACE 2/2 mice showed indices no different from wild-type mice. Thus, the continued low hematocrit present in this animal suggested that any anemia observed in ACE null mice was not the result of chronic renal failure. Another potential explanation for anemia in ACE 2/2 mice is vasodilation associated with volume expansion resulting in a dilutional anemia. We carefully evaluated this by radioactively labeling red blood cells and carefully measuring both red cell mass and intravascular volume. This study showed that ACE deficient mice have a true anemia (a reduction of red blood cell mass) and that this is associated with an elevation of plasma erythropoietin. These animals do not lack iron, nor is there evidence of hemolysis. In considering the anemia, one possible etiology was the elevation of the peptide acetyl-SDKP observed in ACE null mice. This peptide has been associated with bone marrow suppression. Acetyl-SDKP is elevated in ACE null mice because, the peptide is normally degraded by the amino terminal catalytic domain of ACE. However, we now have 2 lines of evidence proving that accumulation of acetyl-SDKP is not the cause of anemia in ACE null or ACE 2/2 mice. One line of proof involves study of the mice line termed ACE 7/7 in which point mutations were introduced into the ACE gene selectively inactivating the catalytic activity of only the amino terminal ACE domain.\textsuperscript{18} While these animals express normal quantities of ACE protein, this protein is catalytically unable to degrade acetyl-SDKP. Despite this, the ACE 7/7 mice present with normal hematocrits. A second line of proof eliminating acetyl-SDKP as the mechanism of anemia was an experiment in which ACE 2/2 mice were implanted with subcutaneous minipumps delivering sufficient angiotensin II to raise blood pressure to near normal levels.\textsuperscript{13} Mice genetically lacking ACE are exquisitely sensitive to the administration of exogenous angiotensin II. Indeed, levels of angiotensin II that normally raise the blood pressures of wild-type mice by 20 mm Hg are sufficient to induce extreme hypertension and rapid death in ACE null mice. However, when infusion rates of angiotensin II were lowered, we were able to achieve systolic blood pressures of \( \approx \)120 mm Hg in ACE 2/2 animals. The advantage of this experiment is that by administering angiotensin II, we now have a model in which only one ACE product (angiotensin II) has been restored to normal while other peptide products (such as acetyl-SDKP) are unaffected. The result of this experiment was that ACE 2/2 mice receiving angiotensin II had an increase of hematocrits to near normal levels. Thus, our hypothesis is that angiotensin II itself plays a direct role in erythropoiesis. In fact, work from our laboratory has implicated angiotensin II as capable of stimulating the Jak-STAT pathway, which is known to play a critical role in erythropoiesis.\textsuperscript{27} The precise mechanisms by which the renin-angiotensin system influences erythropoiesis remains an open and interesting question. A variety of human clinical reports have noted an association between the activation of the renin-angiotensin system and...
increased erythropoiesis.28–30 Also, other studies have suggested a link between ACE inhibitors and worsened anemia in patients with chronic renal failure.31–33

There are 2 isozymes of ACE. The more commonly studied ACE isozyme, termed somatic ACE, is made by endothelium and other somatic tissues. This is a single polypeptide chain composed of 2 separate catalytic domains. The second ACE isozyme is made by developing male germ cells and is called testis ACE. Testis ACE is half as large as somatic ACE and contains only a single catalytic domain, which is identical to that found in the carboxyl terminal half of the somatic ACE protein. Males make abundant quantities of testis ACE; testis has one of the highest overall levels of ACE activity of any tissue type.

In considering testis ACE, there are 2 major questions: how do developing male germ cells produce an isozyme of ACE different from that of somatic tissues and, perhaps more important, and what is the precise functional role of testis ACE? The first question—testis ACE formation—is now well understood. Both isozymes of ACE originate from a single genetic locus containing 2 distinct promoter regions (Figure 2A).34,35 The testis ACE promoter, uniquely recognized by developing male germ cells, is located within the twelfth intron of the somatic ACE gene. Initiation of transcription within the ACE gene results in the smaller testis ACE isozyme.

The precise function of testis ACE is more complicated. We now know that male mice lacking testis ACE reproduce poorly; both the frequency and the size of litters is markedly reduced from what is observed with wild-type mice. An elegant experiment by Ramaraj et al reconstituted testis ACE expression in an animal otherwise null for somatic ACE.36 Restoration of testis ACE restored the fertility of male mice to normal levels. The substrate of testis ACE is unknown. Indeed, it remains unknown whether testis ACE functions as an enzyme or perhaps as some structural protein necessary for proper sperm development. Two recent developments give some insight into this question. Kondoh et al recently discovered that testis ACE may influence reproduction through the action of a new ACE catalytic activity: a glycosylphosphatidylinositol (GPI)-anchored protein releasing activity.37 They found that this activity was necessary for zona pellucida binding of sperm.

Our group is nearing completion of mice genetically modified to selectively inactivate the ACE C-terminal catalytic domain. These mice should produce a testis ACE protein lacking traditional dipeptidase activity and should give insight into whether the mere presence of testis ACE protein or dipeptidase activity is crucial in reproduction.

The Role of ACE in the Heart Is Not Well Understood

As readers of this review know, ACE inhibitors and AT1 receptor antagonists are commonly used in the treatment of heart disease. Indeed, it is “common knowledge” that elevated cardiac levels of angiotensin II are deleterious. This common knowledge derives from both clinical studies and transgenic studies in mice.38–43 Further, a recent publication in Nature described the phenotype of ACE2 knockout mice.44 ACE2 is a zinc-containing monocarboxypeptidase that has 33% homology with testis ACE.45 Knockout mice lacking this enzyme develop cardiac contractile dysfunction as they age. This was attributed to mildly elevated (just less than 2-fold) levels of cardiac angiotensin II. The problem with the “common knowledge” concerning deleterious cardiac levels of angiotensin II is that this view may not be wholly accurate. We say this in light of our experiments studying the phenotype of mice engineered to express ACE selectively in cardiac tissue.46 From a technical perspective, this was achieved by ACE promoter manipulation, similar to that described for the ACE 3/3 mice. However, instead of controlling ACE with an albumin promoter, we created a mouse model in which ACE was under the control of the cardiac-specific, α-myosin, heavy-chain promoter. These mice, termed ACE 8/8, express 100-fold normal cardiac ACE levels, a figure somewhat distorted by the fact that wild-type mice make very little endogenous cardiac ACE. Perhaps it is better to describe ACE 8/8 mice as having cardiac ACE levels 3.9-fold those found in the lung. This ACE is structurally normal and is located on the cell surface of cardiac myocytes. The ACE 8/8 mice are also unique in that endothelial and renal expression of ACE were essentially abolished. Thus, by concentrating ACE expression in the heart, our experimental goal was to focus production of angiotensin II within this tissue. Precisely this was observed in that ACE 8/8 mice have cardiac angiotensin II peptide levels ~4.3-fold those of wild-type mice.

ACE 8/8 mice have systolic blood pressures of ~102 mm Hg. While this is less than wild-type, the magnitude of the reduction (~8 mm Hg) is not great. Also renal development and renal concentrating ability in ACE 8/8 mice were identical to that observed in wild-type. However, ACE 8/8 mice are not normal as indicated by a marked increase of mortality observed after weaning. Specifically, while nearly all wild-type mice survive in excess of 300 days, only 64% of ACE 8/8 mice lived 60 days after birth and only 23% lived 300 days. Surprisingly, we have no evidence that this mortality was due to structural defects in the ventricles. Ventricular weight was no different in ACE 8/8 versus wild-type mice. Further, there was virtually no evidence of increased ventricular fibrosis. Ventricular characterization by echocardiography showed heart dynamics of ACE 8/8 mice nearly equivalent to wild-type mice. Finally, intraventricular catheterization with a pressure transducer showed both ventricular pressure levels and dP/dT to be equivalent to those measures in wild-type mice. Thus, from a purely structural perspective, mice exposed to consistently elevated levels of cardiac angiotensin II showed no apparent structural disorganization or increased fibrosis of ventricular tissue. While mice are not human, and not all conclusions in mice are directly applicable to humans, evaluation of the ACE 8/8 mice do suggest that the relationship between cardiac angiotensin II and heart dysfunction may be more complicated than typically envisioned.

In contrast to ventricular structure, ACE 8/8 mice have a marked and consistent expansion of atrial volume (Figure 5A); careful analysis showed that the atria weight of ACE 8/8 mice was three-fold greater than that of wild-type or het-
homozygous mice (*P*<0.0001). Even more surprising, these mice have a variety of cardiac electrical abnormalities including AV conduction block and a highly reproducible and very significant reduction of cardiac electrical voltage (Figure 5B through 5D). While we believe that these abnormalities may be a result of cardiac angiotensin II, we must add the caveat that the ACE 8/8 mice are different from wild-type mice not only in the angiotensin II levels within cardiac tissue but also in the elevated levels of cardiac ACE. Thus, future work must distinguish between abnormalities of angiotensin II levels and the possibility that abnormally elevated membrane expression of ACE protein may have consequences apart from those due to ACE catalytic activity. We are presently studying ACE 1/8 compound heterozygous mice in which cardiac ACE expression is roughly half that of ACE 8/8 mice. Since these animals also lack ACE in noncardiac tissues, cardiac content of angiotensin II should be elevated. These mice should provide insight into the pathobiology of cardiac enlargement and cardiac electrical abnormality associated with the ACE 8/8 homozygous animal. Whatever the exact cause of the electrical abnormality in the ACE 8/8 mice, this phenomena is extremely interesting. The α-MHC promoter has been used in many instances and we are unaware of a reduced cardiac electrical amplitude and atrial enlargement as nonspecific consequence of protein over-expression. Thus, ACE 8/8 mice represent an opportunity to understand the origins of this unusual phenotype and, as a result, the role of ACE and angiotensin II in the heart.

**Conclusions**

The creation of genetically modified mice has advantages and disadvantages like all experimental approaches. Disadvantages include the fact that a mouse null for all expression of a protein represents an extreme phenotype that is quite different from the situation of most human diseases. Also, most genetic manipulation of a mouse induces a fixed change present from the conception of the animal. Again this is quite different from the clinical situation of selectively blocking an enzymatic pathway in adults using a pharmacologic inhibitor. Nonetheless, the exceptional plasticity of manipulation made possible using gene targeting is remarkable and endows the genetic approach with unique power. As we indicated, mutations that can be envisioned can be created. The application of this methodology to the renin-angiotensin system has
providing further insight into a story that had its beginnings with the crude experiments of Tigerstedt in 1898. Since then, evidence using a variety of approaches has implicated the RAS as probably the central regulator of normal physiology. That angiotensin II influences blood pressure is no surprise. That angiotensin II influences renal development, hematopoiesis, reproduction and cardiac electrical activity is more unexpected. In fact, the power of gene targeting in mice is underlined both by our realization that angiotensin II plays important roles apart from blood pressure control and in providing convenient models to study the details of these processes. In this review, we tried to lay down principals that we hope will be as true in 10 years as today. However, what is most predictable is that the RAS has other presently unknown functions that remain to be discovered.

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