Nontraditional Roles of Angiotensin-Converting Enzyme


Many articles have described the biochemistry of the renin-angiotensin system (RAS). Simply put, renin and angiotensin-converting enzyme (ACE) produce angiotensin II, which affects the brain (increased thirst), the gut (increased salt absorption), the adrenals (aldosterone production), the kidneys (salt and water retention), the heart (increased cardiac output), and vascular smooth muscle (vasoconstriction). Inhibitors of ACE and the angiotensin II type 1 receptor are now widely used to reduce blood pressure and to treat heart failure, and renal disease. The effectiveness of these drugs and abundant data from animal models show that the RAS is the body’s central mechanism for regulating blood pressure.

Modern studies of the RAS have substantially broadened the known roles of this system by showing that angiotensin II influences cellular proliferation and the inflammatory response. Although this wider interpretation of the RAS encompasses more than control of blood pressure, it still focuses on the effects of angiotensin II in the context of vascular injury. This increased understanding of the RAS inevitably leads to the question of whether, even now, we fully understand all of the physiological roles of the RAS. Here, we argue that the answer is no, that nature uses the components of the RAS and, in particular, ACE for a wide variety of physiological tasks.

ACE Is Necessary for Renal Development and Normal Male Fertility

In understanding the function of the RAS, a powerful tool is the ability to genetically alter mouse genes. Knockout studies of angiotensinogen, renin, ACE, or the angiotensin II type 1 receptor have confirmed the important role of these proteins in regulating blood pressure, but they also underline the wider physiological role for the RAS. For example, adult mice lacking ACE show marked abnormalities of renal development characterized by hypertrophy of small arteries, interstitial fibrosis, atrophy of the inner medulla and renal papilla, and dilation of calyces. Renal pathology is attributed to the lack of angiotensin II, because identical pathology is present in mice deficient for either angiotensinogen or renin, and similar renal pathology is seen in rats treated at an early age with an ACE inhibitor. Although the pathogenesis of the hypertrophy of the small renal arteries is not known, Matsusaka et al. has demonstrated that the renal papillary blunting is attributed to a functional hydronephrosis; in the absence of angiotensin II generation, there is abnormal development of the smooth muscle layer along the renal pelvis, lack of normal ureteric peristalsis, and elevation of intrapelvic urinary pressure. Thus, ACE and angiotensin II contribute to normal development of the urinary track, apart from effects on blood pressure.

Although angiotensin II is critical for renal development, this peptide is not the cause of the reproductive defect observed in male ACE knockout (KO) mice. ACE KO mice make phenotypically normal sperm and engage in intercourse but are nearly infertile. Although the exact mechanism is not known, studies have shown that it is the lack of testis ACE enzymatic activity, and not the lack of ACE protein, per se, that is responsible for the phenotype. Because male angiotensinogen KO mice are not reported as infertile, the infertile of male ACE KO mice must be mediated by effects on peptides other than angiotensin II. This emphasizes that the RAS has effects on many important peptides other than angiotensin I. Although renin is enzymatically specific, ACE is much more promiscuous in its substrate specificity. ACE cleaves angiotensin I, but it also cleaves bradykinin, substance P, luteinizing hormone-releasing hormone, acetyl SerAspLysPro (AcSDKP), and β amyloid1-42, among other peptides. ACE has carboxy peptidase activity. It can also cleave triptides and even be endopeptolytic. Although a single polypeptide chain, ACE contains 2 independent catalytic domains, often termed the N- and C-terminal domains. In vitro, the 2 catalytic sites show differences in binding and sensitivity to pharmacological inhibitors. Even more important, the 2 catalytic sites differ in their affinity and effectiveness in cleaving individual peptide substrates. An example is the peptide AcSDKP, which is cleaved almost exclusively by the N-terminal catalytic domain. In contrast, the C-terminal domain is the major catalytic site responsible for the conversion of angiotensin I to angiotensin II.
ACE and Hematopoiesis

It has been known for many years that, in humans, ACE inhibitors induce a small reduction of hematocrit levels. This is reflected in ACE KO mice, which exhibit a normocytic anemia attributed to reduced red cell mass, a phenotype independent of renal function. Short-term administration of angiotensin II to ACE KO mice increases hematocrit to near normal levels. Moreover, a variety of studies have suggested that angiotensin II acts as a regulator of erythropoiesis through its actions on erythroid precursors in the bone marrow and as an erythropoietin secretagogue.

AcSDKP is a 4 amino acid peptide released from the precursor thymosin β4. When normal volunteers were administered ACE inhibitors (which block both ACE domains), plasma and urine levels of AcSDKP rose 5-fold, showing that ACE is the major enzyme responsible for the degradation of this peptide.

Initial investigations of AcSDKP indicated that this peptide inhibited the recruitment of primitive hematopoietic progenitors into active proliferation. Thus, by degrading AcSDKP, ACE may help recruit stem cells into the S phase. AcSDKP has been reported to have several other effects, including promoting angiogenesis.

Other ACE peptides, such as angiotensin II and substance P, also appear to have effects on hematopoietic cell development. This became clear when the analysis of ACE KO mice showed that this enzyme plays a critical role in the development of myeloid cells.

To study the role of ACE in myelopoiesis, ACE activity was eliminated with pharmacological ACE inhibitors in an in vitro myeloid colony-forming assay. When wild-type (WT) bone marrow culture was stimulated with granulocyte-macrophage colony-stimulating factor, macrophage colony-stimulating factor, or granulocyte colony-stimulating factor, the inhibition of ACE consistently led to a significant increase in colony number. Further analysis strongly suggested that this was because of high levels of substance P in the absence of ACE. Evaluation of bone marrow in the ACE KO mice demonstrated elevated levels of substance P. This peptide is normally destroyed by ACE; in the absence of ACE activity, substance P induced bone marrow stromal cells to secrete growth factors that contributed to increased colony formation.

Although substance P appears to influence myeloid precursor number, additional experiments suggest that cellular differentiation is effected by both substance P and angiotensin II. For example, the upregulation of the early myeloid maturation marker FcγRII/III depends on the presence of angiotensin II, whereas the upregulation of the granulocyte marker Gr1 and the macrophage marker F4/80 appears dependent on both angiotensin II and substance P.

Angiotensin II formation is also necessary for the functional maturation of macrophages; macrophages derived from ACE KO mice showed reduced levels of secreted proinflammatory cytokines, surface major histocompatibility complex (MHC) class II protein, and surface density of the costimulatory factors CD80 and CD86. Angiotensin II supplementation fully or partly rescued these defects. Finally, when ACE KO mice were treated by acute intraperitoneal injection of methicillin resistant Staphylococcus aureus, these mice showed deficient bacterial clearance, resulting in greater blood dissemination than in WT mice. Thus, ACE appears necessary for normal myeloid cell differentiation and function.

ACE and MHC Class I Processing

There are several other areas in which research has indicated a role for the RAS during inflammatory injury. For example, Medhora et al have summarized evidence showing that ACE inhibitors and, to a lesser extent, angiotensin II type 1 receptor antagonists are some of the most successful drugs yet found to mitigate radiation-induced pneumonitis and fibrosis. Although many of these studies were performed in animal models, a clinical trial of the ACE inhibitor captopril in patients receiving total body irradiation showed promising results in reducing mortality after hematopoietic stem cell transplantation. Although some of the effect of an ACE inhibitor may be attributed to reduced angiotensin II production, there is also evidence that AcSDKP, which is elevated by ACE inhibition, may play an important role in reducing lung and other organ injury.

Further, angiotensin I-7, which is elevated by ACE inhibitors, has also been associated with antifibrotic, antiangiogenic, and antiproliferative effects.

Several publications have emphasized the role of ACE and angiotensin II in the pathogenesis of experimental autoimmune encephalomyelitis, a model mimicking multiple sclerosis. For example, Platten et al reported that, in experimental autoimmune encephalomyelitis, an ACE inhibitor or an angiotensin II type 1 receptor antagonist suppressed autoimmune T-helper 1 and T-helper 17 T cells and also increased FoxP3 positive T-regulatory cells. This study found that the administration of the ACE inhibitor lisinopril actually reversed paralysis when administered to mice after the establishment of experimental autoimmune encephalomyelitis. Further work by this group identified angiotensin II-induced expression of transforming growth factor-β as being an important pathological mechanism in experimental autoimmune encephalomyelitis. Additional studies documenting a role for the RAS in autoimmune demyelinating diseases are summarized by Lühder et al.

Fundamentally, ACE is a peptidase, and the suggestion that ACE plays a role in the immune response inevitably leads to the question of peptide presentation by MHC class I antigens. All of the nucleated tissues use MHC class I proteins to present peptide antigens on the cell surface. Under normal circumstances, cytoplasmic proteins are degraded by the proteasome into peptides, which are transported into the endoplasmic reticulum where they are trimmed, loaded onto MHC class I molecules, and then transported to the cell surface. Circulating CD8+ T cells are tolerant to the natural repertoire of displayed MHC class I peptides. However, in the presence of viral infection, viral derived peptides also become
were experiments in which a WT mouse was immunized with cells from an ACE KO mouse but then challenged with cells from either a WT mouse or a WT mouse treated for several days with the ACE inhibitor ramipril. When the immunized WT mouse was challenged with WT cells, there was virtually no T-cell reaction. However, when the immunized WT mouse was challenged with cells from a syngeneic WT mouse treated with ramipril, there was a significant CD8+ T-cell response. In other words, treating a mouse with an ACE inhibitor changed the repertoire of displayed MHC class I peptides. Finally, when a cross-immunization study was performed using ACE KO cells pretreated with a blocking antibody specific for the MHC class I protein H-2D\(^b\) (one of the 2 MHC class I proteins present in strain C57BL/6 mice), the ACE KO cells were significantly less efficient as immunogens. Such experiments argue that it is differences in the MHC class I peptide repertoire, and not differences in minor histocompatibility antigens, that are responsible for the immunogenicity of ACE KO versus WT mice. These and other experiments provide very strong evidence that, even under normal physiological conditions, ACE affects the MHC class I peptide repertoire in WT mice. Thus, ACE affects one of the central means by which the immune system detects viral infection and tissue transplantation. It also raises the question as to whether conditions, such as hypertension, may subtly change the repertoire of expressed MHC class I peptides, perhaps leading to some form of immune activation and cytokine release affecting blood pressure.

**ACE Overexpression and Resistance to Tumors**

Additional evidence for a significant role for ACE in the immune response resulted from analysis of mice called ACE 10/10. In this model, targeted homologous recombination was used to place ACE gene expression under the control of the c-fms promoter.\(^{33}\) c-fms is expressed by myelomonocytic lineage cells, where it encodes the receptor for macrophage colony-stimulating factor.\(^{34,35}\) Thus, ACE 10/10 mice markedly overexpress ACE in monocytes, macrophages, and other myelomonocytic lineage cells and lack ACE expression by endothelial cells, which do not recognize the c-fms promoter. Because of the high levels of ACE in monocyteic cells, care must be taken in extrapolating from the ACE 10/10 model to the normal physiological role of ACE.

The basal physiology of the ACE 10/10 mice is very similar to that of WT mice. ACE 10/10 animals have normal blood pressures, renal function, and appearance of both bone marrow and peripheral blood. However, when immunologically challenged, ACE 10/10 mice have a marked enhancement of their innate and adaptive immune responses. This first became apparent when we studied the growth of the B16 melanoma in ACE 10/10 mice.\(^{33}\) B16 is an aggressive mouse neoplasm that is commonly used to evaluate tumor immunology. Tumor growth in ACE 10/10 mice was evaluated by implanting melanoma cells intradermally and then measuring tumor volume 14 days later. Tumors in WT mice averaged 540 mm\(^3\), whereas heterozygous and ACE 10/10 mice averaged only 252 and 90 mm\(^3\), respectively (Figure 2). This difference in tumor size was observed whether the ACE

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*Figure 1. Cross-immunization of wild-type (WT) and angiotensin-converting enzyme (ACE) knockout (KO) mice. WT mice were immunized with peritoneal macrophages from either ACE WT or ACE KO mice. After 10 days, the recipient mice were euthanized, and splenocytes were expanded in vitro by restimulating with macrophages equivalent to those used for immunization. After 7 days, lymphocytes were again restimulated with macrophages equivalent to those used for immunization but for 5 hours. Fluorescence-activated cell sorter analysis was then used to stain for CD8 and interferon (IFN)-\(\gamma\). The percentage of CD8+ cells that are IFN-\(\gamma\)high is indicated.*

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loaded onto MHC class I molecules, leading to CD8+ T-cell recognition, activation, and, ultimately, cell destruction. Substantial work had studied the trimming of peptides within the endoplasmic reticulum and implicated an aminopeptidase called ERAP (endoplasmic reticulum aminopeptidase associated with antigen processing) as critically important. In 1992, Eisenlohr et al\(^{31}\) showed that, when overexpressed in cells, ACE was able to trim the carboxy termini of MHC class I peptides. However, little follow-up work was performed and, until recently, it was not thought that carboxypeptidases played any natural role in MHC class I peptide trimming. Ultimately, it was the development of ACE KO and ACE overexpressing mice that led us to re-examine ACE in MHC class I peptide processing.\(^{32}\) The critical experiment implicating ACE in this process was cross-immunization analysis (Figure 1). If a mouse is immunized with cells from an identical animal, it will not activate CD8+ T cells. However, when WT mice (having WT ACE) were immunized with cells from an ACE KO mouse, there was significant expansion and activation of CD8+ T cells. This phenomenon was also observed when ACE KO mice were immunized with cells from a WT mouse. These experiments were carefully controlled; all of the mice were backbred to a common genetic background (C57BL/6), and the donor and recipient mice were the same sex. Further strengthening the argument...
ACE Overexpression and Bacterial Resistance

The thesis that ACE 10/10 mice show an enhanced immune response led us to test the behavior of the ACE 10/10 model when challenged with bacterial infection. More than tumor models in mice, infections mimic the biological behavior of human disease. Our approach was to challenge ACE 10/10 with *Listeria monocytogenes* (listeria), a mainstay in the study of innate immunity, and with methicillin-resistant *Staphylococcus aureus*. Both systems demonstrated a substantially better innate immune response in ACE 10/10 mice as compared with WT mice. For example, when mice were challenged with an IV injection of listeria and then euthanized 3 days after inoculation, there were >6-fold the bacteria (colony-forming units) in the spleens of WT mice than in the spleens of ACE 10/10 mice. At 5 days after bacterial infection, colony-forming units in the spleens of WT mice were 8-fold those of ACE 10/10 mice. This difference between WT and ACE 10/10 mice is all the more remarkable, because WT mice are fully immunocompetent and very capable of clearing a listeria infection. A similar result was found when mice were challenged by an intradermal injection of methicillin-resistant *S. aureus*. Four days after infection, the quantity of bacteria in the skin of WT mice was >50-fold the colony-forming units found in ACE 10/10 mice. Our study of tumors in the ACE 10/10 mice showed that the enzymatic activity of ACE was critical for an enhanced
immune response. Experiments with ACE inhibitors showed a similar result for resistance to bacteria; when treated with ACE inhibitors, ACE 10/10 mice were equivalent to WT mice in their response to either listeria or methicillin-resistant S aureus.

Many studies have documented that 2 major mechanisms used by macrophages to kill bacteria are the generation of reactive oxygen species by NADPH oxidase and the production of NO by inducible NO synthase (iNOS).37,38 When reactive oxygen species were measured, there was no difference between macrophages from WT or ACE 10/10 mice. However, studies of either iNOS induction after lipopolysaccharide or nitrite production in response to listeria consistently showed a marked increase of iNOS and NO by ACE 10/10 macrophages. This increased iNOS production is critical to the enhanced innate resistance in ACE 10/10 mice, because treating these mice with the iNOS inhibitor 1400W rendered them equivalent to WT mice when challenged with either listeria or methicillin-resistant S aureus.

Great insight into the ACE 10/10 model was obtained from an experiment in which peritoneal macrophages were assessed for their ability to kill listeria in vitro. This showed that, in the absence of immune stimulation, there was no difference between macrophages derived from ACE 10/10 and WT mice. Only after stimulation with interferon-γ were cells from ACE 10/10 mice significantly better in killing bacteria than equivalent cells from WT mice. As discussed previously, the enhanced immune response of ACE 10/10 can be eliminated with an ACE inhibitor. However, this is not an acute effect, meaning that, for an assay such as in vitro killing, the addition of an ACE inhibitor during the 8-hour killing assay does not reverse the increased efficacy of the ACE 10/10 macrophages. Rather, ACE 10/10 mice must be treated with an ACE inhibitor for several days to revert the cellular phenotype to that of cells from WT mice. These and other data are consistent with a model in which ACE overexpression changes the underlying pattern of monocytic cell phenotype to those of cells from WT mice. These and other data are consistent with a model in which ACE overexpression changes the underlying pattern of monocytic differentiation and tilts these cells toward a more proinflammatory phenotype. Although the biochemical sequence of events leading to the ACE 10/10 phenotype is not fully understood, we postulate that the catalytic actions of ACE on an unidentified peptide substrate are responsible for the phenotypic change.

Perspectives
The modern ACE gene resulted from a gene duplication of a primordial ACE having one catalytic site; this duplication is thought to have occurred >300 million years ago.39 The modern clinical emphasis on the role of ACE in blood pressure control often obscures the implications that this protein maintained 2 independent catalytic sites throughout millions of years of evolution. ACE is enzymatically much less specific than renin, but it is involved in many more physiological processes. Understanding the many roles of ACE is important, because the peptide substrates and products of this enzyme have profound physiological effects.

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Disclosures
None.

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