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Regulation of blood pressure and cardiovascular function by renalase

Gary V. Desir[1,2]

[1]Department of Medicine, Yale University School of Medicine, New Haven, Connecticut, USA and [2]Department of Medicine, VACHS Medical Center, West Haven, Connecticut, USA

The renalase pathway is a previously unrecognized mechanism for regulating cardiac function and blood pressure. In this pathway, renalase, a novel secreted amine oxidase that is inactive at baseline, is rapidly turned on (~10 fold increase) by either a modest increase in blood pressure or by brief surges in plasma catecholamines. The active enzyme degrades circulating catecholamines, causing a significant fall in blood pressure. Plasma catecholamines not only activate renalase enzymatic activity but also lead to a 3–4 fold stimulation of renalase secretion. The renalase knockout mouse (KO) is hypertensive and exquisitely sensitive to cardiac ischemia. Abnormalities in the renalase pathway are present in animal models of chronic kidney disease (CKD) and hypertension. Two single-nucleotide polymorphisms (SNPs) in the renalase gene were found to be associated with essential hypertension in man. Blood renalase levels are inversely correlated with glomerular filtration rate (GFR) and are markedly reduced in patients with end-stage kidney disease (ESRD). We hypothesize that renalase is secreted into blood by the kidney (although also expressed in heart, skeletal muscle, and small intestine) and plays a key role in regulating blood pressure and cardiovascular function, and that abnormalities in the renalase pathway contribute to the heightened cardiovascular risks observed in patients with CKD.

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Correspondence: *Gary V. Desir, Section of Nephrology, Department of Medicine, Yale School of Medicine, 333 Cedar Street, LMP 2073, PO Box 208029, New Haven, Connecticut 06520-8029, USA.*
E-mail: gary.desir@yale.edu

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CARDIOVASCULAR COMPLICATIONS IN CHRONIC KIDNEY DISEASE

Although it is clear that renal replacement therapy, along with an aggressive management of infection, hypertension, hyperlipidemia, and secondary hyperparathyroidism, prolongs the life of those with end-stage kidney disease (ESRD), cardiovascular complications and overall mortality remains high. Data obtained from dialysis registries indicate a 6- to 10-fold increase in cardiovascular events compared with that in the non-dialysis population.[1] This increased propensity for cardiovascular disease seems to correlate with extensive arterial calcification, increased oxidative stress,[2] and a heightened sympathetic tone.[3,4] Furthermore, it was not well recognized, until recently, that cardiovascular disease is also prevalent and aggressive in patients with moderate reduction in renal function, such as those with chronic kidney disease (CKD) stage 3–5, who had not reached end stage and who were not receiving dialysis treatment. Compared with healthy controls, patients with CKD are far more likely to develop cardiovascular disease, and this propensity is not fully explained by the presence of traditional risk factors such as hyperlipidemia and hypertension.

Two large recent studies stand out, for they confirm earlier studies showing excess cardiovascular disease in CKD, and dramatically highlight the extent of the problem. A secondary analysis of the VALIANT study indicates that in patients with preexisting cardiac disease, the estimated risk of death from any cause over a 2-year period increased as glomerular filtration rate (GFR) declined, and patients with GFRs of less than 20 ml/min were six times more likely to die than were those with GFRs above 60 ml/min.[5] In another study that examined over 1.1 million enrollees from a single Health Maintenance Organization in northern California over a 2-year period, it was noted that renal function was an independent predictor of subsequent cardiac events, hospitalization, and overall mortality.[6] These findings not only confirm earlier studies but also bring to light the clinical and public health importance of CKD.

As approximately 26 million persons suffer from CKD in the United States alone, it is of utmost importance to gain a more detailed mechanistic understanding of the link between kidney and heart disease. Epidemiological data strongly suggest that although traditional risk factors for cardiac

disease are prevalent in the CKD population, they do not seem to fully account for the observed disease burden. Additional conditions, such as sympathetic activation, inflammation, and disordered calcium and phosphate metabolism, are prevalent in CKD and are believed to increase cardiovascular risk. The development of optimal management protocols ultimately depends on a deeper knowledge of the key functions of the kidney, and on the development of therapeutic tools that can replicate the kidney's critical functions and that are applicable to the management of these non-traditional risk factors.

In the healthy host, extensive cross-talk takes place between the kidney and the sympathetic nervous system to regulate important physiological processes. For instance, renal hemodynamics is regulated by an α1-adrenergic receptor-mediated pathway, and stimulation of both α1 and α2 receptors can increase sodium reabsorption.[7,8] The activation of β1 receptors can stimulate renin release from juxtaglomerular cells. Sympathetic overactivity is prevalent in patients with renal disease, and has been documented by microneurography in those with CKD stage 3 and above.[9–11]

The diseased kidney generates activating afferent signals through the stimulation of baro- and chemoreceptors. It is noteworthy that mild parenchymal injury, as can be achieved by injection of phenol into one pole of the kidney, with no measurable reduction in GFR, is associated with increased renal sympathetic outflow. Plasma norepinephrine (NE) is a valuable, though not perfect, proxy for the activity level of the sympathetic nervous system. NE levels are determined by the degree of sympathetic nerve activity, with a greater overspill of NE into the circulation as nerve activity increases, and by the capacity of peripheral tissues, such as the kidney, to metabolize NE. In the physiological state, the kidney takes up NE and metabolizes it using monoamine oxidase B and catechol-o-methyl transferase, and as will be described below, by renalase, a secreted amine oxidase recently discovered in our laboratory. In patients with ESRD, a significant increase in plasma NE is associated with a worse prognosis.[4,12,13]

DISCOVERY OF RENALASE

The kidney plays a key role in fluid and electrolyte balance, and also serves as an endocrine organ. Indeed, it is the major site for rennin secretion, and an important source of vitamin D and erythropoietin. Renalase was identified in the course of testing the hypothesis that the kidney secretes proteins that impact cardiovascular health, but are yet to be characterized, and could represent novel therapeutic targets.[14] The Mammalian Gene Collection (MGC) database, which at the time contained over 13,000 entries, was screened using an algorithm designed to identify putative secreted proteins of unknown function. The search yielded 114 genes encoding previously uncharacterized proteins, bearing secretory signal peptides. Tissue expression was assessed by northern blot and genes preferentially expressed in the kidney were selected for further study. Protein secretion was tested in cultured cells and putative functional domains were identified by computer analysis. Clone 9, which encodes a protein subsequently named renalase, was chosen for a more detailed analysis because renalase is secreted in human plasma and renalase levels are markedly reduced in patients with ESRD.

The human renalase gene resides on chromosome 10 at q23.33, encompasses 309,469 base pairs (bp), and has 10 exons. There is evidence for the existence of at least four alternatively spliced isoforms (Figure 1). The most highly expressed isoform (renalase1) is 342 aa long, and encoded by exons 1–4, 6–7, and 9. It is the predominant human renalase protein (hRenalase) detected in plasma, kidney, heart, skeletal muscle, and liver. It contains a signal peptide, a flavin adenine dinucleotide (FAD)-binding region, and an amine oxidase domain (Figure 2). hRenalase2 is 315 aa long, encoded by exons, 1–4, 6–7, and 10, and differs from hRenalase1 at the extreme carboxy terminus. hRenalase3 (exons 1, 4, 6–7, 10) and hRenalase4 (exons 5–9) are significantly shorter, with 232 and 138 aa, respectively, and unlikely to have the same function as renalase1 and 2. Indeed, hRenalase3 lacks a significant portion of the amine oxidase domain, as does hRenalase4, which, in addition, has neither a signal peptide nor an FAD-binding site. The functional significance of the spliced isoforms is not known. As only hRenalase1 is detected in plasma, perhaps the others have a paracrine function, or play a role in intracellular substrate metabolism. Unlike hRenalase2, hRenalase1 is efficiently secreted by transfected mammalian cells. hRenalase3 and 4 have not been tested, but they are unlikely to be secreted, and may have different enzymatic function, or none at all.

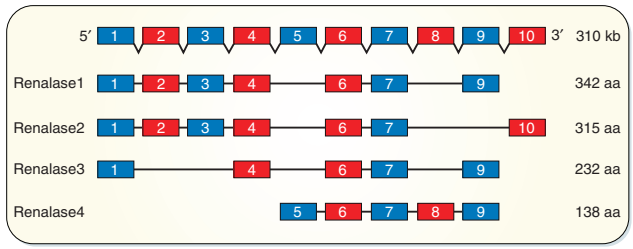

Figure 1 | Renalase isoforms. The 10 exons of the renalase gene are numbered. Of the nine possible isoforms, only those (renalase 1, 2, 3, 4) for which mRNA data are available are shown. aa: amino acids.

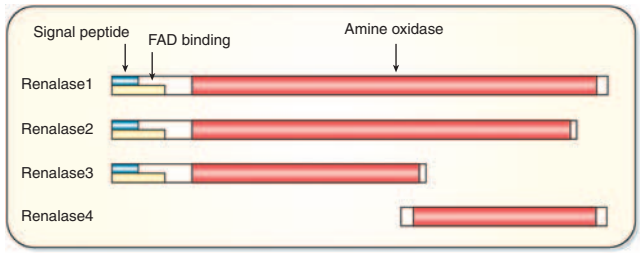

Figure 2 | Functional domains of renalase. Signal peptide (aa 1–17); FAD (flavin adenine dinucleotide) binding site (aa ~3–42); amino oxidase domain (aa ~75–335).

hRenalase1 can be detected in plasma using a polyclonal antibody raised against a glutathione *S* transferase-renalase fusion protein.[14] Plasma levels are markedly reduced in patients with ESRD, suggesting that the kidney is the predominant organ that secretes renalase and regulates circulating levels. It is currently not understood why other tissues, such as the heart, skeletal muscle. and liver, that express renalase do not compensate by increasing production and secretion. It is noteworthy that subtotal nephrectomy is associated with left ventricular hypertrophy, and a significant decrease in heart hRenalase1 levels in neonatal and adult rats.[15,16]

The mouse gene has been cloned and characterized.[17] Mouse *renalase* consists of seven exons and resides in mouse chromosome 19C1. The mouse renalase protein (mRenalase) has 72% amino acid identity with hRenalase, and contains a putative signal peptide, an FAD-binding site, and an amine oxidase domain. mRenalase can be expressed in prokaryotic and eukaryotic cells, and is observed to be secreted out of human cell lines. Interestingly, the *mRenalase* gene is highly expressed not only in the kidney, but also in the testicle, suggesting that the testicle may be a significant source of secreted renalase.

Recombinant hRenalase1 was screened for amine oxidase activity, as it contained an FAD-binding site and an amine oxidase domain. Of the amines tested, only catecholamines were metabolized by recombinant renalase1. On the basis of its amino-acid sequence, and substrate specificity, it is concluded that renalase belongs to a new class of FAD-containing amine oxidases. When injected into rodents, recombinant hRenalase1 leads to a transient, dose-dependent fall in blood pressure, heart rate, myocardial contractility, and peripheral vascular resistance.[14]

RENALASE ACTIVATION

Under basal conditions, the human plasma lacks significant amine oxidase activity.[18] We had confirmed that renalase activity could not be detected under basal conditions, suggesting that unlike refolded recombinant renalase, native renalase either has no amine oxidase activity or may circulate as a proenzyme that requires specific signals for activation. We tested if a sudden increase in plasma catecholamines, achieved by infusing exogenous epinephrine, represents such a signal, and found that renalase activity increased by approximately 10-fold within 30 s, for at least 60 min.[14,15] The rapid increase in activity most likely represents the activation of circulating renalase, and not *de novo* secretion. Within 15 min after epinephrine administration, plasma renalase increases by two- to three-fold, indicating a delayed renalase secretion. Of note, as blood pressure also increases with epinephrine administration, it is difficult to precisely determine if activation and secretion are mediated by an increase in plasma catecholamines, a rise in blood pressure, or by both. The kinetics of activation were examined using doses of epinephrine chosen to increase systolic blood pressure by 2–110 mm/Hg. Plasma renalase is fully activated when systolic blood pressure increases by 7 mm/Hg over baseline, suggesting that renalase plays a role in the minute-to-minute regulation of blood pressure. It is noteworthy that even a 10-fold increase in renalase activity may not significantly alter plasma catecholamine levels.[18] As discussed in more detail below, renalase administration provides significant protection against cardiac ischemia, and it is therefore likely that renalase has additional, and perhaps more relevant, enzymatic functions.

The molecular mechanisms that mediate the acute activation of renalase *in vivo* are not yet understood. One possibility is that increased catecholamines could cause a conformational change in the renalase molecule, with the subsequent dissociation of an inhibitor or binding of a circulating activator. Alternatively, activation may involve the proteolytic cleavage of blood renalase. Given that the FAD-binding site is at the extreme amino terminus (amino acid 3–42), and the amine oxidase domain extends from amino 75 to 335, it is unlikely that a significant portion of the molecule could be cleaved without affecting enzymatic activity. Perhaps a small segment can be removed at either the amino or carboxyl terminus. Finally, active renalase may be a dimer, and enzymatic activity could be modulated through the formation of dimers. This is an attractive hypothesis as the active form of many amine oxidases, including monoamine oxidase-A and monoamine oxidase-B, is a dimer.

RENALASE DEFICIENCY AND HYPERTENSION

As a number of factors are known to contribute to the development of hypertension in patients with CKD and ESRD, it is important to determine the contribution of renalase to that process. Data obtained from different experimental approaches indicate that renalase deficiency, in the absence of significant kidney disease, is associated with hypertension. Renalase gene expression can be downregulated in the rat using small inhibitory RNAs, without affecting renal function.[19] The mean arterial pressure increased by 12 mm/Hg, and the hypertensive response to exogenous catecholamines was markedly increased in the inhibitory RNA-treated animals. Dahl salt-sensitive rats develop moderately severe hypertension, increased sympathetic activity, and elevated catecholamines when maintained on a high-salt diet. Systolic blood pressure increased by 20 mm/Hg after 3 weeks on an 8% salt diet, and renalase expression decreased by 70%.

To further evaluate the role of renalase in the regulation of blood pressure, we generated a renalase knockout (KO) mouse by disrupting the renalase locus using homologous recombination.[20] The targeting construct deletes the promoter region and a large part of the coding region. Gene disruption was confirmed by PCR and western blot. Renalase KO mice weighted 25% less than control animals. Renal function, kidney histology, and plasma aldosterone levels are indistinguishable from those of wild-type littermates. Hemodynamic monitoring by telemetry indicates an increased heart rate and blood pressure in the renalase KO, with a

relatively greater elevation in diastolic pressure. Mean arterial pressure is significantly elevated in KO both during activity and at rest. Taken together, these data suggest that renalase deficiency is associated with tachycardia and hypertension. The relatively greater increase in diastolic pressure suggests significant vasoconstriction in the renalase KO, as would be expected if renalase played a significant role in catecholamine metabolism. Renalase seems to play a key role in the regulation of ambulatory blood pressure in mice.

Finally, Zhao *et al.*[21] tested for association of the renalase gene with essential hypertension by examining eight single-nucleotide polymorphisms of the renalase gene in 2586 individuals (1317 hypertensive cases and 1269 normotensive controls) from the International Collaborative Study of Cardiovascular Disease in Asia (InterASIA in China). Two single-nucleotide polymorphisms (rs2576178 GG genotype and rs2296545 CC) were associated with essential hypertension. Interestingly, rs2296545 CC results in an amino acid change (glutamic to aspartic acid at amino acid 37) within the FAD-binding domain, and it is tempting to speculate that this conservative amino acid change may weaken FAD binding and affect the function of renalase. These findings provide novel genetic susceptibility markers for essential hypertension, as well as novel insights for the mechanism of development of essential hypertension.

RENALASE DEFICIENCY IN CKD

Rats subjected to the removal of approximately 85% of kidney tissue (5/6 Nx) develop chronic renal failure and most of the associated abnormalities, including increased sympathetic activation, hypertension, and left ventricular hypertrophy. The 5/6 Nx rat is considered to be an excellent animal model of CKD. These animals develop severe blood renalase deficiency 2–3 weeks post-surgery.[15] Renalase levels in kidney and heart tissues were examined by immunocytochemistry and found to be markedly reduced. Renalase activation by catecholamines was also shown to be abnormal in 5/6 Nx rats. Although an epinephrine infusion can activate blood renalase in 5/6 Nx rats, the magnitude and duration of the activation were significantly lower than those which were observed in control rats. Taken together, these data demonstrate that there is a marked decrease of renalase protein and renalase activity in 5/6 Nx rats, and renalase deficiency may contribute to elevated plasma catecholamine levels.

As cardiac NE, a renalase substrate, increases by 600-fold during cardiac ischemia, and given that renalase is expressed in the heart, we tested whether recombinant renalase had a protective effect on the myocardium during ischemia. In the isolated perfused mouse heart models, left ventricular function was better preserved in hearts perfused with renalase, with left ventricular pressure being 85 and 47% of baseline at 120 min for renalase and glutathione *S* transferase, a control protein, respectively.[22] Renalase also decreased the myocardial infarct size by 54%. The data indicate that renalase reduces myocardial damage during acute ischemia, and its expression in the heart is decreased in CKD. We speculate that cardiac renalase deficiency may partly explain the increased susceptibility to ischemic myocardial damage and ventricular arrhythmias observed in patients with CKD.

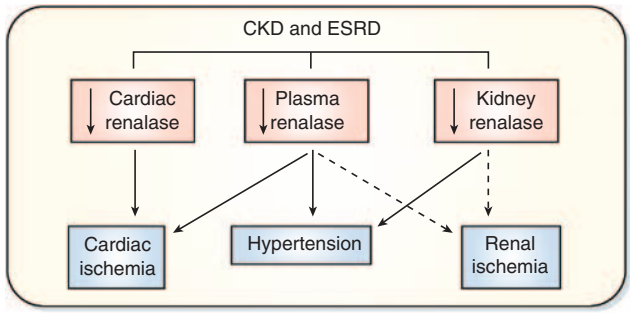

Figure 3 | Pathophysiology of renalase. CKD: chronic kidney disease; ESRD: end-stage renal disease; dotted lines: speculative link.

THE RENALASE PATHWAY

In summary, renalase is a novel FAD-dependent amine oxidase that is secreted into blood by the kidney. Recombinant renalase lowers blood pressure *in vivo* by decreasing cardiac output and peripheral vascular tone. Renalase seems to be secreted as a proenzyme, which can be activated by catecholamines. It is likely that renalase not only degrades catecholamines, but also metabolizes additional substrates. And it is possible that these yet unidentified substrates will turn out to be more relevant to renalase's physiological role and cardioprotective properties. Plasma renalase is significantly reduced in patients with CKD/ESRD, and the activation pathway is defective in rats with CKD. Interestingly, CKD is associated with a marked reduction in cardiac renalase expression, and the renalase KO is more susceptible to cardiac ischemic damage. These data support the notion that renalase plays an important role in the regulation of blood pressure and in the prevention of cardiac ischemic damage, and that its deficiency may account for the increased cardiovascular risk in patients with CKD (Figure 3). We speculate that renalase replacement therapy will improve cardiovascular outcome in CKD.

DISCLOSURE

Patent application submitted for the use of renalase.

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